Nitric Oxide Production in the Mammary Gland

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ABSTRACT

Although the effects of nitric oxide (NO) have been widely studied in many different cell and tissue types, very little is known of the role it plays in the mammary gland. Thus, the production of NO by mammary gland was investigated in a series of experiments. NO is a free radical gas which is produced by a wide variety of cells by the action of the enzyme nitric oxide synthase (NOS) on arginine. This results in the formation of citrulline and NO. The study first examined several methods for their suitability for the detection of NO or NOS. Evaluation of the methods revealed that the indirect measurement of NO production by the detection of nitrate and nitrite (NOx), the spontaneously produced metabolites of NO, was the most valid and reliable. The measurement of NOx was carried out in culture medium using a fluorescent-based assay, which was developed by the modification of published methods, during the course of this study. Comma-D cells (murine mammary epithelial cell line) were used to investigate the production of NOx by the inducible form of NOS (iNOS) following treatment with cytokines and cytotoxins. The cell's response was characterised and showed that mammary epithelial cells produce NOx in a dose dependent manner in response to interferon-γ (IFN-γ). Lipopolysaccharide (LPS), a component of bacterial cell walls, was employed to examine the response of the mammary epithelial cells to cytotoxins and it was found that the treated cells produced more NOx than the untreated ones, however, no dose response was apparent. The specific iNOS inhibitor, aminoguanidine (AG) and general NOS inhibitor N^{ω}-nitro-L-arginine (L-NNA) were both used to confirm that the NOx measured in the medium was produced by NOS. The production of NOx by the mammary gland was also examined in cultured explants of mammary tissue taken from pregnant (D 12-14 of pregnancy) and lactating (D 12-14 postpartum or D 17-18 postpartum) rats. A significant difference was found in the basal production of NOx between the different developmental stages. The method of euthanasia of the rats also affected the amount of NOx produced. The inclusion of prolactin (PRL) also increased the production of NOx from both Comma-D cells and explants of mammary tissue. Xanthine oxidase (XO), an enzyme responsible for the conversion of NOx to NO under anaerobic conditions, does not interfere with the determination

of NO production using the NOx assay. The measurement of NOx was carried out in the milk of cows following the intramammary infusion of *Streptococcus uberis* or interleukin-1β (IL-1β). By comparing the milk NOx concentration with the somatic cell count (SCC) and electrical conductivity (EC) of the milk, it was concluded that the source of the NOx in the milk could not be attributed entirely to the epithelium or the somatic cells. The experiments in this Thesis clearly show that the mammary gland is capable of the production of NO in response to a variety of situations and that the regulation of the production is very complex. The work also identifies some new areas of research, which if completed would further enhance the understanding of the role NO plays in the mammary gland.

For Grant,

My life, my love and my best friend

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LIST OF ABBREVIATIONS

ACh	acetylcholine
AG	aminoguanidine
cAMP	cyclic adenosine monophosphate
ANCOVA	analysis of covariance
ANOVA	analysis of variance
APS	ammonium persulphate
ATP	adenosine triphosphate
BAEC	bovine aortic endothelial cell
BCA	bicinchoninic acid
BMM	bone marrow derived monocytes
BSA	bovine serum albumin
CaM	calmodulin
CHAPS	3-[(3-cholamidopropyl)-dimethylammonio]-1-propane-sulfonate
CHX	cyclohexamide
cfu	colony forming units
D	day
DMEM	Dulbecco's modified Eagle's medium
DMEM:F12	Dulbecco's modified Eagle's medium:nutrient mixture F12
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
cDNA	complementary deoxyribonucleic acid
EC	electrical conductivity
EDRF	endothelium derived relaxing factor
EDTA	ethylenediaminetetraacetic acid
EGTA	ethylene glycol-bis(β-amino-ethyl ether)
EGF	epidermal growth factor
FCS	foetal calf serum
bFGF	basic fibroblast growth factor
GLM	general linear model
HEPES	N-2-(hydroxyethyl) piperazine-N'-2-ethanessulfonic acid

HUVEC	human umbilical vein endothelial cells
IFN-γ	interferon-γ
hIFN-γ	human recombinant interferon-γ
mIFN-γ	mouse recombinant interferon-γ
rIFN-γ	rat recombinant interferon-γ
IL-1β	interleukin-1β
kb	kilobases
kDa	kilodaltons
LDL	lower detection limit
L-NAME	N^G-nitro-L-arginine methyl ester
L-NMMA	N^G-monomethyl-L-arginine
L-NNA	N^ω-nitro-L-arginine
LPS	lipopolysaccharide
MBF	mammary blood flow
MOPS	3-[N-morpholino]propane-sulphonic acid
n	number of samples
NADPH	β-Nicotinamide adenine dinucleotide phosphate, reduced form
NF-κB	nuclear factor-κB
NO	nitric oxide
NOS	nitric oxide synthase
cNOS	constitutive nitric oxide synthase
eNOS	endothelial nitric oxide synthase
iNOS	inducible nitric oxide synthase
nNOS	neuronal nitric oxide synthase
NOx	nitrite plus nitrate
OD	optical density
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PBSE	phosphate buffered Saline & EDTA
$PGF_{2\alpha}$	prostaglandin-$F_{2\alpha}$
PMSF	phenylmethylsulfonyl fluoride
PRL	prolactin
PVP	polyvinylpyrrolidone

R^2	coefficient of determination
RNA	ribonucleic acid
mRNA	messenger ribonucleic acid
rRNA	ribosomal ribonucleic acid
SAS	Statistical Analysis System
SCC	somatic cell count
SDS	sodium dodecyl sulphate
SEM	standard error of the means
TBS	tris-buffered-saline
TEMED	N,N,N',N'-tetramethylethylenediamine
TPA	12-*O*-Tetradecanoylphorbol 13-acetate
Tris	tris(hydroxymethyl)aminomethane
TNF-α	tumor necrosis factor-α
VSMC	vascular smooth muscle cells
x *g*	times gravity
XD	xanthine dehydrogenase
XO	xanthine oxidase
XOR	xanthine oxidoreductase

CHAPTER ONE

INTRODUCTION AND LITERATURE REVIEW

This study investigates the production and regulation of nitric oxide (NO) in the mammary gland. Therefore this review will briefly examine the avenues of work which led to the discovery of NO, as well as its synthesis, roles and regulation. A section on the mammary gland will discuss the current knowledge as well as postulate further roles for NO and nitric oxide synthase (NOS) in the development and regulation of mammary growth and defence. Finally a discussion of the aims of this Thesis will be presented.

1.1 Nitric Oxide

Nitric oxide (NO), which has the lowest molecular weight of any known bioactive product secreted by mammalian cells (Nathan & Xie, 1994), had for many years been known as one of the major components of pollution. Apart from its ability to cure meat, NO held little interest for biologists. Or so they thought! How is it that this molecule, once thought of as unimportant, is now so widely studied? In 1987 four streams of biomedical research converged on nitric oxide as being the elusive molecule responsible for many of the results they were finding. In 1992 the journal Science named nitric oxide as "The molecule of the year" and in 1998 the Nobel Prize in Physiology or Medicine was jointly awarded to Robert F Furchgott, Louis J Ignarro and Ferid Murad for their discoveries concerning "nitric oxide as a signalling molecule in the cardiovascular system".

1.1.1 *History*

During the last century many people took organic nitrates or nitrites such as glyceryl trinitrate (nitroglycerine) for the treatment of coronary heart disease (Knowles, 1997). However, it was only in the last 15-20 years that evidence started to emerge as to the exact nature of the compound responsible for the beneficial effects.

Elucidation of NO's many effects arose from several lines of work. As early as 1916 (Mitchell *et al.,* 1916) it was reported that human beings synthesise oxides of nitrogen. Over half a century later, Green *et al.* (1981a) found that subjects on controlled low level intakes of nitrate actually excreted 4-fold greater amounts than were ingested, thus providing more evidence for the biosynthesis of nitrate in humans. Rats were also found to synthesise nitrate, with both germ-free (those lacking gastrointestinal tract microflora) and conventional rats excreting more nitrate than they ingested, showing that the intestinal nitrifying bacteria were not obligatory for nitrate formation (Green *et al.,* 1981b).

Increase in the levels of nitrate in both the blood and urine occurs in mice following the administration of lipopolysaccharide (LPS) alone or in conjunction with Calmette-Guerin bacillus (Stuehr & Marletta, 1985). Cytotoxins such as LPS, interferon-γ (IFN–γ), lymphokinin or Calmette-Guerin Bacillus also increases the amount of nitrate produced from cultured murine peritoneal macrophages suggesting that the activation of macrophages is a potent stimulator of nitrate biosynthesis (Stuehr & Marletta, 1985; Stuehr & Marletta, 1987).

As macrophages stimulated with cytotoxins synthesise L-citrulline and nitrite from L-arginine, but not D-arginine, L-arginine deiminase activity was proposed as being responsible for the conversion (Hibbs *et al.,* 1987). However, Iyengar *et al.* (1987) disputed this, arguing that since L-arginine deiminase is not found in mammals, then another pathway was responsible for the oxidation of arginine to nitrate and nitrite. Iyengar *et al.* (1987) suggested the intermediate in the arginine to nitrate and nitrite pathway was N-guanido-hydroxylated arginine. Their work further established that nitrate and nitrite were being derived from the terminal guanido nitrogen of L-arginine. Marletta *et al.* (1988) went on to establish that the enzymatic activity

responsible for the conversion of L-arginine to nitrate/nitrite and L-citrulline was only present in the cytosolic fraction of macrophages stimulated with LPS and IFN--γ, and not in unstimulated cells and that NADPH was required for the reaction to occur.

Another area of research that contributed to the elucidation of NO as a biologically active molecule was that which examined the activation of guanylate cyclase. During the mid 1970's an interest in the carcinogenic and mutagenic properties of nitrosamines led DeRubertis & Craven (1976) to investigate their properties in various rat and human tissues. They found that there was an enhancement of guanylate cyclase activity and increased concentrations of cyclic GMP following induction with several nitrosamines. The following year Arnold *et al.* (1977) reported that NO activated guanylate cyclase and increased guanosine 3', 5'-monophosphate (cyclic GMP) levels in various rat preparations.

Finally, studies of vascular smooth muscle relaxation contributed substantially to the emerging knowledge on the biological activity of NO. In the presence of endothelial cells, acetylcholine (ACh) induces relaxation of isolated preparations of rabbit thoracic aorta (Furchgott & Zawadzki, 1980). The authors postulated that ACh acted upon the muscarinic receptors of the endothelial cells releasing a substance(s) that causes relaxation of the smooth muscle. This substance became known as the endothelium derived relaxing factor (EDRF). In 1983 Rapoport *et al.* (1983) suggested that endothelium-dependent relaxation by nitrovasodilators was due to cyclic GMP dependent protein phosphorylation and dephosphorylation of the myosin light chain. Three years later Ignarro *et al.* (1986) reported that EDRF from artery and vein directly activates guanylate cyclase, thus resulting in elevation of cyclic GMP levels and the subsequent relaxation of smooth muscle. In 1987 two reports confirmed that Furchgott and Zawadzki's EDRF was NO. Both Ignarro *et al.* (1987) and Palmer *et al.* (1987) used a variety of biological and chemical assays to determine that NO released from both arteries and veins was identical in terms of biological activity and chemical properties to EDRF. The following year Palmer *et al.* (1988) suggested that the release of NO from porcine endothelial cells, induced by bradykinin (a vasodilator) and calcium ionophore, A23187, was enhanced by infusions of L-arginine, but not D-arginine. Due to this substrate specificity they argued that L-arginine was the precursor for NO synthesis in vascular endothelial

cells. Mass spectrometry analysis indicated that NO was formed from the terminal guanidino nitrogen atom of L-arginine. Later that year Sakuma *et al.* (1988) identified L-arginine as the precursor for EDRF derived relaxation. They suggested that since the inhibition of the synthesis of EDRF from endothelial cells was similar to the inhibition of the synthesis of oxides of nitrogen by macrophages, then macrophages could probably make NO and endothelial cells may respond to LPS and IFN–γ as macrophages had been shown to do.

In 1994 Archer *et al.* (1994) suggested that in rat pulmonary arteries, the mechanism by which NO increased cGMP vascular smooth muscle relaxation, was by a cGMP-dependent protein kinase-dependent activation of calcium sensitive K channels. However, Bolotina *et al.* (1994) showed that rabbit smooth muscle cells exposed to exogenous NO or to native EDRF directly activated calcium-dependent K channels.

The experiments, which led to the elucidation of NO and its effects, have been extensively reviewed by Furchgott & Vanhoutte (1989) and Moncada *et al.* (1991).

1.1.2 *Production of Nitric Oxide*

Nitric oxide synthase (NOS), the enzyme responsible for the catalysis of L-arginine and the production of NO and citrulline, has been described and characterised in many cell types. See Section 1.2 for further information.

Synthesis of NO from L-arginine is now generally regarded as being the result of a five-electron oxidation of the guanidino nitrogen of L-arginine via an N^{ω}-hydroxyl-L-arginine intermediate (Stuehr *et al.*, 1991b). The reaction is catalysed by NOS, which has requirements for molecular oxygen and NADPH as co-substrates (Knowles *et al.*, 1989; Mayer *et al.*, 1989), and for FAD, FMN and haem as co-factors (White & Marletta, 1992). The activity of NOS is enhanced by 5,6,7,8-tetrahydrobiopterin (Pollock *et al.*, 1991) and both free calcium (Ca^{2+}) and calmodulin (CaM) play roles in the regulation of the enzyme (Knowles *et al.*, 1989; Mayer *et al.*, 1989; Bredt & Snyder, 1990; Busse & Mulsch, 1990; Pollock *et al.*, 1991).

1.1.3 *Roles of Nitric Oxide*

NO plays a role in many aspects of physiology. This section reviews the research in a few of these areas and has been restricted to work which is considered relevant background to this Thesis.

Neurotransmission

Although not considered to be a 'classical' neurotransmitter, NO has now been implicated in many roles in both the central and peripheral nervous systems. However, not all researchers originally thought NO was a neurotransmitter. Ignarro *et al.* (1990) postulated that the inhibitory transmitter released from non-adrenergic non-cholinergic neurons may not be NO and could be an unknown transmitter, which in turn stimulates the production of NO from the smooth muscle. This was refuted by Bult *et al.* (1990) who reported NO as being an inhibitory non-adrenergic non-cholinergic neurotransmitter in the canine ileocolonic junction. Evidence for NO's role as a neurotransmitter has also been supplied by Bredt *et al.* (1990) who clearly demonstrated NOS immunoreactivity in numerous peripheral neural systems and in discrete neuronal populations in the brain. Furthermore the rat forebrain also contains NOS which catalyses the NADPH-dependent conversion of L-arginine to NO and citrulline (Knowles *et al.*, 1989). Glutamate (an excitatory neurotransmitter), acting on N-methyl-D-aspartate receptors of cerebellar cells (Garthwaite *et al.*, 1988), enhances the production of NO, suggesting that NO mediates glutamate stimulated cGMP formation (Bredt & Snyder, 1989) and further supporting a role for NO as an intercellular messenger in the brain.

More recently NO has been implicated in the expression of long-term potentiation of synaptic transmission (Schuman & Madison, 1991) as the retrograde messenger, which is liberated from postsynaptic neurons and travels back to presynaptic terminals. This work was supported by the observations of O'Dell *et al.* (1991) who also suggested that NO released from postsynaptic neurons diffuses and acts on presynaptic cells thus inducing long-term potentiation. Together, these studies provided conclusive evidence of an association of NO with neurons.

Blood Flow Regulation

Since the initial discovery by Furchgott & Zawadzki (1980) that rat aorta rings released a substance from their endothelial cells which caused relaxation of the blood vessels, there have been many more reports on endothelium-dependent relaxation of blood vessels. Since then NO has been shown to have roles in the maintenance of vascular tone as well as the distribution of blood flow and regulation of arterial pressure.

Release of NO from endothelial cells can occur through two different pathways. Basal release which can be as much as 20-40 % of the maximum released by ACh activation in the rat aorta, and stimulated release in which NO is released by physiological and pharmacological agonists, and physical forces such as sheer stress (the mechanical force associated with blood flow; Schini-Kerth & Vanhoutte, 1995). In this connection, following a six week period of chronically elevated blood flow in the femoral artery of the dog, there was increased *in vitro* endothelium-dependent relaxation in response to stimulation with either ACh, adenosine diphosphate or α_2-adrenergic stimulation, compared with the artery from the contralateral limb, suggesting that the relaxation may be modulated in response to the chronic alterations in blood flow (Miller *et al.,* 1986). Furthermore, when the steady flow rate through an *in vitro* segment of canine femoral artery was doubled; it caused an increase in both the release of 6-keto-prostaglandin $F_{1\alpha}$ and relaxation of the bioassay ring (Rubanyi *et al.,* 1986). There was no apparent relaxation when the endothelium was absent. Rubanyi *et al.* (1986) reported that the release of prostacyclin was not the primary mediator of flow-induced relaxation, but it was another relaxing substance released which had similar properties to that released following ACh stimulation. This was supported by Miller & Vanhoutte (1988) who showed that the relaxations to ACh in canine arteries when subjected to chronic increases in blood flow are mediated by activation of muscarinic receptors and are due to increased release of EDRF. The increases were not due to a change in the endothelial muscarinic receptor subtype or the sensitivity of the smooth muscle to EDRF (Miller & Vanhoutte, 1988). In human forearm arterial beds, NO is continuously released and plays a major role in the control of both basal and ACh stimulated blood flow. The vasoconstrictor effect of N^G-monomethyl-L-arginine (L-NMMA; an inhibitor of NOS) was investigated and

found to be reversed by L-arginine (Vallance *et al.,* 1989). L-NMMA also causes a dose-dependent increase in mean arterial blood pressure in rabbits and in rats and the administration of L-arginine reverses this hypertension (Rees *et al.,* 1989; Glick, Gehman & Gascho, 1993). This suggests that NO from venous vascular endothelium reduces baseline venous tone.

Sheer stress has also been implicated as a stimulator of NO from vascular endothelial cells. Dull & Davies (1991) investigated the relationship between sheer stress and intercellular calcium concentration ($[Ca^{2+}]i$) in cultured bovine aortic endothelial cells (BAEC). Calcium is of interest as it has been implicated as playing a role in the regulation of NO production (Section 1.1.2). Changes in the flow rate of the media across the cellular monolayer did not significantly alter the basal $[Ca^{2+}]i$, but increases in flow in the presence of adenosine triphosphate (ATP) caused large increases in $[Ca^{2+}]i$ transients, which slowly returned to basal levels. Upon the initiation of flow and in the absence of exogenous ACh, BAEC $[Ca^{2+}]i$ levels increased to approximately four times above basal levels. In contrast, rat aortic smooth muscle cells do not show any flow-mediated increases in $[Ca^{2+}]i$ (Geiger *et al.*, 1992). However, both increases in flow and treatment with ACh caused an increase in the endothelium $[Ca^{2+}]i$ and dilation of rat arterioles isolated from cremaster skeletal muscle. These effects were both abolished by the removal of the endothelium (Falcone *et al.,* 1993).

Cell Defence

Along with its role as an intercellular signal molecule, NO is also a cytotoxic effector molecule in non-specific immune reactions. Cytoprotective roles have also been described for this molecule.

The production of NO from various cell types has been shown to be cytotoxic to both tumor cells and parasites, thus benefiting the host. Many early studies have shown that macrophages stimulated *in vitro* or *in vivo* with LPS or IFN-γ respond with increased levels of nitrate and nitrite (Stuehr & Marletta, 1985; Hibbs *et al.*, 1987; Iyengar *et al.*, 1987; Stuehr & Marletta, 1987). Both activated peritoneal macrophages (Hibbs *et al.,* 1972) and peritoneal macrophages from mice with

parasites (Krahenbuhl & Remington, 1974) are cytotoxic to mouse fibroblasts (L-929 cell line) and mouse mammary adenocarcinoma cells (EMT-6 cells). Activated macrophages also inhibit the mitochondrial energy metabolism of the murine leukaemia cell line, L1210, resulting in inhibition of growth (Granger *et al.*, 1980). NO synthesised from murine peritoneal macrophages induced with recombinant IFN–γ, was responsible for the *in vitro* killing of *Leishmania major*, a protozoal parasite. In contrast, mice infected with *L. major* and injected with L-NMMA into the lesions, had 10^4-fold increases in parasites obtainable from the lesions (Liew *et al.*, 1990). Rat hepatocytes (Billiar *et al.*, 1990) and rabbit vascular smooth muscle cells (Busse & Mülsch, 1990) respond to endotoxins and cytokines respectively with increases in the expression of an inducible cytosolic enzyme activity of the type previously described in macrophages. The activation in a murine macrophage line with LPS resulted in both an increase in nitrite synthesis and an increase in L-arginine transport and the authors suggested that this elevation of transport may provide a mechanism for sustained substrate supply for the generation of NO by macrophages (Bogle *et al.*, 1992).

Therapeutic applications related to the inhibition of NO production have also been explored. In rats, hypotension due to septic shock can be prevented by the partial inhibition of NO synthesis using L-NMMA although complete inhibition reversed this beneficial effect (Nava *et al.*, 1991). Both L-NMMA and N^G-nitro-L-arginine methyl ester (L-NAME; an inhibitor of NOS) were able to reverse the hypotension associated with severe septic shock in two male human patients (Petros *et al.*, 1991). Geroulanos *et al.* (1992) also successfully treated a patient with severe septic shock using N-NMMA. However, the suggestions by both Petros *et al.* (1991) and Geroulanos *et al.* (1992) that L-NMMA and L-NAME may have roles in the treatment of septic shock was disputed as being premature by Hotchkiss *et al.* (1992). Their argument was that further studies should be done to examine the effects of the agents and to determine what role NO actually plays during septic shock.

Protective roles have also been shown for NO. Yates *et al.* (1992) reported that lipid peroxidation of low-density lipoproteins were decreased when incubated with murine peritoneal macrophages stimulated with IFN-γ and tumour necrosis factor-α (TNF-

α). Incubation with the NOS inhibitor L-N^G-aminohomoarginine caused a reduction in NO synthesis and an increase in low-density lipoprotein oxidation. Rubbo *et al.* (1994) went on to show that NO was able to protect soybean phosphatidylcholine membranes and lipoproteins via the redirection of superoxide mediated cytotoxic reactions. Thus NO appears to have antioxidant effects. This antioxidant effect was also shown by Hayashi *et al.* (1995) who reported that the antioxidant efficiency was related to the concentration of NO and the relative importance of reactions with other oxygen radicals. The reaction of NO with oxygen and superoxide and the formation of reactive nitrogen species and the effects these have on biological systems is a complex area of emerging new research. For extensive reviews see Wink & Mitchell (1998) and Patel *et al.* (1999).

Platelet Adhesion/Aggregation

NO release contributes to the non-adhesive properties of vascular endothelium. For example, bradykinin inhibition of the basal and thrombin stimulated adhesion of human platelets to bovine vascular endothelial cells was found to be due to the release of NO (Radomski *et al.*, 1987; Sneddon & Vane, 1988). Human platelets contain an endothelial type NOS which is activated by aggregation of the platelets with collagen. The generation of NO was shown to modulate platelet reactivity by an increase in cGMP, thus leading to inhibition of aggregation (Radomski *et al.*, 1990).

General Comments

This section has clearly demonstrated the role of NO in many areas of physiology although for the sake of brevity many of the details regarding how NO exerts these actions have been omitted. Many excellent review articles now exist which cover these areas in more detail.

1.2 Nitric Oxide Synthase

The previous section examined various roles of NO. This section will examine how NO is produced and where the production occurs.

1.2.1 *Identification*

In 1989 Knowles *et al.* (1989) reported the existence of a soluble enzyme from the forebrain of a rat which catalysed the formation of NO and L-citrulline from L-arginine. The enzyme was dependent on NADPH and Ca^{2+} and was essentially inactive at resting concentrations of Ca^{2+}, but fully active at concentrations above 400 nM. The discovery of this enzyme helped to explain the results of Garthwaite *et al.* (1988), who found that cerebellar cells stimulated with N-methyl-D-aspartate (an excitatory amino acid which increases cGMP in the rat brain), released a messenger with properties very similar to EDRF. Calmodulin (CaM) was also shown to be a requirement of NOS purified from rat cerebella (Bredt & Snyder, 1990).

A novel enzyme from vascular endothelial cells was isolated which appeared to be involved in the formation of NO from L-arginine (Palmer & Moncada, 1989). Partial characterisation showed that the enzyme was similar to that previously reported in macrophages (Marletta *et al.*, 1988). Mayer *et al.* (1989) reported that the L-arginine converting enzyme found in porcine aortic endothelial cells was dependent upon NADPH and Ca^{2+} and Busse & Mulsch (1990) later reported that the activity was mediated by CaM. NOS purified from cultured BAEC shows similar requirements for L-arginine, NADPH, Ca^{2+} and CaM but also requires tetrahydrobiopterin for full activity (Pollock *et al.*, 1991).

Although the production of nitrite and NO from murine macrophages following treatment with cytotoxins such as LPS and lymphokinin had been well characterised (Section 1.1.1), the enzyme responsible for this production was not purified and characterised until 1991 (Hevel *et al.,* 1991; Stuehr *et al.*, 1991a; Yui *et al.*, 1991). The activity of the enzyme either requires, or is enhanced by NADPH, FAD, FMN and tetrahydrobiopterin (Marletta *et al.*, 1988; Hevel *et al.*, 1991; Stuehr *et al.*, 1991a;

Yui *et al.*, 1991; White & Marletta, 1992). Neither of free calcium or CaM are required for full activity (Stuehr *et al.*, 1991a; Yui *et al.*, 1991).

Thus, these different types of NOS were classified into two groups, constitutive NOS's (cNOS), which are continuously present and have a requirement for Ca^{2+}, and inducible NOS (iNOS), which is a Ca^{2+} independent enzyme and is induced by certain cytokines and cytotoxins.

1.2.2 *Different Isoforms*

The above mentioned studies led to the discovery that there were, in fact, three distinct isoforms of NOS; neuronal NOS (nNOS), inducible NOS (iNOS) and endothelial NOS (eNOS), which are the products of separate genes (Morris & Billiar, 1994). The carboxy-terminal two-thirds of the proteins are highly homologous, while the amino terminals are unique to each isozyme (Nishida *et al.*, 1992) resulting in each one having a different molecular weight. The functions of the three enzymes differ and although some similarities are reported in the regulation of the different isoforms, all show some form of unique regulatory systems. A comparison of the main properties of the three isoforms is given in Table 1.1.

Table 1.1: Comparison of characteristics of nitric oxide synthase isoforms. Properties of the three isoforms of NOS including the size and predicted length of the protein and the proposed binding sites on the cDNA are given. Requirements for calcium and the predominant location for the activity are also compared.

Characteristic	Isoform	References
	iNOS	
Size (amino acid length)	~ 1150	(Lowenstein *et al.*, 1992; Lyons *et al.*, 1992; Xie *et al.*, 1992; Charles *et al.*, 1993; Geller *et al.*, 1993a; Chartrain *et al.*, 1994)
Size (kDa)	~ 130	(Hevel *et al.*, 1991; Stuehr *et al.*, 1991a; Baek *et al.*, 1993)
Binding sites (cDNA)	FAD, FMN, NADPH, CaM	(Lowenstein *et al.*, 1992; Lyons *et al.*, 1992; Charles *et al.*, 1993; Chartrain *et al.*, 1994).
Calcium	Independent	(Stuehr *et al.*, 1991a; Yui *et al.*, 1991)
Location of Activity	Cytosolic	(Marletta *et al.*, 1988; Xie *et al.*, 1992)
	eNOS	
Size (amino acid length)	~1200	(Janssens *et al.*, 1992; Lamas *et al.*, 1992; Marsden *et al.*, 1992; Sessa *et al.*, 1992)
Size (kDa)	~135	(Förstermann *et al.*, 1991; Pollock *et al.*, 1991; Pollock *et al.*, 1993; Tracey *et al.*, 1994)
Binding sites (cDNA)	FAD, FMN, NADPH, CaM	(Janssens et al., 1992; Lamas *et al.*, 1992; Nishida *et al.*, 1992; Sessa *et al.*, 1992)
Calcium	Dependent	(Förstermann *et al.*, 1991; Pollock *et al.*, 1991; Pollock *et al.*, 1993; Tracey *et al.*, 1994)
Location of Activity	Particulate	(Pollock *et al.*, 1991; Sessa *et al.*, 1992; Busconi & Michel, 1993; Michel *et al.*, 1993; Pollock *et al.*, 1993; Hecker *et al.*, 1994; Tracey *et al.*, 1994)
	nNOS	
Size (amino acid length)	~ 1430	(Bredt *et al.*, 1991; Hall *et al.*, 1994)
Size (kDa)	~ 155	(Schmidt *et al.*, 1991)
Binding sites (cDNA)	FAD, FMN, NADPH, CaM	(Bredt *et al.*, 1991; Nakane *et al.*, 1993)
Calcium	Dependent	(Garthwaite *et al.*, 1988; Knowles *et al.*, 1989)
Location of Activity	Cytosolic	(Schmidt *et al.*, 1991)

Inducible Nitric Oxide Synthase (iNOS)

Early studies showed that murine macrophages could be induced with cytotoxins such as LPS, IFN–γ and lymphokinin to produce nitrite (Stuehr & Marletta, 1985; Stuehr & Marletta, 1987). Further research showed that L-arginine was the substrate (Hibbs *et al.*, 1987) and N-guanido-hydroxylated arginine the intermediate in this reaction (Iyengar *et al.*, 1987). NADPH, FAD, FMN and tetrahydrobiopterin have all been shown to be either required for, or to enhance macrophage enzyme production of NO (Marletta *et al.*, 1988; Hevel *et al.*, 1991; Stuehr *et al.*, 1991a; Yui *et al.*, 1991; White & Marletta, 1992). The enzyme activity is independent of free calcium or CaM (Stuehr *et al.*, 1991a; Yui *et al.*, 1991). Cultured murine macrophages express cytosolic NOS activity and synthesise iNOS mRNA (approximately 4.4 kb in length) only after exposure to IFN–γ and LPS (Marletta *et al.*, 1988; Xie *et al.*, 1992). The induced macrophage enzyme is catalytically active as a dimer of approximately 250 kDa, but can also be dissociated into two inactive monomers, each of approximately 130 kDa (Hevel *et al.*, 1991; Stuehr *et al.*, 1991a; Baek *et al.*, 1993). Haem, tetrahydrobiopterin and L-arginine are also required for dimerization leading to full enzyme activity and the production of NO (Baek *et al.*, 1993). Both LPS and IFN-γ induced murine macrophage iNOS and human iNOS have been cloned and have predicted lengths of 1144 amino acids and 1152 amino acids, respectively, (Lowenstein *et al.*, 1992; Lyons *et al.*, 1992; Xie *et al.*, 1992; Charles *et al.*, 1993; Geller *et al.*, 1993a; Chartrain *et al.*, 1994). Analysis of the cDNA suggests binding sites for FAD, FMN, NADPH and CaM for both clones (Lowenstein *et al.*, 1992; Lyons *et al.*, 1992; Charles *et al.*, 1993; Chartrain *et al.*, 1994).

Some insights into the regulation of iNOS have been suggested following analysis of the murine iNOS clone. The transcription initiation site is preceded 30 bp upstream by a TATA box and the promoter region also contains consensus binding sequences for IFN response elements, LPS-related response elements, nuclear factor-κβ (NF-κβ) and TNF response elements (Lowenstein *et al.*, 1993; Xie, Whisnant & Nathan, 1993). These sites may explain the synergy between LPS and IFN-γ in the induction of iNOS apparent in some cell types (Section 1.2.5).

Endothelial Nitric Oxide Synthase (eNOS)

Both native and cultured BAEC, porcine kidney tubular epithelial cells, human endothelial cells and COS cells (monkey kidney cell line) transfected with eNOS cDNA express eNOS enzyme activity predominately in the particulate fraction of the cells (Pollock *et al.*, 1991; Sessa *et al.*, 1992; Busconi & Michel, 1993; Michel *et al.*, 1993; Pollock *et al.*, 1993; Hecker *et al.*, 1994; Tracey *et al.*, 1994). Examination of cultured and freshly isolated endothelial cells found no difference in the localisation of the enzyme activity (Hecker *et al.*, 1994). The 135 kDa enzyme requires L-arginine, NADPH, calcium, CaM, FAD and tetrahydrobiopterin for full activity (Förstermann *et al.*, 1991; Pollock *et al.*, 1991; Pollock *et al.*, 1993; Tracey *et al.*, 1994). Both BAEC eNOS and human eNOS have been cloned and encode proteins of predicted lengths of 1205 amino acids and 1250 amino acids respectively (Janssens *et al.*, 1992; Lamas *et al.*, 1992; Marsden *et al.*, 1992; Sessa *et al.*, 1992). Analysis of both cDNA sequences suggests binding sites for FMN, FAD and NADPH (Janssens *et al.*, 1992), with analysis of the bovine clone also suggesting a binding site for CaM (Lamas *et al.*, 1992; Nishida *et al.*, 1992; Sessa *et al.*, 1992). As well as an N-myristylation (Lamas *et al.*, 1992; Sessa *et al.*, 1992) and N-linked glycosylation sites, (Sessa *et al.*, 1992), a site for phosphorylation by cAMP-dependent protein kinase was also evident on the bovine gene (Lamas *et al.*, 1992).

Some speculations about the regulation of eNOS can be made based upon analysis of the bovine eNOS gene. There are two transcription start sites, at 170 and 240 bp upstream from the methionine translational initiation codon. In comparison to the murine iNOS gene, the bovine eNOS promoter and human eNOS genes appear to lack a TATA box (Marsden *et al.*, 1993; Venema *et al.*, 1994). Analyses of the promoter regions show sheer stress responsive elements as well as estrogen half-palindromic motifs. There are also potential Sp1 binding sites and GATA motifs, which are consistent with a constitutively expressed gene found in endothelial cells (Marsden *et al.*, 1993; Venema *et al.*, 1994).

The location of eNOS within the cell may play a role in its regulation. Wild type eNOS (myristoylated and palmitoylated) is localised at the cytoplasmic face of the golgi, whereas the mutant non-acylated variant is not (Sessa *et al.*, 1995). Golgi compartmentalisation is necessary for eNOS to respond to intracellular signals and

produce NO (Sessa *et al.*, 1995) and cells expressing the wild type eNOS release substantially more NO than those cells expressing the mutant (non-acylated) form (Sessa *et al.*, 1995).

The effect that myristolation and palmitoylation have on the intracellular location of eNOS has been examined in detail. Endothelial NOS is the only NOS isoform to contain an N-myristoylation consensus sequence. This allows recognition by the enzyme myristoly-CoA:protein N-myristoyl transferase (Liu & Sessa, 1994) and the incorporation of the saturated fatty acid myristate (Pollock *et al.*, 1992). When mutagenesis of the myristoylation consensus sequence occurs in COS cells, greater than 94 % of the activity is expressed in the cytosol (Busconi & Michel, 1993; Sessa *et al.*, 1993; Liu & Sessa, 1994), although cytosolic eNOS has also been shown to be myristolated (Liu *et al.*, 1995). Myristoylation is required for palmitoylation of eNOS (Liu *et al.*, 1995) as the myr- mutant (myristoylation site deficient) does not undergo palmitoylation (Robinson *et al.*, 1995). Palmitoylated wild type eNOS is located exclusively in the particulate fraction (Liu *et al.*, 1995; Robinson *et al.*, 1995) although there is also a cytosolic myristolated non-palmitoylated pool (Liu *et al.*, 1995). Bradykinin treatment stimulates the depalmitoylation of eNOS. Robinson *et al.*, (1995) suggested that this loss could be the mechanism of release of eNOS from the membrane and translocation into the cytoplasm. In contrast, Liu *et al.*, (1995) showed that treatment with bradykinin did not affect the rate or extent of eNOS palmitoylation, and although an increase in NO release was measured, stimulation did not significantly effect the translocation from the membrane to cytosol. Liu *et al.*, (1995) also reported that mutation of cystine-15 or cystine-26 resulted in a 95 % decrease in the incorporation of ^{3}H-palmitic acid into the eNOS compared with wild type incorporation. The mutation did not effect the membrane association of the eNOS thus suggesting that palmitoylation is not required for membrane association.

However, experiments suggest that acylation targets eNOS to the caveolae (Shaul *et al.*, 1996). Caveolae are specialised microdomains of the plasma membrane. Caveolins are a family of transmembrane proteins, which form a key structural component of these microdomains (Feron *et al.*, 1996). Palmitoylation is necessary for targeting of eNOS into the caveolin-rich domains of the plasma membrane (Garcia-Cardena *et al.*, 1996), which is mediated by interactions with cell specific

caveolin isoforms (Feron *et al.*, 1996). In bovine lung microvascular endothelial cells, mature eNOS resides in the luminal plasma and intracellular membranes (Garcia-Cardena *et al.*, 1996) and the primary location of eNOS in the plasma membrane is in the caveolae (Shaul *et al.*, 1996). Therefore myristoylation is required for initial targeting to the cell membrane and subsequent palmitoylation may stabilise the eNOS-membrane association (Robinson *et al.*, 1995).

Neuronal Nitric Oxide Synthase (nNOS)

Cerebellar cells release a diffusible messenger, which has similar Ca^{2+} dependent properties to EDRF (Garthwaite *et al.*, 1988). The soluble enzyme responsible has been isolated from rat brain and shows dependence upon free calcium with no synthesis of NO apparent below 80 nM free Ca^{2+} (Knowles *et al.*, 1989). SDS PAGE analysis of nNOS purified from rat and porcine cerebellar revealed an enzyme of between 150 and 160 kDa. The cytosolic enzyme (Schmidt *et al.*, 1991) is dependent upon L-arginine, NADPH, Ca^{2+}, tetrahydrobiopterin and CaM for the formation of NO (Bredt & Snyder, 1990; Mayer *et al.*, 1990; Schmidt *et al.*, 1991; Schmidt *et al.*, 1992b). Analysis of rat cerebella and human brain nNOS cDNA indicates a protein of 1429 and 1434 amino acids respectively (Bredt *et al.*, 1991; Hall *et al.*, 1994).

Analysis of the nNOS gene may give some insights into its regulation. The human nNOS gene consists of 28 exons and like the iNOS gene, contains a TATA box 28 bp downstream of the initiation site (Hall *et al.*, 1994). Analysis of the structure of rat and human nNOS cDNAs revealed binding sites for CaM, FAD, FMN and NADPH (Bredt *et al.*, 1991; Nakane *et al.*, 1993). There is also a binding site for cAMP-dependent protein kinase phosphorylation in rat nNOS (Bredt *et al.*, 1991). NOS purified from rat cerebella is stoichiometrically phosphorylated by protein kinase A, protein kinase C and calcium/CaM dependent protein kinase. Each enzyme predominantly phosphorylates an individual serine in the protein (Bredt *et al.*, 1992).

Neuronal NOS does not contain the N-myristoylation consensus sequence (which allows recognition by the enzyme myristoly-CoA:protein N-myristoyl transferase and the incorporation of the saturated fatty acid myristic acid), that is in eNOS, and yet it is membrane bound. However, the amino acid sequence of nNOS contains a 230

amino acid N-terminal domain, which is not present in eNOS, and as both nNOS and eNOS show similar activities, this suggests that this domain may not be required for catalytic activity (Brenman *et al.*, 1995). Thus, the N-terminal domain may play a role in membrane binding for nNOS.

Other factors may also influence the membrane binding of nNOS. For example, in skeletal muscle, the association of nNOS with the protein dystrophin results in its membrane association (Brenman *et al.*, 1995). Humans who have Duchenne muscular dystrophy and *mdx* mice, both lack dystrophin, which results in the translocation of nNOS from the sarcolemma to the cytosol (Brenman *et al.*, 1995). In contrast, in the brain, dystrophin does not appear to be required for the membrane association of nNOS (Brenman *et al.*, 1995). In skeletal muscle, the interaction of nNOS and dystrophin complex is through the direct interaction of the PDZ domain of nNOS with α1-syntrophin (dystrophin associated protein; Brenman *et al.*, 1996). In the brain, the PDZ protein motif of nNOS interacts with postsynaptic density-95 protein. However, mice who carry an nNOS isoform lacking the PDZ domain, do not exhibit either a postsynaptic density-95 protein/nNOS complex in the brain, nor an association of nNOS with the sacrolemma in skeletal muscle. This therefore suggests that the PDZ domain is important for the synaptic association of nNOS (Brenman *et al.*, 1996).

The three NOS isoforms discussed in this section (Section 1.2.2) will be examined further in subsequent sections where the tissue specificity and regulation of the individual isoforms will be discussed.

1.2.3 *Tissue Specificity of NOS Isoforms*

Even though the different isoforms are named for the tissue from which they were first isolated, it has now been established that they can be found in other tissues as well. For example both eNOS and nNOS have been clearly demonstrated in epithelial cells (Asano *et al.*, 1994; Iizuka *et al.*, 1998) and NOS activity induced by cytokines has been demonstrated in the cytosolic fraction of vascular smooth muscle cells (Busse & Mülsch, 1990).

Inducible Nitric Oxide Synthase (iNOS)

Induction of nitrite production in macrophages using cytotoxins such as LPS, IFN–γ and lymphokinin was reported as early as 1985 (Stuehr & Marletta, 1985; Stuehr & Marletta, 1987). However, it is now known that the calcium-independent, inducible form of NOS is expressed in more than just macrophages (Table 1.2). For example, rabbit thoracic aorta smooth muscle cells (Busse & Mülsch, 1990), the murine mammary epithelial cell Comma-D (Low *et al.*, 1997), and rat aortic endothelial cells (Suschek *et al.*, 1993) all express iNOS when stimulated with cytokines or cytotoxins.

Endothelial Nitric Oxide Synthase (eNOS)

Although it was in 1980 when Furchgott & Zawadzki (1980) identified EDRF and noted its dependence upon the endothelium, it was not until the early 1990s that research was carried out characterising the enzyme responsible for the production of EDRF in endothelial cells (Förstermann *et al.*, 1991; Pollock *et al.*, 1991; Pollock *et al.*, 1993; Tracey *et al.*, 1994). Research now shows that eNOS is expressed in more than just endothelial cells (Table 1.3), for example epithelial cells of the rat lung (Kawai *et al.*, 1995), bovine and rat mammary gland alveoli (Lacasse & Prosser, 1995a; Iizuka *et al.*, 1998) and in smooth muscle cells of the rat mammary gland (Iizuka *et al.*, 1998) and goat pudic artery (Lacasse & Prosser, 1995a).

Neuronal Nitric Oxide Synthase (nNOS)

The first NOS to be isolated and characterised was that from porcine and rat brain (Bredt & Snyder, 1990; Mayer *et al.*, 1990; Schmidt *et al.*, 1991). Now, nNOS has been clearly demonstrated in several tissues other than neuronal tissue (Table 1.4), for example, in human bronchial epithelial cells (Asano *et al.*, 1994), mammary gland alveoli epithelium (Onoda & Inano, 1998) and skeletal muscles of rats, mice and guinea pigs (Weiner *et al.*, 1994; Brenman *et al.*, 1995; Silvagno *et al.*, 1996). Alternative splicing of the nNOS gene also occurs in rat skeletal muscle, where it results in the production of nNOS-μ, which is the result of a 102 bp insert. Neuronal NOS-μ, which migrates at 164 kDa under SDS-PAGE conditions (c.f. nNOS which is 160 kDa) is only expressed in skeletal and heart muscles (Silvagno *et al.*, 1996).

Table 1.2: Location of inducible/calcium independent nitric oxide synthase in selected tissues and cells. The tissue or cell type along with species and the method of stimulation and detection are given.

Tissue or Cell	Species	Stimulus	Detection	Reference
A549 (alveolar type 2 epithelium-like cells)	Human	IFN-γ+IL-1β+TNF-α+LPS	iNOS RT-PCR/iNOS mRNA	(Asano *et al.*, 1994)
Aortic endothelial cells	Rat	IL-1β+IFN-γ	iNOS mRNA/media nitrite	(Suschek *et al.*, 1993)
BEAS 2B (transformed bronchial epithelial cells)	Human	IFN-γ+IL-1β+TNF-α+LPS	iNOS RT-PCR/iNOS mRNA	(Asano *et al.*, 1994)
Bone marrow derived macrophages	Bovine	*S. dublin*	Media nitrite/iNOS mRNA	(Adler *et al.*, 1995)
Bronchial epithelial cells	Human	IFN-γ+IL-1β+TNF-α+LPS	iNOS RT-PCR/iNOS mRNA	(Asano *et al.*, 1994)
Comma-D (mammary epithelial cell)	Murine	IFN-γ+TNF-α	Media nitrite/iNOS mRNA	(Low *et al.*, 1997)
Internal mammary artery (endothelium denuded)	Human	LPS	Attenuation of phenylephrine induced contractile responses	(Thorin-Trescases *et al.*, 1995)
J774 macrophage cell line	Murine	LPS	Media nitrite	(Bogle *et al.*, 1992)
Liver	Rat	*K. pneumoniae*	Activity/SDS Page	(Evans *et al.*, 1992)
Liver	Rat	*P. acnes* +LPS	Activity/SDS Page	(Iida *et al.*, 1992)
Liver	Rat	LPS	Haemoglobin assay/NOS activity	(Knowles *et al.*, 1990)
Lung	Rat	LPS	Haemoglobin assay/NOS activity	(Knowles *et al.*, 1990)
Mammary gland - endothelial cells of blood vessels	Rat	LPS	Immunohistochemistry iNOS Ab	(Onoda & Inano, 1998)
Mammary gland - myoepithelium of alveoli	Rat	LPS	Immunohistochemistry iNOS Ab	(Onoda & Inano, 1998)
Mammary gland - myoepithelium of lactiferous ducts	Rat	LPS	Immunohistochemistry iNOS Ab	(Onoda & Inano, 1998)
Mammary gland (cultured)	Rat	LPS	Western iNOS	(Onoda & Inano, 1998)
Monocyte derived macrophages	Murine	*S. dublin* ± IFN-γ	Media nitrite	(Adler *et al.*, 1995)
Peritoneal macrophage	Murine	IFN-γ	Western iNOS Ab	(Vodovotz *et al.*, 1993)
Primary hepatocytes	Rat	12-*O*-tetradecanoylphorbol 13-acetate	iNOS mRNA	(Menegazzi *et al.*, 1996)
Somatic cells	Bovine	*S. uberis*	Milk nitrite	(Lacasse *et al.*, 1997)
Somatic cells (cultured)	Bovine	LPS	Media nitrate+nitrite	(Lacasse *et al.*, 1997)
Thoracic aorta (endothelium denuded)	Rabbit	IFN-γ+TNF-α	Attenuation of noradrenalin induced contractions/increase in cGMP	(Busse & Mülsch, 1990)
Thoracic aorta smooth muscle cells	Rabbit	IL-1β	Increase in cGMP in cultured cells	(Busse & Mülsch, 1990)

Table 1.3: Location of endothelial nitric oxide synthase in selected tissues and cells. The tissue or cell type along with species and the method of detection are given.

Tissue or Cell	Species	Detection	Reference
Aorta	Rat	Western eNOS Ab	(Xu *et al.*, 1996)
Aortic endothelial cell	Bovine	eNOS mRNA	(Sessa *et al.*, 1995; Feron *et al.*, 1996;
		Immunofluroescence eNOS Ab Western eNOS Ab	Garcia-Cardena *et al.*, 1996; Harrison *et al.*, 1996; Goetz *et al.*, 1999)
Aortic endothelial cell	Human	Western eNOS Ab	(Hishikawa *et al.*, 1995)
Lung	Rat	eNOS mRNA	(Kawai *et al.*, 1995)
Lung alveolar and serosal epithelial cells	Rat	Insitu eNOS RNA probe	(Kawai *et al.*, 1995)
Lung endothelial cells of blood vessels	Rat	Insitu eNOS RNA probe	(Kawai *et al.*, 1995)
Lung microvascular endothelial cells	Bovine	Western eNOS Ab	(Garcia-Cardena *et al.*, 1996)
Mammary gland - cultured	Rat	Western eNOS Ab	(Onoda & Inano, 1998)
Mammary gland - endothelial cells of blood vessels	Rat	Immunohistochemistry eNOS Ab	(Onoda & Inano, 1998)
Mammary gland - myoepithelium of alveoli	Rat	Immunohistochemistry eNOS Ab	(Onoda & Inano, 1998)
Mammary gland - myoepithelium of lactiferous ducts	Rat	Immunohistochemistry eNOS Ab	(Onoda & Inano, 1998)
Mammary gland-alveoli & ducts (epithelium)	Bovine	Immunohistochemistry eNOS Ab	(Lacasse & Prosser, 1995)
Mammary gland-alveoli (epithelium)	Rat	Western eNOS Ab	(Iizuka *et al.*, 1998)
Mammary gland-endothelium of blood vessel	Rat	Western eNOS Ab	(Iizuka *et al.*, 1998)
Mammary gland-endothelium of lactiferous ducts	Rat	Western eNOS Ab	(Iizuka *et al.*, 1998)
Mammary gland-epidermis	Rat	Western eNOS Ab	(Iizuka *et al.*, 1998)
Mammary gland-sebaceous glands	Rat	Western eNOS Ab	(Iizuka *et al.*, 1998)
Mammary gland-smooth muscle	Rat	Western eNOS Ab	(Iizuka *et al.*, 1998)
Mesenteric artery	Rat	Western eNOS Ab	(Xu *et al.*, 1996)
Pudic artery - smooth muscle	Caprine	Immunohistochemistry eNOS Ab	(Lacasse & Prosser, 1995)
Skeletal muscle	Guinea Pig	eNOS mRNA	(Weiner *et al.*, 1994)
Skeletal muscle	Murine	Western eNOS Ab	(Brenman *et al.*, 1995)
Umbilical vein endothelial cell	Human	Immunofluroescence eNOS Ab	(Sessa *et al.*, 1995)
Ventricular myocytes	Rat	Western eNOS Ab	(Feron *et al.*, 1996)

Table 1.4: Location of neuronal nitric oxide synthase in selected tissues and cells. The tissue or cell type along with species and the method of detection are given.

Tissue or Cell	Species	Detection	Reference
A549 (alveolar type 2 epithelium-like cells)	Human	nNOS RT-PCR	(Asano *et al.*, 1994)
BEAS 2B (transformed bronchial epithelial cells)	Human	nNOS RT-PCR	(Asano *et al.*, 1994)
Bronchial epithelial cells	Human	nNOS RT-PCR	(Asano *et al.*, 1994)
Heart	Rat	Western nNOS-m Ab	(Silvagno *et al.*, 1996)
HIT-TI5 (glucose responsive clonal pancreatic B-cell)	Hamster	Western nNOS Ab	(Schmidt *et al.*, 1992)
Hypothalamus	Rat	Western nNOS Ab	(Xu *et al.*, 1996)
Mammary gland - epithelium of alveoli	Rat	Immunohistochemistry nNOS Ab	(Onoda & Inano, 1998)
Mammary gland - epithelium of lactiferous ducts	Rat	Immunohistochemistry nNOS Ab	(Onoda & Inano, 1998)
Mammary gland-alveoli (epithelium)	Rat	Western nNOS Ab	(Iizuka *et al.*, 1998)
Mammary gland-sebaceous glands	Rat	Western nNOS Ab	(Iizuka *et al.*, 1998)
Mammary gland-smooth muscle	Rat	Western nNOS Ab	(Iizuka *et al.*, 1998)
Pancreas - Islets of Langerhans	Rat	Immunohistochemistry nNOS Ab	(Schmidt *et al.*, 1992)
Paraventricular nucleus - hypothalamus	Rat	Insitu nNOS RNA probe Immunocytochemistry nNOS Ab	(Luckman *et al.*, 1997)
Skeletal muscle	Guinea Pig	nNOS mRNA	(Weiner *et al.*, 1994)
Skeletal muscle	Murine	Western nNOS Ab	(Brenman *et al.*, 1995)
Skeletal muscle	Rat	Western nNOS-m Ab	(Silvagno *et al.*, 1996)
Supraoptic nucleus - hypothalamus	Rat	Insitu nNOS RNA probe Immunocytochemistry nNOS Ab	(Luckman *et al.*, 1997)

1.2.4 *Regulation of Endothelial Nitric Oxide Synthase (eNOS)*

Although described as one of the constitutively expressed NOS isoforms (nNOS is the other), the expression of eNOS has now been shown to be controlled in some part by factors such as sex steroids, sheer stress (the mechanical force associated with blood flow) and cytokines. Table 1.5 shows the effects of reproductive status and the hormone estradiol on NO production in various tissue types. This table is by no means exhaustive.

Hormonal

Increased concentrations of eNOS are apparent in the aorta and mesenteric artery of pregnant rats (Xu *et al.*, 1996), and there is increased NOS activity in the uterine artery, heart, kidney, skeletal muscle and oesophagus in pregnant guinea pigs. Increases in eNOS mRNA is also apparent in the skeletal muscle (Weiner *et al.*, 1994). There is little formation of NO in uterine tissues from non-pregnant rats and in tissues collected during delivery and immediately postpartum, but there is a four-fold increase in the formation of NO in tissues collected during pregnancy (Yallampalli *et al.*, 1994). NOS activity stays constant through the oestrous cycle in the fallopian tube of the rat until the late night proestrous stage where there is a 10-fold fall in activity (Bryant *et al.*, 1995). This stage is characterised by low estradiol and high progesterone concentrations in the circulation (Smith *et al.*, 1975).

Studies to examine the effects of specific sex steroids on NOS have found that treatment with estradiol increases its activity in the heart, kidney and skeletal muscle of the guinea pig (Weiner *et al.*, 1994) and in ovine fetal pulmonary artery endothelium (Lantin-Hermoso *et al.*, 1997). There are also increases in eNOS mRNA concentration in skeletal muscle following treatment with estradiol (Weiner *et al.*, 1994). Endothelial NOS protein levels in human aortic endothelial cells were also increased by estradiol treatment (Hishikawa *et al.*, 1995). Treatment of human umbilical vein endothelial cells (HUVEC) with 17β-estradiol increases the release of nitrate and nitrite (NOx) with maximal release of NOx apparent following one hour of pre-treatment of the cells with estradiol. Levels of NOx released from cells treated for one hour and 24 hours with estradiol were similar (Caulin-Glaser *et al.*, 1997).

Human AEC pre-treated with estradiol for at least 8 hours showed a significant increase in ionomycin-stimulated production of NO (Hishikawa *et al.*, 1995). Estrogen treatment, which increases acetylcholine stimulated coronary blood flow in postmenopausal women, is inhibited when L-NMMA was infused concomitantly (Guetta *et al.*, 1997). In comparison, prepubertal rats treated with estradiol were found to produce lower levels of nitrites when compared to the non-treated controls (Yallampalli *et al.*, 1994). Treatment of BAEC with estradiol induces a redistribution of eNOS from the membrane to an intracellular site near the nucleus. The estradiol treatment also induced a rapid rise in the levels of intra-cellular calcium (Goetz *et al.*, 1999) However, Caulin-Glaser *et al.*, (1997) found that HUVEC treated with estrogen did not result in the mobilisation of cytosolic calcium. Interestingly, the study also showed that cells stimulated with estrogen did not require calcium and CaM for activity (Caulin-Glaser *et al.*, 1997).

Neither testosterone nor progesterone appears to have an effect on NO production suggesting that the effects of estrogen are hormone specific (Yallampalli *et al.*, 1994; Hishikawa *et al.*, 1995; Goetz *et al.*, 1999). The effect also appears to be receptor mediated as the use of an estrogen receptor antagonist inhibits the estrogen stimulated release of NO (Caulin-Glaser *et al.*, 1997; Lantin-Hermoso *et al.*, 1997; Goetz *et al.*, 1999). The response is not transcriptionally regulated as the release of NOx from HUVEC is the same in 5,6-dichloro-1β-D-ribofuranosylbenzimidazole (an RNA polymerase 2 inhibitor) treated and untreated estrogen stimulated cells (Caulin-Glaser *et al.*, 1997).

Growth factors have also been shown to stimulate NOS. Treatment of bovine endothelial cells with basic fibroblast growth factor (bFGF) results in increases in eNOS at both the mRNA and protein levels (Kostyk *et al.*, 1995).

Table 1.5: Hormonal control of endothelial nitric oxide synthase. The effect of reproductive status and estradiol on the NO production by selected tissues is given. Nitrite indicates the tissue was cultured, protein refers to eNOS protein and mRNA refers to eNOS mRNA.

Hormone/State	Tissue/Cell	Species	Result	Reference
Estradiol	Aortic endothelial cell	Human	Increased protein	(Hishikawa *et al.*, 1995)
Estradiol	Heart	Guinea pig	Increased activity	(Weiner *et al.*, 1994)
Estradiol	Kidney	Guinea pig	Increased activity	(Weiner *et al.*, 1994)
Estradiol	Skeletal muscle	Guinea pig	Increased activity	(Weiner *et al.*, 1994)
Estradiol	Skeletal muscle	Guinea pig	Increased mRNA	(Weiner *et al.*, 1994)
Non-pregnant	Uterine tissue	Rat	Decreased nitrite	(Yallampalli *et al.*, 1994)
Parturition	Uterine tissue	Rat	Decreased nitrite	(Yallampalli *et al.*, 1994)
Postpartum	Uterine tissue	Rat	Decreased nitrite	(Yallampalli *et al.*, 1994)
Pregnancy	Aorta	Rat	Increased protein	(Xu *et al.*, 1996)
Pregnancy	Heart	Guinea pig	Increased activity	(Weiner *et al.*, 1994)
Pregnancy	Kidney	Guinea pig	Increased activity	(Weiner *et al.*, 1994)
Pregnancy	Mesenteric artery	Rat	Increased protein	(Xu *et al.*, 1996)
Pregnancy	Oesophagus	Guinea pig	Increased activity	(Weiner *et al.*, 1994)
Pregnancy	Skeletal muscle	Guinea pig	Increased activity	(Weiner *et al.*, 1994)
Pregnancy	Skeletal muscle	Guinea pig	Increased mRNA	(Weiner *et al.*, 1994)
Pregnancy	Uterine artery	Guinea pig	Increased activity	(Weiner *et al.*, 1994)
Pregnancy	Uterine tissue	Rat	Increased nitrite	(Yallampalli *et al.*, 1994)
Proestrous	Fallopian tube	Rat	Increased activity	(Bryant *et al.*, 1995)

Cytokines

Treatment of HUVEC or BAEC with either TNF–α or interleukin-1β (IL-1β) decreases the levels of eNOS mRNA which falls to 50 % of the control level by 4 hours post-treatment and to a barely detectable level after 24 hours (Yoshizumi *et al.*, 1993; Alonso *et al.*, 1997). The addition of TNF-α, IL-1β or IFN–γ alone to HUVEC had no effect on NOS activity, but it increased when IFN–γ was added together with either TNF–α or IL-1β. When all three cytokines were added together there was an additional increase in activity even though the steady state levels of eNOS mRNA were decreased (Rosenkranz-Weiss *et al.*, 1994). The inhibitory effect of TNF–α is blocked by treatment with cyclohexamide (CHX) suggesting that the effect is dependent upon the synthesis of new protein. The transcription rate of the eNOS gene is unaffected by TNF–α treatment suggesting that the decrease in mRNA levels is not mediated by decreases in gene transcription rate. Treatment with TNF–α appears to decrease mRNA levels by destabilisation of the mRNA as the half life of untreated eNOS mRNA is about 48 hours, whereas in the presence of TNF–α this drops to 3 hours (Yoshizumi *et al.*, 1993). A *cis* element has been described in the 3' UTR of the eNOS gene, which binds to cytosolic proteins in a time-dependent manner when stimulated with TNF–α. This binding between the cytosolic proteins and the eNOS mRNA may result in the destabilisation of the mRNA shown in TNF–α treated cells (Alonso *et al.*, 1997).

Bacterial stimulation also effects the production of eNOS. Treatment of rats with LPS or lipoteichoic acid resulted in the induction of eNOS mRNA in the liver with significantly higher levels 12 hours after treatment than pre-treatment levels. By 24 hours post-treatment, the levels had returned to near basal levels (Bucher *et al.*, 1997).

Others

Other physiological factors have also been implicated in the regulation of eNOS. Exposure of BAEC to sheer stress leads to the increased induction of eNOS mRNA and protein which can be inhibited by actinomycin D (inhibitor of mRNA synthesis). Cells exposed to sheer stress are found to be more responsive to the calcium

ionophore A-23187, with even greater amounts of nitrite produced (Uematsu *et al.*, 1995). Chronic exercise in dogs can increase the nitrite levels in both large coronary arteries and the microvessels and increases in eNOS gene expression are also apparent in AEC cultured from exercised dogs (Sessa *et al.*, 1994).

BAEC were found to exhibit higher basal levels of nitrate and nitrite accumulation, NOS immunoreactive protein (135 kDa) and NOS mRNA during proliferation, than following the attainment of confluence. Those stimulated with calcium ionophore A-23287 were also found to produce more nitrate and nitrite while growing than after reaching confluence (Arnal *et al.*, 1994). The release of NO did not have any effect on cell growth. However, in comparison Garg & Hassid (1989) found that vasodilator drugs that release NO inhibited mitogenesis and proliferation in vascular smooth muscle cells (VSMC) and this occurred independent of cell damage.

Hypoxia in HUVEC and bovine pulmonary artery endothelial cells results in decreases in eNOS mRNA levels following 48 hours of treatment. Hypoxia decreased eNOS mRNA levels by both decreasing the transcription rate of the eNOS gene, and the stability of the mRNA (McQuillan *et al.*, 1994).

1.2.5 *Regulation of Inducible Nitric Oxide Synthase (iNOS)*

Although cytokines are the main stimulants of iNOS expression, research now shows that other physiological factors can influence the induction of the gene.

Hormonal

As described earlier for eNOS, sex steroids can also influence the expression of iNOS. There is a decrease in calcium independent NOS activity during the late night proestrous in the rat (Bryant *et al.*, 1995) when blood estrogen concentrations are low and progesterone concentrations are high (Smith *et al.*, 1975). Increased amounts of iNOS mRNA are apparent in the uterus of pregnant rats when compared to non-pregnant controls in diestrus. There is a rapid decrease in iNOS mRNA levels during labour and the first day postpartum (Dong *et al.*, 1998).

In comparison to eNOS where no effect of progesterone is apparent on NO production (Yallampalli *et al.*, 1994; Hishikawa *et al.*, 1995; Goetz *et al.*, 1999), treatment of pregnant rats on day 20 of gestation with progesterone inhibited the iNOS mRNA levels decreasing during spontaneous labour on day 22 of gestation (Dong *et al.*, 1998). Treatment with prostaglandin-$F_{2\alpha}$ ($PGF_{2\alpha}$) significantly decreased the expression of iNOS in the uterus of pregnant rats and resulted in premature labour. Co-treatment with both $PGF_{2\alpha}$ and progesterone inhibited pre-term labour and the decrease in iNOS that occurred with $PGF_{2\alpha}$ alone. Treatment with RU-486 (progesterone antagonist) induced preterm labour and decreased the iNOS mRNA content of the uterus in a time-dependent manner. However, the estrogen receptor antagonist ICI 164384 increased iNOS mRNA levels in the uterus, which reached maximum levels 48 hours after treatment (Dong *et al.*, 1998). This is in direct contrast to hormonal control of eNOS where the use of estrogen receptor antagonists decreases the increase in eNOS in response to estrogen (Caulin-Glaser *et al.*, 1997; Lantin-Hermoso *et al.*, 1997; Goetz *et al.*, 1999). Serum progesterone levels in pregnant rats treated with ICI 164384 increased indicating a possible role for estradiol in the regulation of serum progesterone levels (Dong *et al.*, 1998). Together these results suggest that the uterine production of NO may play a role in the maintenance of uterine quiescence during pregnancy. However, these results are likely to be species specific, as both Barber *et al.*, (1999) and Bartlett *et al.*, (1999) provide evidence to suggest that NO is not involved in modulation of myometrial tone in women during pregnancy. NO does not appear to be involved in the regulation of uterine contractility in ewes either (Mirabile *et al.*, 2000).

In pathological conditions resulting in excess NO generation, estrogens may be beneficial in moderating the effects. For example, LPS treatment of ovariectomised rats results in increased levels of plasma nitrite, however, pre-treatment with estradiol significantly decreases the plasma nitrite levels of LPS treated rats (Kauser *et al.*, 1997).

Growth factors may also play a role in the regulation of NO production. EGF enhances the production of nitrite by Comma-D cells following incubation with TNF–α and IFN–γ or IFN–γ alone, however, it was without effect in the presence of

TNF–α alone (Low *et al.*, 1997). IGF-1 treatment of rat thoracic aorta smooth muscle cells resulted in decreased concentrations of nitrite in the media, decreased activity of NOS and decreased iNOS mRNA levels. Insulin and IGF-2 decreased the production of nitrite from IL-1β stimulated cells (Schini *et al.*, 1994).

Glucocorticoids have also been shown to inhibit the production of NO and the induction of iNOS. Dexamethasone inhibits cytokine stimulated induction of iNOS in rat hepatocytes and decreases the 8-bromo-cAMP stimulated production of nitrate and nitrite in rat VSMC (Geller *et al.*, 1993b; Imai *et al.*, 1994).

Cytokine

Cytokines stimulate increases in NO in many different cell types and the effects appear to be very cell and/or cytokine specific (Table 1.6). For example, treatment with IFN–γ increases the production of nitrite from murine bone marrow derived monocytes (BMM), but in contrast it has no effect on either bovine BMM or blood monocyte derived macrophages (Adler *et al.*, 1995). Increases in NO production are also apparent in bovine BMM treated with *S. dublin* (Adler *et al.*, 1995) and in IL-1β stimulation of rat aortic endothelial cells (Suschek *et al.*, 1993). Treatment of rat thoracic aorta smooth muscle cells with either IL-1β, or TNF–α, increased levels of nitrite in the media (Schini *et al.*, 1994).

Different effects are also seen when different concentrations and combinations of cytokines are used. In many cases the effects of the cytokines produce synergistic responses when added in conjunction with other cytokines or cytotoxins. Stimulation of A549 cells (alveolar type II epithelium-like) with a combination of IFN-γ, IL-1β, TNF-α and LPS results in an increase in NOS activity which is maximal 8 hours following treatment. All four were required for maximal induction of iNOS (Asano *et al.*, 1994). Rat hepatocytes also respond synergistically to IFN-γ, IL-1β, TNF-α and LPS (Geller *et al.*, 1993b). The increased expression of iNOS mRNA in murine macrophages following treatment with LPS and IFN–γ in comparison to LPS alone is the result of an increased rate of transcription of the iNOS gene and not as a result of increased stability of the mRNA (Lorsbach *et al.*, 1993). The combination of TNF-α

and IFN–γ resulted in the inhibition of noradrenaline induced contractions of deendothelialised rabbit aorta (Busse & Mülsch, 1990). TNF–α and IFN–γ also increase the concentration of nitrite in the medium of Comma-D cells. The combination of TNF–α and IFN–γ together doubled the production of nitrite compared to that produced by cells when treated with IFN–γ alone, while TNF–α alone did not influenced the production of nitrite (Low *et al.*, 1997). Neither TNF–α nor IFN–γ when added alone to primary rat AEC had an effect on NO production, however, co-stimulation of the cells with IFN–γ and IL-1β results in higher levels of both nitrite in the medium and iNOS mRNA (Suschek *et al.*, 1993). In contrast, the co-stimulation of primary rat AEC with TNF–α and IL-1β led to decreased production levels if low concentrations of TNF–α were used, however, if high concentrations of TNF–α were used there was an increase in the production of nitrite (Suschek *et al.*, 1993). Stimulation of human vascular endothelial cells with TNF-α leads to the induction of NF-κB. NO was shown to inhibit the activation of NF-κB by both inducing and stabilising the NF-κB inhibitor IκBα (Peng *et al.*, 1995).

In some cases cytokines have been found to inhibit the production of NO. For example, pre-treatment of murine BMM with TGF–β and bovine BMM with recombinant human IL-4 prior to treatment with *S. dublin* results in a significant decrease in iNOS mRNA and media nitrite (Adler *et al.*, 1995). LPS has also been shown to have a suppressive effect on IFN–γ stimulated activity in murine macrophages. Pre-treatment with LPS levels between 50 and 200 pg/ml LPS was required for at least 4-6 hours for a suppressive effect to become apparent. However, addition of LPS and IFN–γ together resulted in the increase of nitrite levels in an LPS concentration dependent manner. NOS enzyme activity, iNOS immunoreactive protein and iNOS mRNA were all decreased in the LPS pre-treated cells (Bogdan *et al.*, 1993). Experiments to examine the timing of the suppression revealed that pre-treatment with LPS resulted in the enhancement of production of iNOS following 5 hours treatment with IFN–γ, but following 18 hours treatment with IFN–γ there was a down regulation. Thus, pre-treatment with LPS does not appear to interfere with the initial synthesis of mRNA, but it is likely that it decreases the levels during the following stimulatory period (Bogdan *et al.*, 1993). IFN–γ stimulated activity and

iNOS protein levels in murine peritoneal macrophages were suppressed by TGF-β. The level of transcription was unaffected by incubation with TGF–β, however, it decreased mRNA translation. As the use of actinomycin D led to iNOS mRNA levels being decreased more in TGF–β treated cells than in untreated cells, this suggests that TGF–β reduces the stability of mRNA (Vodovotz *et al.*, 1993).

The induction of iNOS mRNA is rapid and transient. Stimulation results in maximal iNOS mRNA transcripts being present between 6-8 hours following treatment (Asano *et al.*, 1994; Adler *et al.*, 1995; Menegazzi *et al.*, 1996; Low *et al.*, 1997), however, maximal amounts of nitrite do not appear in the culture media until 24-48 hours following treatment (Adler *et al.*, 1995). Both LPS and lipoteichoic acid induce iNOS mRNA in the liver and kidney of the rat, with levels returning to near undetectable 24 hours after treatment (Bucher *et al.*, 1997). The rapid induction of iNOS mRNA in primary neonatal rat hepatocytes apparent following treatment with 12-*O*-Tetradecanoylphorbol 13-acetate (TPA) peaks at 2-3 hours, then falls, returning to basal levels 24 hours after treatment. The addition of TPA not only causes an induction in the expression of the iNOS gene, but also results in the decreased half life of the transcript (7.2 hours for control versus 1.9 hours for hepatocytes pre-treated for 3 hours with TPA; Menegazzi *et al.*, 1996).

The production of NO following induction of iNOS by cytokines and cytotoxins requires new protein synthesis as cyclohexamide (CHX) has been shown to inhibit the induction of iNOS (Busse & Mülsch, 1990; Geller *et al.*, 1993b; Lorsbach *et al.*, 1993; Low *et al.*, 1997). When added with TPA to hepatocytes, CHX enhanced the steady state levels of the iNOS transcript. Both TPA and CHX also induce the nuclear translocation of NF-κB (Menegazzi *et al.*, 1996). Studies with actinomycin D have shown that the production of NO following cytokine stimulation actually requires new synthesis of mRNA. The addition of CHX induces the expression of the iNOS gene in both primary neonatal hepatocytes and VSMC in the rat (Imai *et al.*, 1994; Menegazzi *et al.*, 1996). Actinomycin D inhibits iNOS mRNA induction in rat hepatocytes (Geller *et al.*, 1993b) and when added to CHX and 8-bromo-cAMP treated VSMC, there is a decrease in the CHX induced iNOS mRNA (Imai *et al.*, 1994). As there is no decay of the CHX stimulated iNOS mRNA (cAMP alone

treated VSMC mRNA has a half life of approximately 2 hours) this indicates that the superinduction of iNOS by CHX is actually due to an increase in the mRNA stability (Imai *et al.*, 1994).

Regulatory sequences in the iNOS gene appear to be required for induction. Maximum expression of iNOS requires the presence of two discrete regions up stream of the TATA box, as shown by deletion mutants. Region 1 (-48 to -209 bp) contains LPS-related response elements. Region 2 (-913 to -1029 bp) alone does not increase the expression, but in conjunction with Region 1 causes an additional induction. Region 2 was shown to contain binding motifs for IFN-related transcription factors (Lowenstein *et al.*, 1993). The transfection of reporter constructs containing truncated promoter regions of the iNOS gene revealed that the NF-κB site is required for LPS induction. The blocking of NF-κB activation with a specific inhibitor prevented LPS inducing the nuclear binding activity of NF-κB binding proteins as well as the macrophages' ability to produce nitrite (Xie *et al.*, 1994). Following treatment with IFN–γ or LPS, no production of nitrite or increases in the levels of iNOS mRNA were apparent in the macrophages of IRF-1 -/- mice (contain a targeted disruption of IFN regulatory factor-1 binding site) although the macrophages from the wildtype mice exhibited a dose dependent induction of nitrite production and increases in iNOS mRNA in response to IFN–γ (Kamijo *et al.*, 1994).

Others

In vivo exposure of rats to ozone, followed by the *in vitro* addition of LPS, IFN–γ or LPS/IFN–γ to recovered macrophages led to increased amounts of iNOS produced when compared to rats that were not exposed to the ozone (Pendino *et al.*, 1993).

Table 1.6: Cytokine and cytotoxin effects on inducible nitric oxide synthase production of NO. Comparison between the effects of various cytokines and cytotoxins on the induction of iNOS and production of NO in various tissue and cell types.

Stimulation	Result	Cell/Tissue Type	Species	Reference
EGF + TNF-α	No effect	Comma-D	Murine	(Low *et al.*, 1997)
EGF + TNF-α + IFN-γ	Increased nitrite	Comma-D	Murine	(Low *et al.*, 1997)
Pre LPS then IFN-γ	Decreased mRNA/protein/activity	Macrophages	Murine	(Bogdan *et al.*, 1993)
IFN-γ	No effect	Aortic endothelial cells	Rat	(Suschek *et al.*, 1993)
IFN-γ	No effect	Blood monocyte derived macrophages	Bovine	(Adler *et al.*, 1995)
IFN-γ	No effect	Bone marrow derived monocytes	Bovine	(Adler *et al.*, 1995)
IFN-γ	Increased nitrite	Bone marrow derived monocytes	Murine	(Adler *et al.*, 1995)
IFN-γ	Increased nitrite	Comma-D	Murine	(Low *et al.*, 1997)
IFN-γ	No effect	Polymorphonuclear leukocyte	Human	(Yan *et al.*, 1994)
IFN-γ + LPS	Increased nitrite	Macrophages	Murine	(Bogdan *et al.*, 1993)
IFN-γ + IL-1β	Increased nitrite & mRNA	Aortic endothelial cells	Rat	(Suschek *et al.*, 1993)
IFN-γ + IL-1β + TNF-α + LPS	Increased NOS activity	A549 (alveolar type 2 epithelium-like cells)	Human	(Asano *et al.*, 1994)
IFN-γ + IL-1β + TNF-α + LPS	Increased NOS activity	BEAS 2B (transformed bronchial epithelial cells)	Human	(Asano *et al.*, 1994)
IFN-γ + IL-1β + TNF-α + LPS	Increased mRNA	Hepatocytes	Rat	(Geller *et al.*, 1993)
IFN-γ + LPS	Increased mRNA/protein	C3-L5 mammary-adenocarcinoma	Murine	(Orucevic *et al.*, 1999)
IFN-γ + LPS	Increased nitrite	EMT-6 breast cancer cells	Murine	(Cendan *et al.*, 1996)
IFN-γ + LPS	Increased mRNA	Macrophages	Murine	(Lorsbach *et al.*, 1993)
IFN-γ + TGF-β	Decreased protein	Macrophages	Murine	(Vodovotz *et al.*, 1993)
IL-1β	No effect	Comma-D	Murine	(Low *et al.*, 1997)
IL-1β	No effect	Polymorphonuclear leukocyte	Human	(Yan *et al.*, 1994)
IL-1β	Increased nitrite	Aortic endothelial cells	Rat	(Suschek *et al.*, 1993)
IL-1β	Increased nitrite	Vascular smooth muscle cells	Rat	(Schini *et al.*, 1994)
LPS	Increased mRNA	Kidney	Rat	(Bucher *et al.*, 1997)
LPS	Increased protein	Mammary tissue explants	Rat	(Onoda & Inano, 1998)
LPS	No effect	Polymorphonuclear leukocyte	Human	(Yan *et al.*, 1994)
Pre IL-4 then *S. dublin.*	Decreased nitrite and mRNA	Bone marrow derived monocytes	Bovine	(Adler *et al.*, 1995)
PreTGF-β then *S. dublin.*	Decreased nitrite and mRNA	Bone marrow derived monocytes	Murine	(Adler *et al.*, 1995)
TNF-α	No effect	Aortic endothelial cells	Rat	(Suschek *et al.*, 1993)
TNF-α	No effect	Comma-D	Murine	(Low *et al.*, 1997)
TNF-α + IFN-γ	Increased nitrite	Comma-D	Murine	(Low *et al.*, 1997)
TNF-α + IL-1β	Increased nitrite - if TNF-α conc high	Aortic endothelial cells	Rat	(Suschek *et al.*, 1993)
TNF-α + IL-1β	Decreased nitrite - if TNF-α conc low	Aortic endothelial cells	Rat	(Suschek *et al.*, 1993)

1.2.6 *Regulation of Neuronal Nitric Oxide Synthase (nNOS)*

Hormonal

Treatment of pregnant guinea pigs with tamoxifen (estrogen receptor antagonist) decreased cerebral levels of calcium-dependent NOS activity compared to the activity of non-pregnant animals. Although progesterone had no effect on NOS activity, treatment with either estrogen or testosterone increased activity in the cerebellum. Both pregnancy and treatment with estradiol were associated with increases in nNOS mRNA in skeletal muscle (Weiner *et al.*, 1994). The levels of both nNOS protein and mRNA in the hypothalamus of pregnant rats were also higher (Xu *et al.*, 1996). Using specific antibodies, Dong *et al.* (1996) showed that in the rat uterus, nNOS was only present in prepubertal and non-pregnant animals. This contrasts with the apparent pregnancy-related up-regulation of both eNOS and iNOS in the uterus (Yallampalli *et al.*, 1994; Dong *et al.*, 1998), and pregnancy associated increases in nNOS in other tissues (as described above).

Cytokine

The treatment of cultured rat tissues with IFN–γ decreased nNOS mRNA concentrations, but slightly increased the concentrations of nNOS protein. In comparison, treatment with vasoactive intestinal peptide increased both nNOS mRNA and protein levels. The most substantial increases (with either vasoactive intestinal peptide or IFN-γ) were in the brain (Bandyopadhyay *et al.*, 1997).

Other

Both spinal injury and lactation have been shown to result in the up-regulation of nNOS in the rat. Following transection of the right sciatic nerve in rats, a dramatic up-regulation of nNOS mRNA was found in the L4 and L5 ganglia with levels remaining high for up to two months (Verge *et al.*, 1992). Using an mRNA probe for amino acids 151-164 of NOS, Ceccatelli & Eriksson (1993) showed that the levels of NOS mRNA increase significantly in the paraventricular nucleus of the hypothalamus of lactating rats when compared to non-lactating controls.

1.3 Nitric Oxide and the Mammary Gland

From the proceeding Sections it is clear that much is known of the biology and regulation of NO and NOS in other tissues. The role NO plays within the mammary gland has been investigated by several research groups, although comparatively very little is known in this area. NOS has been localised to various structures and tissue types within the mammary gland and cultured cells and explants have been shown to be capable of the production of NO following treatment with various common NOS stimulators. This section of the introduction will cover the current level of understanding of what controls NO production and the roles it may play within the normal mammary gland.

1.3.1 *Effect of Nitric Oxide on Mammary Blood Flow*

The supply of substrates to the mammary gland for the synthesis of milk components is largely determined by the rate of mammary blood flow (MBF) and the blood concentration of substrates (Davis & Collier, 1985). The regulation of MBF therefore appears to be important for the production of milk. Indeed there have been many reports of the correlation between MBF and milk yield in goats and cows (Linzell, 1974). However, there are a number of other factors, which can influence the MBF, for example adrenaline, serotonin, acetylcholine, histamine, IGF-1 and prostacyclin. For reviews on these and other factors and their effects of MBF see (Linzell, 1974; Prosser *et al.*, 1996). NO plays an important role as a vasorelaxant in many blood vessels (Section 1.1.3). The relationship between NO and mammary blood flow was investigated in lactating goats where oxytocin and the nitric oxide donor diethylamine NONOate increased the blood flow through the gland and the nitric oxide inhibitors L-N^G-methylarginine and L-NNA reduced MBF (Fleet *et al.*, 1993; Lacasse *et al.*, 1995; Lacasse *et al.*, 1996). Although these studies in goats provide evidence that NO plays a role in the control of MBF they do not address what controls the production and release of NO by the gland, nor under what conditions and states it occurs.

1.3.2 *Location of Nitric Oxide Synthase and Production of Nitric Oxide within the Mammary Gland*

The local production of NO by the mammary gland has been reported, and NOS, the enzyme responsible for the production of NO has been localised to many structures within the mammary gland. For example, NADPH-diaphorase activity is found in both the secretory epithelium and vascular endothelium of the mammary glands of lactating goats and cows (Lacasse *et al.*, 1996). The NADPH-diaphorase histochemical technique is commonly used in neurological studies and the enzyme responsible has recently been reported as being NOS (Hope *et al.*, 1991). The epithelia of both alveoli and ducts showed NADPH-diaphorase activity, as well as the endothelium and smooth muscle of the pudic artery. These structures also showed immunoreactivity to an eNOS antibody (Lacasse *et al.*, 1996). A similar pattern of staining to that apparent in tissue from goats and cows was detected in mammary tissue collected from lactating rats, three to four days postpartum. NADPH-diaphorase activity and immunoreactivity to eNOS were detected in mammary glandular cells, the endothelium of blood vessels and lactiferous ducts (Iizuka *et al.*, 1998). In contrast, in explants of mammary tissue from progesterone and 17β-estradiol treated (hormone primed) rats, both NADPH-diaphorase staining and immunoreactive signal to eNOS were localised to the myoepithelial cells of the glandular epithelium. There was also an immunoreactive signal to eNOS in the endothelial layer of mammary blood vessels (Onoda & Inano, 1998) as described in the cow and goat (Lacasse *et al.*, 1996) and in the lactating rat (Iizuka *et al.*, 1998).

There was no specific staining of any structures when an nNOS antibody was used on goat and bovine mammary tissue (Lacasse *et al.*, 1996). In contrast, there was positive staining for nNOS in mammary glandular cells of lactating rats (Iizuka *et al.*, 1998) and weak staining for nNOS in the alveoli and lactiferous ducts in explants of mammary tissue from hormone primed rats (Onoda & Inano, 1998).

Following culture of explants of mammary tissue, from hormone primed virgin rats, and treatment with LPS, iNOS immunoreactive signal was localised to the myoepithelial cells and the endothelial layer of mammary blood vessels. A similar pattern of signal was apparent in explants that were not cultured with LPS although

the signal was reported as being less intense (Onoda & Inano, 1998). In contrast there was slight staining for iNOS only in the muscle tissue surrounding the lactiferous ducts in mammary tissue from lactating rats (Iizuka *et al.*, 1998). Although there is some conflict as to the location of the various NOS isoforms within the mammary gland these could be due to the different species studied or the differences in mammary development/stage of lactation.

NO production by mammary cells has also been reported. Comma-D cells (murine mammary epithelial cells) produce nitrite in culture and the concentration is decreased by 75-85 % by the inclusion of L-NNA or L-N^5-iminoethyl ornithine (Lacasse & Prosser, 1995b). Cytokines and cytotoxins also stimulate NO production as previously described in other cell and tissue types (Table 1.6). Nitrite concentration in the medium is increased following treatment of Comma-D cells with IFN-γ and the production is doubled when the cells are co-treated with IFN-γ and TNF-α, with increases in iNOS mRNA also apparent. In contrast, IL-1β has no effect on the production of nitrite by Comma-D cells (Low *et al.*, 1997).

EGF enhanced the production of nitrite when the cells were treated with it in conjunction with IFN-γ or IFN-γ and TNF-α (Low *et al.*, 1997). Explants of mammary tissue from hormone primed rats respond to treatment with LPS with increased production of nitrite in the medium and accompanied by increases in iNOS immunoreactive protein. The increase was inhibited with the glucocorticoids, hydrocortisone and corticosterone. Treatment of the LPS stimulated explants with hydrocortisone also resulted in decreases in iNOS immunoreactive protein (Onoda & Inano, 1998). Glucocorticoids also decrease NO production in rat hepatocytes and rat VSMC (Geller *et al.*, 1993b; Imai *et al.*, 1994). Transient increases in the production of NOx in the medium of primary cultures of mouse mammary glands are also apparent after treatment with prolactin (Bolander, 2001).

These studies all clearly demonstrate that the mammary gland is capable of the production of NO under a variety of conditions, however, the precise roles that this release of NO may play is still under investigation.

1.3.3 *Production of Nitric Oxide during Mastitis*

Given the recent reports that mammary epithelial cells and explants of mammary tissue respond to cytokines and cytotoxins with the increased production of NO, it has been postulated that the release of NO may play a role in the defence of the mammary gland (Low *et al.*, 1997; Onoda & Inano, 1998). This is supported by the finding that the concentrations of nitrite are elevated in the milk of cows following intramammary infusion of *Streptococcus uberis* (Lacasse *et al.*, 1997), *Escherichia coli* (Blum *et al.*, 2000) or LPS (Bouchard *et al.*, 1999; Blum *et al.*, 2000). In addition, somatic cells isolated from the milk of infected and non-infected quarters of cows with spontaneous mastitis, when cultured, respond to treatment with LPS with significant increases in the amount of NOx released (Lacasse *et al.*, 1997). Furthermore, monocytes and neutrophils isolated from healthy lactating cows respond to *in vitro* treatment of IFN-γ and LPS with the increased expression of iNOS mRNA. However, unlike the monocytes, this was not accompanied by an increase in NOx in the media of the neutrophils (Boulanger *et al.*, 2001). Goff *et al.* (1996) also report that NO production could not be induced in bovine neutrophils under a variety of stimulatory conditions. During mastitis, over 95 % of the somatic cells in the milk are neutrophils (Kehrli & Shuster, 1994). Thus, this suggests that the NOx in the milk is from the monocytes and other populations of cells apart from neutrophils. Given that the mammary epithelium also releases NOx following stimulation it is likely that some of the NOx in the milk is from this source also. Other sources for NOx in milk have also been considered. The integrity of the mammary epithelium is often adversely affected during mastitis, thus allowing paracellular passage of various molecules (Wheelock *et al.*, 1966). In unstimulated cows the concentrations of NOx are four times higher in the milk than in the blood. Following intramammary infusion of LPS or *E. coli*, the concentrations of NOx in the milk increase significantly with no apparent effect on plasma NOx (Blum *et al.*, 2000). Furthermore, in healthy women, no correlation was found between the concentration of NOx in milk and plasma collected on days four or five postpartum (Iizuka *et al.*, 1997). Thus the NOx measured in milk is unlikely to be due to movement from the blood into the milk. Further studies are required to fully elucidate the source of NOx in the milk and the role it may play in intramammary defence.

1.4 Objectives

The aim of this Thesis was to investigate and characterise the production of NO by the mammary gland. Although the mammary gland clearly produces NO, both in a basal state and in response to stimulators of NO production (Section 1.3), little is known as to the role NO plays. Despite a clear effect of reproductive hormones on NOS, and the large changes in blood flow in the mammary gland, there are no comparative studies of NOS activity in the mammary gland between pregnancy and lactation. Experiments to address this specific aspect of NOS activity were therefore carried out.

The objectives of each experiment are addressed in detail at the beginning of each section. Briefly, the initial objective was to utilise several known techniques for the measurement of NOS and NO production and to determine if they could be successfully used within the mammary gland. Following the development of a successful technique, the production of NO by Comma-D cells was to be characterised which would then allow the use of these cells to examine other regulatory controls of NO production.

However, as the Comma-D cell line has some limitations as it is derived from a mid-pregnant mouse (Danielson *et al.*, 1984), the use of explants of mammary tissue would provide a system in which direct comparisons could be made between pregnancy and lactation. The production of NO during these states had not previously been compared. Explants of mammary tissue allowed the investigation of NO production in a controlled *in vitro* environment, and also provided the opportunity for *in vivo* manipulation and control over the reproductive status of the animals prior to culture of the explants.

The role of prolactin in the production of NO by the mammary gland was also investigated. PRL functions as both a cytokine and a hormone, thus, given the responsiveness of the mammary gland to cytokines (Section 1.3.2), and the role of PRL in milk secretion, further investigation of PRL's effects on NO production in the mammary gland was warranted.

As these studies were being conducted, it was suggested that xanthine oxidase generates NO from NOx (Godber *et al.*, 2000; Stevens *et al.*, 2000). By this stage the use of an assay system for the indirect measurement of NO production had been validated. The assay relied upon the measurement of NOx. Thus, the role XO plays in the mammary gland was investigated by examining the effect of allopurinol, an XO inhibitor on NO production. This, together with an investigation of the effects of NOS inhibitors would allow confirmation that mammary NOS was responsible for the production of NOx, and whether the activity of XO affected its measurement.

The production of NO by the mammary gland as a defence mechanism was further investigated. Both mammary cells (Low *et al.*, 1997; Onoda & Inano, 1998) and milk somatic cells (Goff *et al.*, 1996; Lacasse *et al.*, 1997; Boulanger *et al.*, 2001) produce NOx but what regulates this production and for what reason it occurs is largely unknown. Comparisons of milk NOx concentrations with the somatic cell count and electrical conductivity of the milk would allow some theories to be developed as to the source of NOx measured in the milk and why it was produced.

Thus, the objectives of this Thesis were to further examine the production of NO within the mammary gland to determine what regulates the release of NO with special interest in the comparison between pregnancy and lactation.

CHAPTER TWO

GENERAL METHODS

This chapter contains methods and general information related to experiments described in this Thesis. Specific information related to individual experiments is described at the beginning of relevant chapters.

2.1 Animals

All rats used in these studies were of the Sprague-Dawley strain and were housed at the Ruakura Small Animal Colony (Hamilton, NZ). Animals were fed *ad libitum,* a commercially available diet (Diet 86; pelleted rodent food, Sharps Grains and Seeds, Carterton, NZ), and had constant access to water. Lighting was on a 12:12, light:dark daily photoperiod. All experimental animals were used with the approval of the Ruakura Animal Ethics Committee (DDS 0032/98 approval #2991 and #3287).

Both pregnant (n=7; approximately 12-14 days) and lactating (n=12; between 12-18 days postpartum) rats were used in the studies described in this Thesis. The approximate stage of pregnancy of the rats was determined by Mr R. Broadhurst, (Ruakura Small Animal Colony, Hamilton, NZ) by palpation of the uterus and estimation based upon recording the day that the male was introduced. Examination of the pups *in utero* following euthanasia of the dam suggested that all pregnancies were of a similar developmental stage. Stage of lactation was recorded as the number of days postpartum.

2.2 Materials and Equipment

Suppliers of chemicals and equipment are listed alphabetically below. All other chemicals used were analytical grade.

2.2.1 *Chemicals*

Agarose gel DNA extraction Kit (Roche, Cat # 15290-018)

Agarose, SeaKem LE (FMC Bioproducts)

Allopurinol (4-hydroxypyrazolo[3,4-d] pyrimidine, Acros, Cat # 121830050)

Aminoguanidine hemisulphate salt (Sigma, Cat # A-7009)

Ampicillin (AppliChem, Cat # A0839)

Amphotericin B (GibcoBRL, Cat # 15290-018)

AquaSil (Pierce, Cat # 42799)

L-[2,3,4,5-^{3}H] arginine monohydrochloride (Amersham, Cat # TRK698)

bisBenzamide (Hoechst No. 33258, Sigma, Cat # B-2883)

Bicinchoninic acid Assay Kit (Pierce)

BSA, Bovine serum albumin (Sigma, Cat # A-7030)

CHAPS, 3-[(3-cholamidopropyl)-dimethylammonio]-1-propane-sulfonate (Sigma, Cat # C-3023)

p-Coumsaric acid (Sigma, Cat # C-9008)

2,3-diaminonapthalene (Molecular Probes, Cat # D-7918)

DH5α competent *E. coli* cells (GibcoBRL, Cat # 18 258-012)

DMEM:F12; 1:1, Dulbecco's modified Eagle's medium:Nutrient mixture F12 (GibcoBRL, Cat # 12 400-024)

DMEM, Dulbecco's modified Eagle's medium (GibcoBRL, Cat # 12 100-046)

DMSO, Dimethyl sulphoxide (Sigma, Cat # D-4540)

DNA, Calf thymus (Sigma, Cat # D-3664)

Donkey anti-rabbit IgG horseradish peroxidase conjugated whole antibody (Amersham Cat # NA 934)

Dowex beads 50W-X8 20-5 mesh (BDH)

EGF, Epidermal growth factor (Sigma, Cat # E-4127)

FCS, Fetal bovine serum, NZ origin (GibcoBRL, Cat # 10 091-148)

D-Glucose-6-phosphate monosodium salt (Sigma, Cat # G-7879)

D-Glucose-6-phosphate dehydrogenase from *Leuconostoc mesenteroides* Type XXIII (Sigma, Cat # G-5760)

Goat anti-rabbit-IgG horseradish peroxidase conjugated whole antibody (Sigma, A-6154)

Igepal CA-630 (Sigma, Cat # I-3021)

Insulin (Sigma, Cat # I-1882)

Interferon-γ, human, recombinant from *Escherichia coli* (hIFN-γ, Roche, Cat # 1 040 596)

Interferon-γ, mouse, recombinant from *E coli* (mIFN-γ, Roche, Cat # 1 276 905)

Interferon-γ, rat, recombinant from CHO cells (rIFN-γ, GibcoBRL, Cat # 13283-023)

Lambda DNA (λ-DNA, Roche, Cat # 745 782)

LB Broth, Lennox L broth base (GibcoBRL, Cat # 12 780-052)

LPS, lipopolysaccharide from *E. coli* Serotype 0111:B4 (Sigma, Cat # L-2630)

Luminol, 5-amino-2, 3-dihydro-1, 4-phthalalinedione (Sigma, Cat # A-8511).

Mouse anti-human ecNOS monoclonal antibody (Transduction, Cat # N30020)

NR, Nitrate reductase from *Aspergillus species* (Roche, Cat # 981 249)

Nitro blue tetrazolium (Sigma, Cat # N-6876)

NADPH, β-nicotinamide adenine dinulceotide phosphate, reduced form (Sigma, Cat # N-1630)

iNOS positive control from induced mouse macrophages (Transduction, Cat # I25900)

eNOS positive control, human endothelial cell lysate (Transduction, Cat # E15900)

L-NNA, N^{ω}-nitro-L-arginine (Sigma, Cat # N-5501)

OptiPhase 'HiSafe' 3 liquid scintillation fluid (Wallac)

PBS, Phosphate buffered saline Dulbecco A tablets (Oxoid)

Penicillin-Streptomycin (GibcoBRL, Cat # 15140-122)

Phenazine Methosulphate (Sigma, Cat # P-9625)

PMSF, phenylmethylsulfonyl fluoride (Roche Cat # 837 091)

Prestained SDS-PAGE High Range Molecular weight markers (Bio-Rad Cat #161-0309)

Rabbit anti-mouse iNOS polyclonal antibody (Biomol, Cat # SA-200)

Rabbit anti-human ecNOS polyclonal antibody (Transduction, Cat # N30030)

Rabbit anti-bovine eNOS polyclonal antibody (Biomol, Cat # SA-201)

Rabbit anti-eNOS control peptide (Biomol, Cat # SP-201)

Rediprime™II (random prime labelling system; Amersham)

Redivue [α-^{32}P]-dCTP (Amersham, Cat # AA0005)

Restriction enzyme *Eco* RI (Roche, Cat # 1 175 084)

Restriction enzyme *Hin*d III (Roche, Cat # 656 321)

Restriction enzyme *Sma* I (Roche, Cat # 656 348)

Restriction enzyme buffer SuRE/Cut Buffer A (Roche, Cat # 1 417 959)

Restriction enzyme buffer SuRE/Cut Buffer H (Roche, Cat # 1 417 991)

RiboGreen™ RNA quantification Reagent and Kit (Molecular Probes)

RNA Ladder 0.25-9.5 kb (GibcoBRL, Cat # 15 620-016)

Sheep anti-mouse IgG horeseradish peroxidase conjugated whole antibody (Amersham, Cat # NXA 931)

Sodium pentabarbitone (Sagital)

STBL2™ competent *E. coli* cells (GibcoBRL, Cat # 10 268-019)

TEMED, N,N,N',N'-tetra-methyl-ethylenediamine (Bio-Rad, Cat # 161-0800)

TRIzol® reagent (GibcoBRL, Cat # 15596-026)

Xanthine sodium salt (Sigma, Cat # X-2502)

2.2.2 *Equipment*

Acrodisk syringe filter (Pall Corporation)

ELx800 UV Microtitre plate reader (Bio-Tek)

Eppendorf Centrifuge 5417C (Pierce)

FL500 Fluorescent plate reader (Bio-Tek)

Gel Doc 1000 (Bio-Rad)

GS-690 Imaging Densitometer (Bio-Rad)

Hybond N membrane (Amersham)

High Intensity Ultrasonic Processor, 50-watt model (Sonics & Materials Inc)

1409 Liquid Scintillation β-counter (LKB Wallac)

Mini-protean II (Bio-Rad)

Muliphore II Nova Blot electrophoresis unit (LKB-Produkter)

Nalgene™ cryo 1 °C freezing container (Nalge Nunc)

Nitrocellulose membrane, NC-Extra 0.45 mm (Sartorius)

Nunclon™ flasks and multidishes (Nalge Nunc)

Radiographic film (Eastman Kodak)

Spectra/Por molecularporous membrane; (Spectrum, Cat # 132 706)

Ultra-Turrax® homogeniser (Janke & Kunkel)

2.2.3 *Addresses of Suppliers*

ACROS Organics, NJ, USA

Amersham Pharmacia Biotech, Buckinghamshire, England

AppliChem, Darmstadt, Germany

BDH Chemicals, Poole, England

Biomol Research Laboratories, Inc. Plymouth Meeting, PA, USA

Bio-Rad Laboratories, Richmond, CA, USA

Bio-Tek Instruments Inc, Winooski, VT, USA

Eastman Kodac Company, Rochester, NY, USA

FMC Bioproducts, Rockland, ME, USA

GibcoBRL/Life Technologies, Grand Island, NY, USA

Janke & Kunkel; Ika®-Labortechnic, Staufen, Germany

LKB-Produkter, Bromma, Sweden

LKB-Wallac, Finland

Molecular Probes, Eugene, OR, USA

Nalge Nunc International, Napperville, IL, USA

Oxoid Ltd, Hampshire, England

Pall Corporation, Ann Arbor, MI, USA

Pierce, Rockford, IL, USA

Roche Molecular Biosciences, Mannheim, Germany

Sagital; May & Baker, Lower Hutt, NZ

Sartorius, Göttingen, Germany

Sigma Chemical Co. St Louis, MO, USA

Sonics & Materials Inc, Danbury, CT, USA

Spectrum Laboratories Ltd, Laguna Hills, CA, USA

Transduction Laboratories, Lexington, KY, USA

Wallac Scintillation Products, Milton Keynes, England

2.3 Murine Mammary Epithelial Cell Culture

The Comma-D (murine mammary epithelial) cells, used in the studies in this Thesis were a generous gift from Dr D. Medina (Baylor College of Medicine, Houston, TX, USA). Cells were used at passages between 17 and 27 and all cultures were maintained at 37 °C in humidified 5 % CO_2:95 % air atmosphere.

2.3.1 *Cell Culture*

Comma-D cells were routinely grown to confluence in Nunclon™ 75 cm^2 flasks in growth media [Dulbecco's Modified Eagle's Medium:Nutrient Mixture F12 (DMEM:F12; 1:1) supplemented with 2 % (v/v) foetal calf serum (FCS), 5 μg/ml insulin, 5 ng/ml epidermal growth factor (EGF), 0.3 mg/ml bovine serum albumin (BSA), 100 U/ml penicillin, 100 μg/ml streptomycin and 3.7 g/l sodium bicarbonate ($NaHCO_3$), pH 7.4]. Once confluent, the cells were passaged by dissociation from the flask using 2.4 mg/ml dispase in passaging solution (1.8 g/l glucose, 17.6 g/l NaCl, 0.22 g/l KCl, 0.14 g/l Na_2HPO_4, 7.15 g/l HEPES, pH 7.3). The cells were then centrifuged at 180 x *g* for 5 minutes and the pellet resuspended in growth media. The cells were then either, reseeded into flasks, used for experiments (Section 2.3.2) or frozen for storage in liquid nitrogen. To store cells, confluent flasks were passaged and the pellet resuspended in freeze medium (65 % quiescent media; [DMEM:F12 with 100 U/ml penicillin, 100 μg/ml streptomycin and 3.7 g/l $NaHCO_3$, pH 7.4], 25 % FCS and 10 % dimethyl sulphoxide [DMSO]) to give approximately 2 x 10^6 cells/ml. The cells were then aliquoted into 1 ml cryovials, which were placed into a Nalgene™ Cryo 1 °C Freezing container and frozen overnight at -80 °C. The cryovials were then transferred to liquid nitrogen for long term storage.

2.3.2 *Production of Nitrite and Nitrate by Comma-D Cells*

To set up experiments to investigate the production of nitrite and nitrate (NOx) by Comma-D cells, stock flasks of cells were passaged as described and the cells were counted using a haemocytometer. Each well of a 24 well Nunclon™ multidish was then seeded with 2 x 10^5 cells in 1 ml growth medium. Unless otherwise stated, the

cells were grown for 4 days in growth medium then cultured for 2 days in serum and growth factor free medium (quiescent medium). Treatments (e.g. cytokines, inhibitors etc) were applied to the culture dissolved in quiescent medium and were prepared as required. For those chemicals that were not sterile, the solution (media and chemical) was sterile filtered using a 0.2 μm Acrodisk syringe filter.

Treatments were applied to triplicate wells, which were grouped together as described by Bennett (1995).

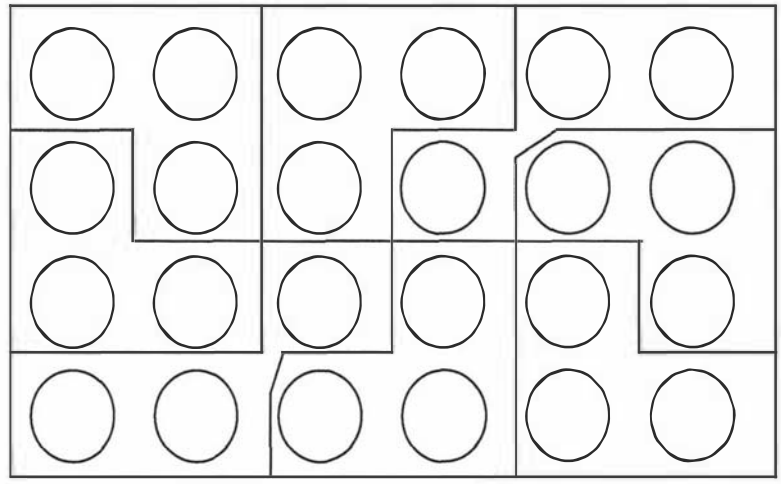

Figure 2.1: Grouping of treatment triplicates on tissue culture plates. Treatments were applied in triplicate in the groupings shown to allow ease of application and consistency.

2.3.3 *Collection of Cells and Media*

Medium samples were collected from the wells and stored at -20 °C until assayed. Following the removal of medium, the cells were collected by rinsing the well with approximately 1 ml of phosphate buffered saline (PBS). The saline was removed then 500 μl PBS was pipetted into the well. The recovered cells were then sonicated (two 15 second bursts using a high intensity ultrasonic processor with the output control set on 30) to detach the cells from the well and to lyse the cell membranes. The resulting suspension was transferred into a microfuge tube and stored at -20 °C.

2.4 Nitrate and Nitrite Assay

Nitrate plus nitrite (NOx) in culture media and milk was measured using a modification of the methods of Misko *et al.* (1993) and Verdon *et al.* (1995).

2.4.1 *Standard Preparation*

Standards of sodium nitrite ($NaNO_2$) and sodium nitrate ($NaNO_3$), ranging from 1000 pmol to 20 pmol (in 50 μl; 20 μM to 0.4 μM) were prepared in either culture media or 1/10 dilution of milk in Milli-Q water. Experiments not presented in this Thesis showed that standards prepared in whole milk and assayed did not exhibit full conversion of nitrate to nitrite.

2.4.2 *Sample Preparation*

Culture Medium

Conditioned medium samples from cell and explant culture experiments were assayed undiluted or were diluted in the relevant medium as required (for example, if the sample was collected from cells during the growth phase, growth medium was used as the diluent).

Milk Samples

Milk samples were centrifuged at 1150 x *g* for 10 minutes at 4 °C. The skim milk was collected and diluted 1/10 with Milli-Q water.

2.4.3 *Assay Method*

Briefly, 50 μl of sample was mixed with 10 μM β-nicotinamide adenine dinucleotide phosphate, reduced form (NADPH) and 40 μl assay mix (nitrate reductase, glucose-6-phosphate and glucose-6-phosphate dehydrogenase in sodium phosphate buffer, pH 7.4) and incubated for 1 ½ hours at room temperature. The concentrations of components in the final reaction mixture were as follows: nitrate reductase, 80 U/l;

glucose-6-phosphate, 500 μM; glucose-6-phosphate dehydrogenase, 160 U/l; sodium phosphate, 14 mM; NADPH, 1 μM. Each sample then received 10 μl of 50 μg/ml 2,3-diaminonaphthalene in 0.62 M HCl. Following incubation for 10 minutes at room temperature and in the dark, the reaction was stopped with the addition of 10 μl of 2.8 M NaOH to each sample. The samples were read on a FL500 Bio-Tek fluorescent plate reader at 360 nm excitation and 460 nm emission. Experiments not presented in this Thesis showed that the NADPH and glucose-6-phosphate dehydrogenase solutions must be made immediately prior to use.

Assays in which $NaNO_2$ in standards was not fully reduced to $NaNO_3$ were repeated (in practice this rarely occurred). This was detected by comparing the absorbance versus concentration curves for both $NaNO_2$ and $NaNO_3$. NOx concentrations in samples were calculated by subtracting the blank (diluent only) from the samples and then calculating the concentration using the linear regression equation derived from the $NaNO_2$ standard curve.

2.4.4 *Effect of FCS on the Measurement of Nitrite and Nitrate*

Introduction

Both Comma-D cells and mammary explants were used to investigate NOx production in this Thesis and it is important to be able to compare results as both systems can reveal different information about the control of NO production in the mammary gland. For example, Comma-D cells will be useful to examine the action of various stimulants directly on epithelial cells, whereas the use of mammary explants provides a system in which interaction between cell types can be assessed. Although FCS and EGF are removed to induce quiescence in Comma-D cells, both have effects on NOx production. For example, EGF, a mitogen of Comma-D cells, also stimulates nitrite accumulation in the medium, although only following treatment with IFN-γ (Low *et al.*, 1997). FCS also increases NOx production from confluent and sub-confluent bovine aortic endothelial cells when cultured with increasing concentrations of FCS (Lopez-Farre *et al.*, 1997). In addition to the use of Comma-D cells, explants of mammary tissue from rats were investigated for their ability to produce NO. Initial explant culture experiments (data not shown) followed the

method of Onoda & Inano (1998) in which explants were cultured without EGF and in the presence of 10 % FCS in Dulbecco's modified Eagle's medium. This is in contrast to the stimulation of NOx production in Comma-D cells, which occurs after the removal of serum and growth factors (Low *et al.*, 1997). Analysis of the media from these initial experiments revealed that there was interference in the NOx assay to such an extent that an estimate of NO production was not possible. Given the reported effects of FCS and EGF on NOx production a direct comparison of the production of NOx by explants and Comma-D cells would have to occur following culture in similar environments. However, as the measurement of NOx in explant culture medium containing 10 % FCS was not possible, and measurement of NOx in medium from growing Comma-D cells was required (growth medium contains 2 % FCS) a further investigation of the cause of the interference was undertaken.

Experimental Design

In order to determine the effect of various media components on the measurement of NOx, $NaNO_2$ and $NaNO_3$ standards were prepared (Section 2.4.1) in the following media.

Comma-D growth medium (G) - Dulbecco's modified Eagle's medium:Nutrient Mixture F12 (DMEM:F12; 1:1) with 2 % (v/v) foetal calf serum (FCS), 5 μg/ml insulin, 5 ng/ml epidermal growth factor (EGF), 0.3 mg/ml bovine serum albumin (BSA), 100 U/ml penicillin, 100 μg/ml streptomycin and 3.7 g/l sodium bicarbonate ($NaHCO_3$), pH 7.4.

Quiescent medium (Q) - DMEM:F12 with 100 U/ml penicillin, 100 μg/ml streptomycin and 3.7 g/l $NaHCO_3$, pH 7.4.

Quiescent medium + 2 % FCS (Q + 2 %) - DMEM:F12 with 2 % (v/v) FCS, 100 U/ml penicillin, 100 μg/ml streptomycin and 3.7 g/l $NaHCO_3$, pH 7.4.

Quiescent medium + 10 % FCS (Q + 10 %) - DMEM:F12 with 10 % (v/v) FCS, 100 U/ml penicillin, 100 μg/ml streptomycin and 3.7 g/l $NaHCO_3$, pH 7.4.

Explant Growth medium (EG) - Dulbecco's modified Eagle's medium (DMEM) with 10 % (v/v) FCS, 100 U/ml penicillin, 100 μg/ml streptomycin 0.25 μg/ml amphotericin B and 3.7 g/l $NaHCO_3$, pH 7.4.

The standards were then assayed for the presence of NOx following the conversion of nitrate to nitrite as described in Section 2.4.3.

Results

The inclusion of FCS in the medium increased the fluorescence of the standards and the blanks (medium containing no $NaNO_2$). The blanks were significantly ($P < 0.0001$) different for the various media (G: 19620 ± 562, Q: 15282 ± 386, Q + 2 %: 19494 ± 507, Q + 10 %: 33284 ± 818 and EG: 32672 ± 936 fluorescent units). Significant differences ($P < 0.0001$) were seen between the slopes of the Q, EG and Q + 10 % curves, before and after adjustment for the blanks (data not shown). No significant differences were seen between the slopes for standards in Q, Q+2 % or G media, following adjustment for the blanks (data not shown). As described in Section 2.4, the measurement of NOx in media requires firstly the conversion of nitrate to nitrite then the subsequent measurement of nitrite. Standard curves of $NaNO_2$ and $NaNO_3$ are employed and the conversion of the nitrate to nitrite is monitored using these. For example, full conversion of nitrate should result in a standard curve with the same profile as that for nitrite as apparent when the standards were prepared in Q (Figure 2.2 A), G or Q + 2 % media (data not shown). However, when the standards were prepared in EG or Q + 10 % media the full conversion of the nitrate to nitrite did not occur (Figure 2.2 B and C). The standard curves shown in Figure 2.2 A, B and C are adjusted for the relevant blank.

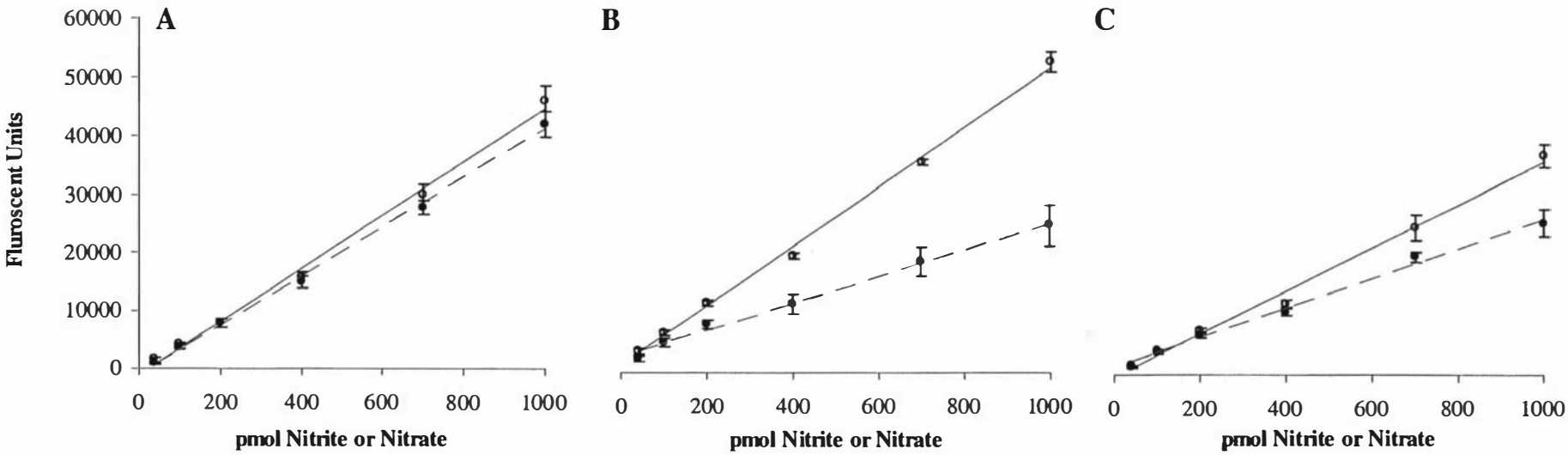

Figure 2.2: Effect of media type on the reduction of nitrate to nitrite. $NaNO_2$ (-o-) and $NaNO_3$ (-•-) standards were prepared in various media and nitrate was reduced to nitrite by nitrate reductase. Total nitrite was then measured using 2,3-diaminonapthalene. **A**: Quiescent medium (DMEM:F12). **B.** Explant growth medium (DMEM + 10 % FCS). **C.** Quiescent medium + 10 % FCS. Standard curves presented were adjusted using the blank for each media.

Discussion

To determine the source of the interference on the NOx assay apparent in initial culture experiments (data not shown), standard curves were generated in various different media, where the presence of FCS significantly increased ($P < 0.0001$) the intensity of the fluorescence. In contrast, the addition of 10 % fetal bovine serum to Earle's minimum essential medium decreased the intensity of the fluorescence signal generated after incubation of the standards or samples with 2,3-diaminonapthalene by 60 % (Misko *et al.*, 1993). Alone, the increased fluorescence generated by the FCS and other unknown components is not a real problem if the goal is to measure nitrite only, as curves in all media generate linear standard curves. Thus, nitrite concentrations in samples of different medium can be estimated from the relevant standard curve (this was standard practice throughout this Thesis). However, it is the lack of conversion of nitrate to nitrite in the presence of FCS that is a concern. Although the $NaNO_3$ standards in either EG or Q + 10 % media produced linear curves (Figure 2.2), the lack of conversion of nitrate to nitrite is a problem as it suggests that it is very likely that nitrate in the samples is also not being fully converted, thus leading to under-estimation of the NOx in the media. However, in this Thesis, the majority of the induction of NOx production was done in quiescent media (no serum or growth factors), thus avoiding the problem. Boulanger *et al.* (2001) also report that FCS is left out of culture medium as it interferes with the NOx assay they use. At this stage it is still unclear as to why the presence of high concentrations of FCS interferes with the conversion of nitrate to nitrite by nitrate reductase. Further work would be required to determine this, if the measurement of NOx in media containing high (10 %) FCS concentrations were required. Thus, in order to allow the direct comparison between Comma-D cells and mammary explants in their responses to various stimuli, both were cultured during the treatment period in the absence of FCS and EGF (Sections 2.3.2 and 2.5.2).

2.5 Explant Culture

Explants of mammary tissue were cultured for up to two days at 37 °C in humidified 5 % CO_2:95 % air environments. The organ culture methods were based upon those described by Topper *et al.* (1975) and Onoda & Inano (1998).

2.5.1 *Siliconising Lens Paper*

Lens paper for use as rafts in explant culture were prepared by a method based upon that of Topper *et al.*, (1975). Briefly, Whatman 105 lens cleaning tissue was washed 3 x 20 minutes in di-ethyl ether with aspiration of the fluid between washes. The tissue was washed 3 x 20 minutes with 95 % ethanol, then 3 x 5 minutes in Milli-Q water and dried overnight at 37 °C. Sheets were carefully separated and siliconised with 1:100 w/v AquaSil/Milli-Q water. Following 30 minutes in the siliconising solution the tissue was washed 3 x 5 minutes with Milli-Q water and dried overnight at 37 °C.

2.5.2 *Preparation and Culture of Explants*

Following euthanasia either by the intraperitoneal administration of 500 µl of 60 mg/ml sodium pentabarbitone, or by CO_2 euthanasia followed by cervical dislocation, the right abdominal mammary gland was excised and diced into approximately 1 mm^3 pieces. Four explants were placed onto siliconised lens paper rafts in the wells of a 24 well tissue culture plate. Media (DMEM:F12 with 100 U/ml penicillin, 100 µg/ml streptomycin, 0.25 µg amphotericin B/ml and 3.7 g/l $NaHCO_3$, pH 7.4) was added (1 ml/well). The explants were cultured for 24 hours then the media were collected and replaced with media containing the appropriate treatments. The treatments were applied to triplicate wells (Figure 2.1). Following a second 24 hours in culture, media were collected. Explants were collected and pooled across the treatment triplicates, weighed, placed into cryovials, snap frozen in liquid nitrogen and stored at -80 °C.

Explant weights are given as the total weight of each treatment triplicate. NOx in the media was determined by measuring the nitrite and nitrate as specified in Section 2.4 for each well. The total amount (pmol) of NOx produced in each treatment was found by adding the amount from each well within that triplicate. Production of NOx (pmol/mg tissue) was calculated from the total amount produced across the treatment triplicates divided by the pooled weight of tissue.

2.6 DNA Assay

The method used to measure DNA in mammary tissue and in cell culture homogenates is based upon that of Labarca & Paigen (1980).

2.6.1 *Sample Preparation for Comma-D Cells*

Comma-D cells were collected and sonicated in PBS. As the assay required a high salt phosphate buffered saline plus EDTA buffer, cellular solutions were diluted prior to assay. Briefly, 37.5 µl of cellular solution (Section 2.3.3) was combined with 37.5 µl PBS and 75 µl 2X PBSE (0.1 M sodium phosphate; 4 M NaCl and 0.004 M EDTA, pH 7.4).

2.6.2 *Sample Preparation for Mammary and other Tissues*

Samples of mammary tissue were prepared for chemical analyses by homogenising 100 mg of tissue in 1 ml of PBS using an Ultra-Turrax® homogeniser. The homogenate was then diluted 1:1 with 2X PBSE.

2.6.3 *DNA Standard Preparation*

DNA standards were prepared using calf thymus DNA. The contents of the vial were reconstituted in Milli-Q water to make a 200 µg/ml solution. The DNA was then diluted in Milli-Q water and the optical density (OD) measured at 260 nm to estimate the actual concentration of DNA in the solution assuming that 50 µg/ml double

stranded DNA has a OD of 1. The initial solution was then suitably diluted to give a standard stock solution of 100 μg/ml. The 100 μg/ml stock was stored at 4 °C and used to make the DNA assay standards as required. The 100 μg/ml stock was diluted in PBSE (0.05 M sodium phosphate; 2 M NaCl and 0.002 M EDTA, pH 7.4) to provide a range of standards from 20 to 2 μg/ml.

2.6.4 *DNA Assay*

DNA contents of the samples were assay by mixing 50 μl of diluted sample with 250 μl of bisBenzamide (Hoechst No. 33258) buffer solution (0.1 μg/ml bisBenzamide in PBSE for cell culture; 1 μg/ml bisBenzamide in PBSE for tissue homogenates) in a 96 well microplate. The fluorescence was then measured using a FL500 Bio-Tek fluorescent plate reader at 360 nm excitation and 460 nm emission.

2.7 Bicinchoninic acid (BCA) Assay for Total Protein

Protein content of mammary tissue (Section 2.6.2, subsample taken prior to dilution with 2X PBSE) and cultured Comma-D cells (Section 2.3.3) were determined by a bicinchoninic acid assay according to the instructions of the manufacturer of the assay kit. Briefly, a set of BSA standards, ranging in concentration from 2 mg/ml to 0.2 mg/ml, were prepared by diluting the stock solution of BSA (supplied with the assay kit) with PBS. The standards and samples (10 μl) were pipetted into separate wells of a 96 well microtitre plate and 200 μl of working reagent (supplied with the assay kit) were added to each well. The plate was mixed, incubated at 37 °C for 1 ½ hours and the absorbance was read on an ELx800 UV Bio-Tek microplate reader at 595 nm.

2.8 Polyacrylamide Gel Electrophoresis

The method used for the separation of proteins was based on that of Laemmli (1970).

2.8.1 *Preparation of the Polyacrylamide Gel*

The 15 % resolving gel solution was made by mixing 5 ml of 30 % (w/v) acrylamide/bis-acrylamide with 2.5 ml 1.5 M Tris-HCl (pH 8.8), 100 µl 10 % (w/v) sodium dodecyl sulphate (SDS) and 2.34 ml of Milli-Q water. To set the gel, 10 µl of N,N,N',N'-tetramethylethylenediamine (TEMED) and 50 µl of a 10 % (w/v) solution of ammonium persulphate (APS) were added. This mixture was then pipetted into a Bio-Rad mini-protean II apparatus (either 0.75 mm or 1.5 mm spacers were used) and overlaid with about 200 µl of water delivered from a syringe via a hypodermic needle. After the gel had polymerised, the water was tipped off and any free water remaining was removed by blotting the gel with filter paper. Stacking gels were made from 0.67 ml of 30 % acrylamide/bis-acrylamide, 1.25 ml of 0.5 M Tris-HCl (pH 6.8), 50 µl 10 % (w/v) SDS and 3 ml of Milli-Q water. To set the gel, 5 µl TEMED, and 25 µl of a 10 % (w/v) solution of APS were added. The stacking gel was pipetted onto the resolving gel and a Teflon comb was inserted.

To prepare a 7.5 % monomer concentration resolving gel, the method was modified by using 2.5 ml of 30 % (w/v) acrylamide/bis-acrylamide and increasing the amount of water to 4.84 ml, all other volumes of reagents remained the same.

2.8.2 *Sample Preparation*

Preparation of the samples was carried out in a manner appropriate for the protein of interest as described in the relevant chapters.

2.8.3 *Gel Electrophoresis*

Gels were loaded with samples, standards or molecular weight markers, placed in the running chamber with approximately 400 ml of electrode buffer (0.025 M Tris; 0.192

M glycine and 0.10 % [w/v] SDS) and then run at 150 V constant voltage for approximately 1 hour. Gels were stained in Coomassie blue stain (25 % [v/v] methanol and 10 % [v/v] glacial acetic acid with 0.5 g/l Bio-Rad Brilliant Blue R) for approximately 2 hours, then destained (in 45 % [v/v] methanol and 10 % [v/v] glacial acetic acid) overnight at room temperature. Staining was omitted if the proteins were transferred to nitrocellulose for Western analysis.

2.9 Electrophoretic Transfer of Proteins

Transfer of proteins from polyacrylamide gels onto nitrocellulose membranes was performed following the method of McLaren *et al.* (1994). Briefly, following electrophoresis, the stacker was removed and the gel was immersed in Tris/glycine buffer (48 mM Tris, 39 mM glycine with 20 % [v/v] methanol, pH 9.2) for approximately 15 minutes. Paper wicks cut from Whatman 3 mm chromatography paper and the nitrocellulose membrane (NC-Extra 0.45 mm) were also soaked in the same buffer. Half of the wicks were laid on the cathode of the LKB Muliphore II Nova Blot electrophoresis unit, followed by the nitrocellulose membrane, then the gel and the remainder of the wicks. The anode was fitted and the gel was electroblotted for 50 minutes at 0.8 mA per cm^2. The membrane was rinsed with TBS-Igepal buffer (Tris-buffered-saline-Igepal; 0.15 M NaCl, 0.1 M Tris, pH 7.6 containing 0.05 % [w/v] Igepal) and air dried overnight at room temperature.

2.10 Immunoblotting

Following the transfer of proteins, the nitrocellulose membrane was probed with the relevant antibodies after the method of Ventling & Hurley (1989). Briefly, nitrocellulose membranes were blocked with TBS-Igepal buffer containing 1 % polyvinylpyrrolidone (PVP) and 0.1 % BSA for 2 hours at room temperature. The membrane was rinsed with TBS-Igepal, and 15 ml of antibody; (diluted to the relevant dilution in TBS incubation buffer (TBS-Igepal buffer containing 0.1 % PVP

and 0.1 % BSA) was added. Following incubation for 90 minutes at room temperature, the membrane was washed three times by soaking in approximately 15 ml TBS-Igepal for 15 minutes.

A horseradish peroxidase conjugated secondary antibody was diluted in TBS incubation buffer and 15 ml was added to the membrane, which was then incubated for 90 minutes at room temperature. The membrane was washed as previously described, and developed by the addition of 15 ml of ECL (15 ml 20 mM Tris, pH 8.6 containing 36 µl of 91.7 mg luminol/ml DMSO, 6 µl of 28 mg p-coumsaric acid/ml DMSO and 5 µl of 30 % H_2O_2). Following a 5 minute incubation, the membrane was enclosed in plastic and exposed to radiographic film. Films were developed, fixed and washed in a 100 Plus All-Pro Imaging automatic X-ray film processor (Hicksville, NY), then scanned using a Bio-Rad GS-690 Imaging Densitometer.

2.11 Isolation of RNA

RNA was extracted from tissues and cells using TRIzol®, which is a commercially available reagent, based upon the method of Chomczynski & Sacchi (1987).

2.11.1 *Isolation from Tissue*

Briefly, 150 mg of tissue was homogenised with 1.5 ml of TRIzol® using an Ultra-Turrax® homogeniser. The homogenate was centrifuged at 12 000 x *g* for 10 minutes and RNA was then extracted from the supernatant using chloroform and isopropanol as described by the manufacturer of TRIzol®. Extracted RNA was dissolved in 100 µl of formamide (Chomczynski, 1992).

2.11.2 *Isolation from Cultured Cells*

For the isolation of RNA from Comma-D cells, the TRIzol® was pipetted directly onto the cells in the flask, mixed and then removed into a safelock eppendorf tube.

Isolation continued using chloroform and isopropanol as described by the manufacturer of TRIzol®. Extracted RNA was dissolved in 40 µl formamide (Chomczynski, 1992).

2.12 Quantification of RNA

Following extraction from tissue or cells, RNA was quantified using RiboGreen™ RNA quantification Reagent and Kit following the manufacturer's instructions. RNA extracted from mammary tissue (Section 2.11.1) or Comma-D cells (Section 2.11.2) were diluted to between 1/1000 and 1/10 000 in TE buffer (Kit 20 X diluted 1:20 with RNAse free Milli-Q water). The RNA standard (supplied with the kit) was diluted to provide a range of standards from 1000 to 20 ng/ml using TE buffer. Standards and samples (100 µl) were mixed with 100 µl of diluted RiboGreen™ reagent (supplied with the kit). Fluorescence was measured at 485 nm (excitation) and 530 nm (emission) on a FL500 Bio-Tek fluorescent plate reader.

2.13 Formaldehyde Gel Electrophoresis

2.13.1 *Preparation of the Formaldehyde/Agarose Gel*

Formaldehyde (0.6 M)/agarose (1 %) gels were prepared as follows: 1 g of agarose (SeaKem LE) was added to 85 ml of Milli-Q water and microwaved on 100 % power for approximately 2 minutes. The agarose was cooled to approximately 50 °C and 5 ml deionised formaldehyde and 10 ml of concentrated running buffer (containing 0.2 M MOPS, 50 mM sodium acetate and 10 mM EDTA, pH 7.0 [10 X RNA buffer]) were added. The formaldehyde/agarose mixture was poured into a gel former to cast either an 11 cm by 23 cm or 11 cm by 15 cm gel. Wells were formed using 10 or 14 well Teflon combs. The gel was set in a fumehood for approximately 1 hour.

2.13.2 *RNA Sample Preparation*

Samples of 20 μg of total RNA extracted from mammary tissue (Section 2.11.1) or Comma-D cells (Section 2.11.2) were prepared as follows: Based upon the results of quantification (Section 2.12) 20 μg of RNA dissolved in formamide was made up to a volume of 20 μl (1 μg/μl) with formamide then 2 μl formaldehyde, 4 μl 10 X RNA running buffer and 14 μl of Milli-Q water were added. Samples were mixed, incubated at 65 °C for 10 minutes and then cooled on ice. Loading dye (50 % glycerol; 1 mM EDTA; 0.4 % bromophenol blue) was then added (2 μl per sample).

2.13.3 *Gel Electrophoresis*

Samples (20 μg total RNA) were loaded onto the gel which was submerged in RNA running buffer (52.5 ml formaldehyde; 892.5 ml Milli-Q water; 105 ml 10 X RNA running buffer) and electrophoretically separated at 100 V constant voltage for approximately 1 hour. A RNA ladder, ranging from 0.25-9.5 kb was also run alongside the samples.

2.14 Northern Transfer

After electrophoresis the RNA was transferred overnight to Hybond N membrane by a passive transfer process. Following transfer, the RNA was visualised by staining the membrane with 0.02 % methylene blue in 0.3 M sodium acetate (pH 5.5) and then destained with Milli-Q water.

2.15 Preparation of Probes

The murine iNOS cDNA and bovine eNOS cDNA's used in this Thesis were generous gifts from Dr C. Rick Lyons, University of New Mexico, Albuquerque, NM, USA and Dr David G. Harrison, Emory University, Atlanta, GA, USA.

2.15.1 *Transfection of E. coli*

STBL2™ Competent E. coli Cells

A plasmid containing the full length clone of iNOS cDNA (iNOS-pGEM.3) was transfected into STBL2™ competent *E. coli* cells. Briefly, 10 μl of the plasmid (diluted to 0.5 ng/μl in Milli-Q water) was mixed with 100 μl of STBL2™ Competent Cells, heat shocked at 42 °C for 25 seconds and placed on ice for 2 minutes. The heat shocked cells were incubated in 900 μl Lennox L broth (LB), containing 25 g/l broth base, for 90 minutes at 30 °C with shaking. A sample (100 μl) of the broth/cell mix was spread on a LB agar/ampicillin plate (25 g/l LB broth base; 15 g/l agar; 50 μg/ml ampicillin) and incubated at 30 °C overnight.

DH5α™ Competent E. coli Cells

A plasmid containing the full length clone of eNOS cDNA (pBOS 13 in pBluescript SK+) was transfected into DH5α™ competent *E. coli* cells. Briefly, 5 μl of a solution containing the plasmid (0.5 ng/μl in Milli-Q water) was mixed with 100 μl of DH5α™ Competent Cells, heat shocked at 42 °C for 30 seconds and placed on ice for 2 minutes. Three hundred microlitres of LB broth (25 g/l Broth base) was added and the cells were incubated for 30 minutes at 37 °C with shaking. The broth/cell mix (405 μl) was spread on a LB agar/ampicillin plate and incubated at 37 °C overnight.

2.15.2 *Overnight Cultures*

Bulk cultures were grown up by selecting a single colony from the LB/ampicillin plates and mixing with approximately 3 ml of LB broth containing 50 μg/ml ampicillin. The tube was incubated overnight at 30 °C (STBL2™ Competent Cells) or 37 °C (DH5α™ Competent Cells) with shaking.

2.15.3 *Mini-Preparations*

Overnight cultures (1.5 ml) were poured into microfuge tubes and spun at 6500 rpm (Eppendorf Centrifuge 5417C) for 3 minutes. Media were poured off and the pellets were resuspended in 200 µl TGE buffer (0.9 % glucose, 0.01 M EDTA, 0.025 M Tris, pH 8.0) and vortexed for approximately 2 minutes. Lysis buffer (400 µl; 0.2 M NaOH, 1 % SDS) was added, the contents mixed by inversion, and incubated on ice for 5 minutes. Neutralising solution was added (300 µl; 3 M potassium acetate, 11.5 % glacial acetic acid, pH 4.8), the tube was vortexed and incubated on ice for 5 minutes. The tubes were then centrifuged at 12 000 rpm for 10 minutes. The supernatant (850 µl) was transferred to a new tube containing 600 µl of isopropanol, mixed by inversion of the tubes and centrifuged at 12 000 rpm for 5 minutes. The supernatant was aspirated and discarded and the pellet was washed with 500 µl 75 % ethanol. Tubes were centrifuged at 12 000 rpm for 2 minutes, the supernatant discarded and the pellets air dried. The plasmid containing the cDNA was resuspended in 50 µl TE/RNAse A buffer (10 mM Tris, 1 mM EDTA, 40 µg/ml RNAse A), incubated at 37 °C for 5 minutes, vortexed then stored at -20 °C.

2.15.4 *Agarose Gel Electrophoresis*

Gel Preparation

The plasmid containing the cDNA (Section 2.15.3) was run on 1 % agarose gels. Briefly, 1 g agarose was added to 100 ml TAE buffer (0.04 M Tris, 5 mM sodium acetate, 1 mM EDTA, pH 7.8) and microwaved on high for 2 minutes. The agarose was cooled to approximately 65 °C and 2 µl of ethidium bromide (10 mg/ml stock solution) was added. Approximately 30 ml was poured into a gel caster, an 8 lane comb was added and the gel was set.

Sample and Standard Preparation

Two microlitres of the plasmid containing the cDNA (dissolved in TE/RNAse A buffer; Section 2.15.3) was mixed with 8 µl of DNA loading dye (40 % sucrose, 0.25 % bromophenol blue, 0.25 % xylene cyanol).

Lambda DNA standard (λEH) was prepared by digesting λ-DNA with *Eco* RI and *Hin*d III, restriction enzymes. Briefly, 40 μl of λ-DNA was mixed with 10 μl SuRE/Cut restriction Buffer H, 5 μl of *Eco* RI, (10 U/μl), 5 μl of *Hin*d III, (10 U/μl) and 40 μl Milli-Q water then incubated for 2 hours at 37 °C. As a standard, 5 μl of the digested DNA was run per lane on the gel.

Electrophoresis

The gel was covered with TAE buffer, the samples were loaded and electrophoretically separated at 55 volts for 45 minutes. The gel was photographed under UV light using a Bio-Rad Gel Doc 1000.

2.15.5 *Restriction Digest of cDNA from Plasmid*

iNOS

To digest the iNOS cDNA from the plasmid, 5 μl of the DNA extract containing the plasmid and insert DNA (Section 2.15.3) was mixed with 4 μl of SuRE/Cut restriction buffer A, 2.5 μl of *Sma* I (10 U/μl) and 28.5 μl Milli-Q water. The digest was incubated at 37 °C for 2 ½ hours.

eNOS

To digest the eNOS cDNA from the plasmid, 40 μl of plasmid and insert DNA (Section 2.15.3) was mixed with 5 μl of SuRE/Cut restriction buffer H, 2.5 μl of *Eco* RI (10 U/μl) and 2.5 μl Milli-Q water. The digest was incubated at 37 °C for 2 ½ hours.

2.15.6 *Isolation and Purification of cDNA*

The digested sample (Section 2.15.5) was loaded onto a 1 % agarose gel and electrophoretically separated at 68 V for 1 hour. Under a UV light, the 4 kb band was cut from the gel using a scalpel blade. The cDNA was purified using an Agarose Gel DNA Extraction Kit according to the manufacturer's instructions. The pellet was

resuspended in 25 μl of TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0), incubated at 55 °C for 10 minutes then centrifuged at 12 000 rpm (Eppendorf Centrifuge 5417C). The supernatant containing the cDNA was transferred to a new tube and stored at -20 °C.

2.15.7 *Quantification of cDNA Concentration*

To determine the concentration of the purified cDNA in the TE buffer (Section 2.15.6), 1 μl was electrophoretically separated alongside 5 μl of λEH (Section 2.15.4) on a 1 % agarose gel and the intensity of the bands compared using a Bio-Rad GS-690 Imaging Densitometer.

2.15.8 *Radiolabelling of Probe*

Radiolabelling of probes was performed using Rediprime™II, and following the manufacturer's instructions. Briefly, 1 μl of purified cDNA in TE buffer (Section 2.15.6; 14.5 ng iNOS cDNA or 19 ng eNOS cDNA) was diluted with 46 μl of TE buffer and placed into a boiling water bath for 5 minutes. The samples were transferred to an ice bath for 5 minutes and then the contents were transferred to a Rediprime™II tube. Two and a half microlitres of Redivue [α-^{32}P]-dCTP (10 mCi/ml, specific activity 3000 Ci/mmol) was added and the probe was incubated for 30 minutes at 37 °C in a lead-lined container. To denature the sample, 14 μl of 4M NaOH was added and the probe was incubated for a further 10 minutes.

2.16 Northern Hybridisation

Pre-hybridisation was carried out by soaking the membrane in 25 ml Church and Gilbert solution (0.5 M sodium phosphate pH 7.2; 1 mM EDTA; 7 % SDS) for at least 20 minutes at 60 °C. The radiolabelled cDNA probe was added and the membrane incubated overnight at 55 °C (eNOS) or 65 °C (iNOS). The membrane was washed in the following solutions for 20 minutes with each wash, 2X SSC (0.3 M NaCl; 0.03 M sodium citrate) + 0.1 % SDS, 1X SSC + 0.1 % SDS and 0.5X SSC +

0.1 % SDS. Following the final wash the membranes were sealed in plastic and exposed to radiographic film at -80 °C for varying lengths of time. Films were then developed, fixed and washed in a 100 Plus All-Pro Imaging automatic X-ray film processor, and scanned using a Bio-Rad GS-690 Imaging Densitometer.

CHAPTER THREE

MEASUREMENT OF NITRIC OXIDE SYNTHASE IN THE MAMMARY GLAND

3.1 Abstract

The aim of this study was to find a method that could be used to routinely detect either nitric oxide synthase (NOS) or nitric oxide (NO) production within the mammary gland, so that further investigations into what controls the production of NO by the mammary gland could occur. This chapter reports the investigation of three different methods; Western analysis using specific antibodies for the detection of iNOS and eNOS protein, an activity assay which measures the conversion of ^{3}H-arginine to ^{3}H-citrulline by NOS, and the measurement of nitrite and nitrate, stable metabolites of NO. The results indicate that iNOS immunoreactive protein is not detected in the mammary gland, heart, spleen or liver of lactating rats or in the lung or mammary gland tissues excised from a lactating rat following an intraperitoneal injection of lipopolysaccharide (LPS). Polyclonal antibodies were unable to detect eNOS protein in the mammary gland and spleen of a lactating rat but did detect many small molecular weight proteins. The source of these small fragments is not known, however, it is possible that they are degradation products of NOS. The use of a monoclonal antibody to eNOS was also inconclusive. High NOS activity was apparent in the brain of a lactating rat with levels of activity in the lung and mammary gland almost undetectable. Further purification to obtain the particulate fractions of the mammary gland and lung did not increase the activity substantially. Measurement of nitrite plus nitrate (NOx) in culture medium utilising the conversion of nitrate to nitrite by nitrate reductase and the subsequent measurement of total nitrite using 2,3-diaminonapthalene proved to be a reliable method for the indirect measurement of NO production by cultured cells.

3.2 Introduction

Many different methods for the measurement of NO production have been reported and include both direct (for example, electrochemical) and indirect (for example, immunohistochemistry, production of nitrite and nitrate) methods. NO has a very short half-life and thus direct detection is often difficult. For a review on these different techniques and their associated advantages and disadvantages see Archer (1993), Kiechle & Malinski (1993) and Kiechle & Malinski (1996).

NO is produced when any of the three isoforms of the enzyme NOS converts arginine to citrulline. Western analysis using specific antibodies to the various isoforms of NOS have been used to detect the presence of NOS in various tissues including cultured bovine aortic endothelial cells (Pollock *et al.*, 1993), rat skeletal muscle (Silvagno *et al.*, 1996), rat uterus (Dong *et al.*, 1996) and in extracts of cultured rat mammary glands (Onoda & Inano, 1998). Many antibodies are now commercially available and therefore Western analysis provides a means of detecting the presence of the NOS enzyme within various tissues taken from different developmental stages. However, the presence of the enzyme does not necessarily mean that it is actively producing NO.

Thus, the activity of NOS is often measured. As NOS produces NO during the conversion of arginine to citrulline, the enzyme activity can be determined by measuring the appearance of ^{3}H-citrulline following incubation of the tissue or purified enzyme with ^{3}H-arginine under controlled conditions (Bredt & Snyder, 1989; Pollock *et al.*, 1991; Ribiere *et al.*, 1996). However, often the activity of the enzyme is low, therefore to increase the activity to detectable levels, NOS can be partially purified from various tissues (Pollock *et al.*, 1991). In many cell types, eNOS enzyme activity is predominately associated with the particulate fraction (Pollock *et al.*, 1991; Sessa *et al.*, 1992; Busconi & Michel, 1993; Michel *et al.*, 1993; Pollock *et al.*, 1993; Hecker *et al.*, 1994; Tracey *et al.*, 1994) thus a series of ultra centrifugation steps and 3-[(3-cholamidopropyl)-dimethylammonio]-1-propane-sulfonate [CHAPS] solubilisation of the enzyme preparations can enhance the detectable activity (Pollock *et al.*, 1991). Thus, although NOS purification from tissues can lead to the increased

sensitivity in the detection of the enzyme activity, it can make routine analysis of more than just a few samples difficult.

Nitric oxide production can also be determined by measuring the production of nitrite and nitrate. When NO reacts with O_2, it produces NO_2 which then forms nitrite and nitrate (Palmer *et al.*, 1987), and cultured macrophages have long been shown to increase the production of nitrite and nitrate in the medium using LPS and IFN-γ (Stuehr & Marletta, 1987). Measurement of this increase in NOx in the medium of cultured cells and explants is now widely used for reporting the production of NO, however, it is often just the production of nitrite that is reported (Lacasse & Prosser, 1995b; Gill, Low & Grigor, 1996; Low *et al.*, 1997; Onoda & Inano, 1998; Orucevic *et al.*, 1999). Although this method provides an indirect measurement of the production of NO, when combined with tissue and explant culture, it allows for a high throughput of samples and experiments.

Thus, the aim of this work was to determine a valid and reliable method for the routine detection of NOS and NO production in the mammary gland. This would then allow further investigation into the control of the regulation of NO within the gland.

3.3 Materials and Methods

3.3.1 *Animals*

Three lactating female rats of the Sprague-Dawley strain were used in these experiments. Animals were cared for as described in Section 2.1.

Following euthanasia by an intraperitoneal infusion of an overdose of sodium pentabarbitone, the right abdominal mammary gland, spleen, heart and liver of one female rat (D 15 postpartum), and the right abdominal mammary gland, lung and brain of a 2nd female rat (D 14 postpartum) were excised and homogenised as described below (Section 3.3.2). A lactating female rat (D 12 postpartum) was

infused intraperitoneally with 4 mg/kg LPS in sterile saline and sacrificed by CO_2 euthanasia 5.5 hours later. The right abdominal mammary gland, and a sample of lung were excised and homogenised as described below (Section 3.3.2).

Following euthanasia using CO_2, the brains of four female mice (supplied by Mr R Broadhurst, Ruakura Small Animal Colony, Hamilton, NZ) were excised, pooled and homogenised as described below (Section 3.3.2).

3.3.2 *Partial Purification of Nitric Oxide Synthase from Rat and Mouse Tissues*

The method used for partial purification of NOS from rat and mouse tissues is based upon that of Pollock *et al.* (1991).

Following removal, the tissue was cut into several pieces and washed in PBS. The pieces of tissue were then blotted to remove excess buffer, weighed and 2 g were homogenised using an Ultra-Turrax® homogeniser in 10 ml of ice cold NOS buffer A (pH 7.4; 50 mM Tris; 0.1 mM ethylene glycol-bis[β-amino-ethyl ether], EGTA; 0.1 mM ethylenediaminetetraacetic acid, EDTA; 0.1 % 2-mercaptoethanol; 2 μM leupeptin; 1 mM phenylmethylsulfonyl fluoride, PMSF, added immediately prior to use). The crude homogenate was centrifuged at 3 000 x *g* for 10 minutes, the supernatant was removed and re-centrifuged at 100 000 x *g* for 60 minutes. The resulting supernatant contained the cytosolic fraction. The pellet from the 100 000 x *g* centrifugation was resuspended in 2 ml ice cold NOS buffer B (pH 7.4; 50 mM Tris; 0.1 mM EGTA; 0.1 mM EDTA; 0.1 % 2-mercaptoethanol; 2 μM leupeptin; 20 mM CHAPS; 10 % glycerol; 1 mM PMSF [added immediately prior to use]). Following incubation at 4 °C for 20 minutes, the sample was centrifuged at 100 000 x *g* for 30 minutes. The supernatant contains the particulate fraction. Both fractions (particulate and cytosolic) as well as a sample of the crude homogenate were either used fresh or stored at -20 °C or -80 °C.

3.3.3 *BCA Assay for Total Protein*

Total protein content of samples (Section 3.3.2) were determined using the BCA assay as previously described (Section 2.7). Samples were prepared for analysis by diluting with Milli-Q water. NOS buffer A and NOS buffer B, also diluted with Milli-Q water, were used as the blanks.

3.3.4 *SDS-PAGE and Western Analysis of Nitric Oxide Synthase*

Samples were diluted prior to electrophoresis using either NOS buffer A for homogenate samples, or NOS buffer B for cytosolic and particulate samples, such that 20 μl contained 30 or 50 μg total protein. Samples (20 μl) were reduced by the addition of 2-mercaptoethanol and diluted with sample loading buffer (5 μl; 2.5 ml 0.5 M Tris; pH 6.8, 1.5 ml Milli-Q water, 8 ml glycerol, 1.0 g SDS, 10 % 2-mercaptoethanol and 1 % bromophenol blue). Following electrophoresis on 7.5 % SDS-PAGE gels and transfer of proteins as described in Sections 2.8 & 2.9, the nitrocellulose membranes were probed with the relevant antibodies after the method of Ventling & Hurley (1989) and as described in Section 2.10 with the following exceptions. Nitrocellulose membranes were blocked with 5 % (w/v) bovine serum albumin in TBS-NP40 (Tris buffered-saline-Nonidet-P40; 0.15 M NaCl, 0.1 M Tris, pH 7.6 containing 0.05 % Nonidet P-40 [w/v]) and rinsed with TBS-NP40. Primary and secondary antibodies were diluted to relevant dilutions (Table 3.1) in TBS-NP40 containing 1 % (w/v) bovine serum albumin, with TBS-NP40 used for all washes.

To confirm specificity of the rabbit anti-bovine eNOS antibody, the commercially available anti-eNOS blocking peptide was used. Briefly, 5 μl rabbit anti-bovine eNOS antibody, 50 μl PBS and 20 μl of anti-eNOS blocking peptide were mixed and incubated for 1 hour at 4 °C. The sample was centrifuged at 10 000 x *g* for 15 minutes and the supernatant was collected, diluted 1/2000 and used to probe a nitrocellulose membrane in the place of the primary antibody.

Table 3.1: Primary and secondary antibody dilutions used for Western analysis of rat and mouse tissues. The primary antibodies used are given along with the corresponding horseradish peroxidase conjugated secondary antibody that was used. Dilutions of the manufacturer's stocks are also given.

Primary	Dilution	Secondary - HRPO	Dilution
Rabbit anti-mouse iNOS polyclonal	1/5000	Donkey anti-rabbit	1/2000
Rabbit anti-human ecNOS polyclonal	1/1000	Donkey anti-rabbit	1/2000
Rabbit anti-bovine eNOS polyclonal	1/2000	Donkey anti-rabbit	1/2000
Mouse anti-human eNOS monoclonal	1/2500	Sheep anti-mouse	1/2000

3.3.5 *Nitric Oxide Synthase Activity Assay*

To measure the activity of the NOS enzymes, an assay which monitored the conversion of arginine to citrulline was employed. This method is based upon those of Pollock *et al.* (1991) and Ribiere *et al.* (1996).

Column Preparation

Columns were prepared as follows. Glass wool was compressed into 14.6 cm long glass disposable pasteur pipettes to a depth of approximately 2 mm. Dowex beads were added (2 ml, 1:1 with Milli-Q water). The column was equilibrated with stop buffer (20 mM HEPES, pH 5.5; 2 mM EGTA; 2 mM EDTA).

Sample Preparation

Samples were prepared as described in Section 3.3.2 and either left undiluted, or were diluted with NOS buffer A such that 50 µl contained between 100-400 µg total protein (Section 3.3.3).

Assay Method

To 50 µl of sample, either 50 µl of Reaction buffer A (50 mM HEPES pH 7.4; 50 mM L-valine; 1 mM $CaCl_2.2H_2O$; 20 µM L-arginine; 1 mM NADPH; 131.92 nM L-[2,3,4,5-^{3}H] arginine monohydrochloride; 1.0 mCi/ml; specific activity 50 Ci/mmol) or 50 µl of Reaction buffer B (50 mM HEPES pH 7.4; 50 mM L-valine; 1 mM $CaCl_2.2H_2O$; 20 µM L-arginine; 1 mM NADPH; 2 mM EGTA; 2 mM N^{ω}-nitro-L-arginine; 131.92 nM L-[2,3,4,5-^{3}H] arginine monohydrochloride) were added. The samples were incubated for 0, 10, 20 or 30 minutes and 400 µl stop buffer (20 mM HEPES, pH 5.5; 2 mM EGTA; 2 mM EDTA) was added. The sample was applied to a Dowex column and washed through with 0.5 ml Milli-Q water and the flow through collected. The column was then washed again with 1 ml Milli-Q water and the flow through collected. Three ml of scintillation fluid (OptiPhase 'HiSafe' 3) was mixed thoroughly with the individually collected flow-throughs and the radioactivity of the samples were counted on a 1409 Liquid Scintillation β-counter. Activity was expressed in pmol ^{3}H-citrulline/minute/mg protein.

3.3.6 *Nitrate and Nitrite Assay*

For the measurement of nitrite only, 50 µl of sample was mixed with 10 µl of NADPH and 40 µl of Milli-Q water and the assay continued as described in Section 2.4.3. NOx was measured as described in Section 2.4. The concentration of nitrate in a sample was calculated as the difference in the results obtained with these two methods.

3.4 Results

3.4.1 *Western Analysis of Nitric Oxide Synthase*

Western analysis using an iNOS antibody shows no evidence of iNOS immunoreactive protein at the expected molecular weight of 130 kDa in the cytosolic and particulate fractions of the mammary gland, spleen, heart and liver of a lactating

rat (Figure 3.1). There was iNOS protein apparent in the positive control (induced murine macrophages). The antibody also bound to smaller molecular weight proteins in both the positive control and the heart cytosolic fraction.

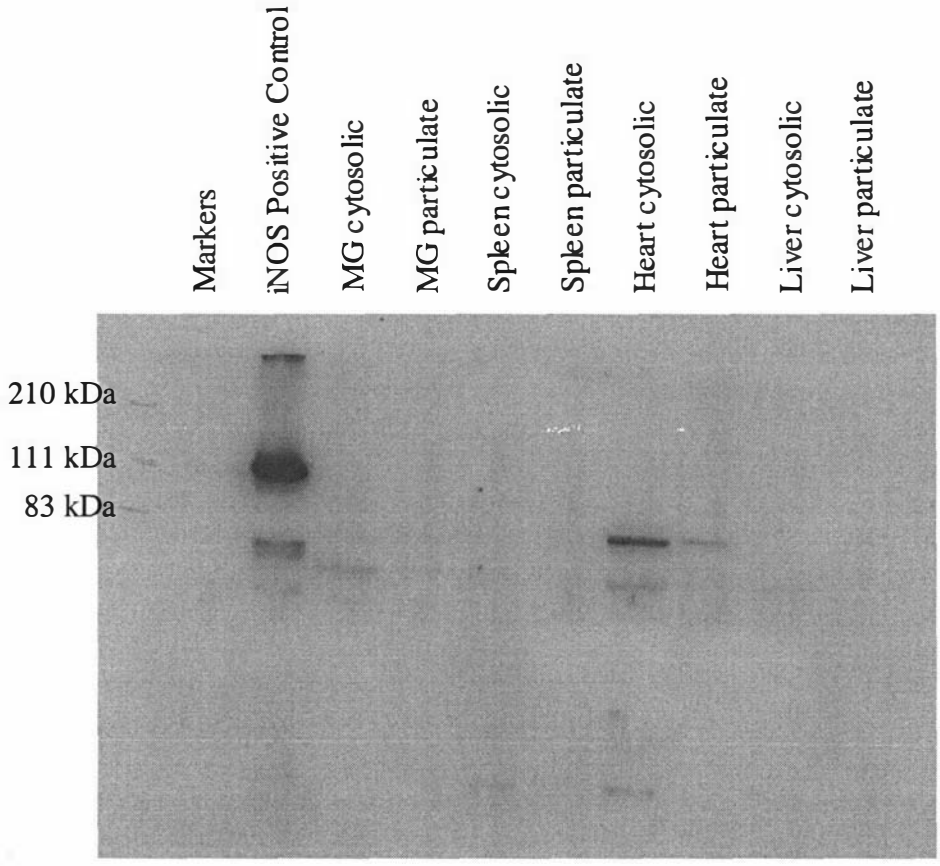

Figure 3.1: Western blot analysis of rat tissues using an anti-iNOS polyclonal antibody. Samples of the mammary gland (MG), spleen, heart and liver were collected from a lactating (D 15 postpartum) rat, homogenised then ultra centrifuged to obtain the cytosolic and particulate fractions. Samples (30 μg protein per lane) were electrophoretically separated on a 7.5 % SDS-PAGE gel. A commercial iNOS positive control (from induced mouse macrophages) was also run. Proteins were transferred to a nitrocellulose membrane, which was probed with a rabbit anti-mouse iNOS antibody.

When an anti-human eNOS polyclonal antibody was used to probe the membrane, there was no evidence of eNOS protein at the expected molecular weight of 140 kDa in any of the mammary gland or spleen samples (Figure 3.2). However, eNOS was apparent in the positive control at ~140 kDa. The antibody also detected many small molecular weight proteins in both the samples and controls.

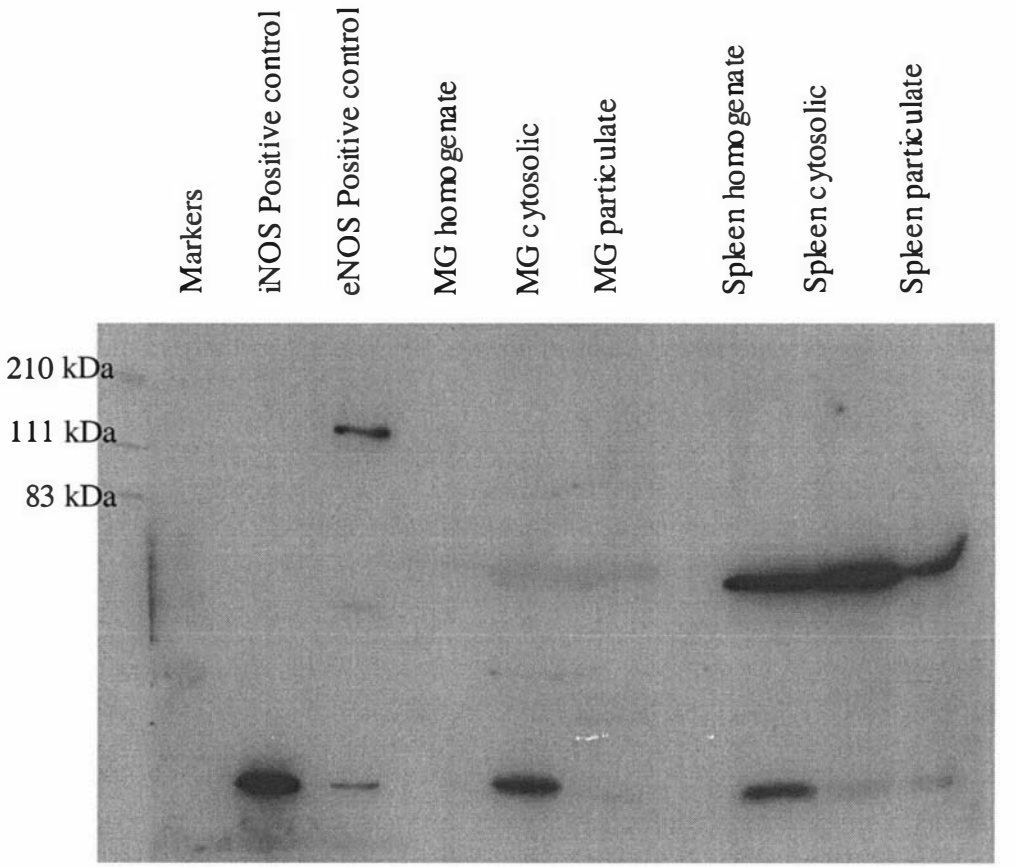

Figure 3.2: Western blot analysis of rat tissues using an anti-human eNOS polyclonal antibody. Samples of the mammary gland (MG) and spleen were collected from a lactating (D 15 postpartum) rat, homogenised then ultra centrifuged to obtain the cytosolic and particulate fractions. Samples (30 μg protein per lane) were electrophoretically separated on a 7.5 % SDS-PAGE gel. A commercial iNOS positive control (from induced mouse macrophages) and a commercial eNOS positive control (human endothelial cell lysate) were also run. Proteins were transferred to a nitrocellulose membrane, which was probed with a rabbit anti-human ecNOS antibody.

When the membrane was probed with an anti-bovine eNOS polyclonal antibody there was no eNOS apparent at the expected molecular weight of 140 kDa (Figure 3.3) in any of the samples. Apart from the band apparent at a molecular weight below 83 kDa, there was no eNOS apparent in the eNOS positive control lane. The antibody also detected many small molecular weight products in the samples. There was also strong cross reactivity between the antibody and the 210 (myosin) and 111 (β-galactosidase) kDa molecular weight markers.

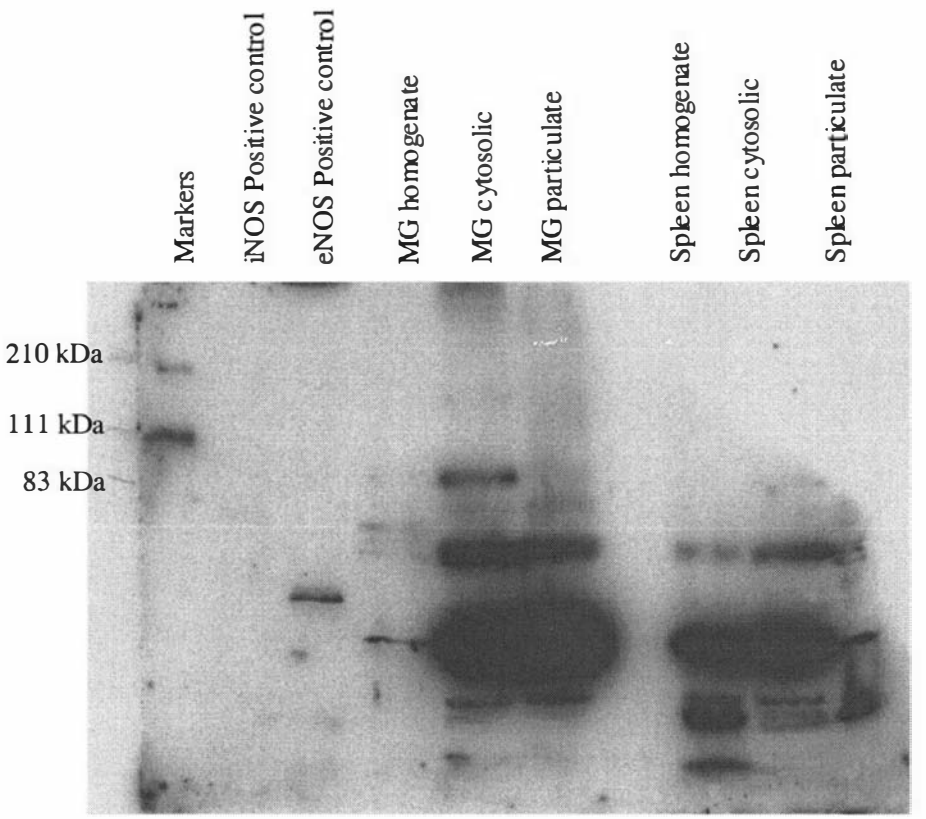

Figure 3.3: Western blot analysis of rat tissues using an anti-bovine eNOS polyclonal antibody. Samples of the mammary gland (MG) and spleen were collected from a lactating (D 15 postpartum) rat, homogenised then ultra centrifuged to obtain the cytosolic and particulate fractions. Samples (30 μg protein per lane) were electrophoretically separated on a 7.5 % SDS-PAGE gel. A commercial iNOS positive control (from induced mouse macrophages) and a commercial eNOS positive control (human endothelial cell lysate) were also run. Proteins were transferred to a nitrocellulose membrane, which was probed with a rabbit anti-bovine eNOS antibody.

Incubation of the membrane with the supernatant of a reaction between the anti-bovine eNOS polyclonal antibody and the anti-eNOS blocking peptide (Figure 3.4) reveals extensive cross reactivity and was almost indistinguishable from the membrane incubated with the anti-bovine eNOS antibody alone (Figure 3.3).

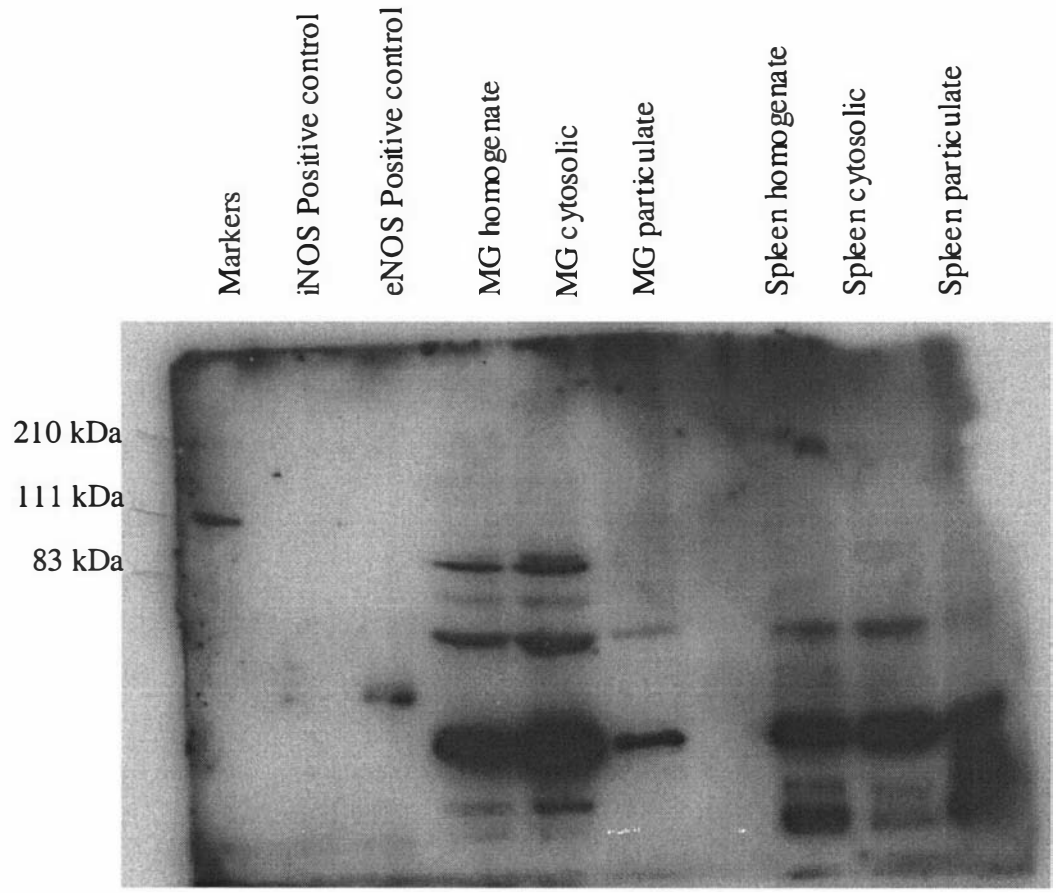

Figure 3.4: Western blot analysis of rat tissues using an anti-bovine eNOS polyclonal antibody and a blocking peptide. Samples of the mammary gland (MG) and spleen were collected from a lactating (D 15 postpartum) rat, homogenised then ultra centrifuged to obtain the cytosolic and particulate fractions. Samples (30 μg protein per lane) were electrophoretically separated on a 7.5 % SDS-PAGE gel. A commercial iNOS positive control (from induced mouse macrophages) and a commercial eNOS positive control (human endothelial cell lysate) were also run. Proteins were transferred to a nitrocellulose membrane, which was probed with a rabbit anti-bovine eNOS antibody, which had been incubated with an anti-eNOS control peptide.

Incubation of the membrane with the donkey anti-rabbit horseradish peroxidase conjugated secondary antibody without prior incubation with a primary antibody demonstrated very little cross-reactivity with the samples on the membrane (Figure 3.5).

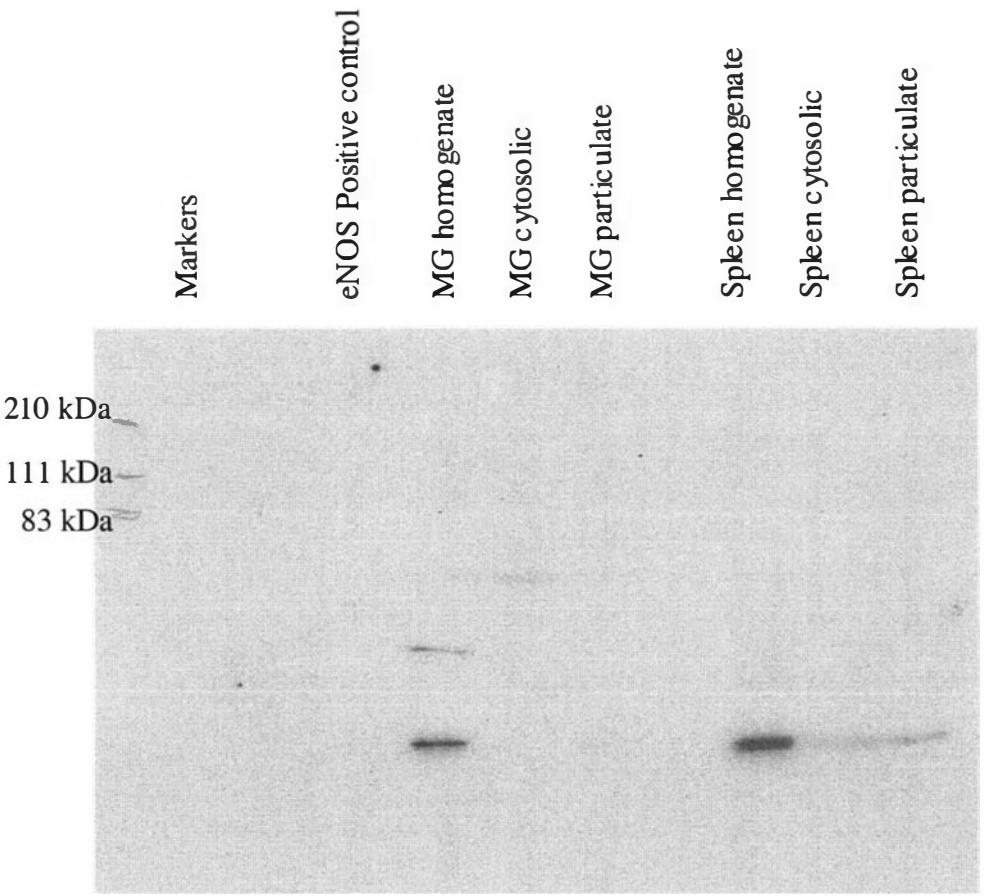

Figure 3.5: Western blot analysis of rat tissues using no primary antibody. Samples of the mammary gland (MG) and spleen were collected from a lactating (D 15 postpartum) rat, homogenised then ultra centrifuged to obtain the cytosolic and particulate fractions. Samples (30 μg protein per lane) were electrophoretically separated on a 7.5 % SDS-PAGE gel. A commercial eNOS positive control (human endothelial cell lysate) was also run. Proteins were transferred to a nitrocellulose membrane, which was probed with a donkey anti rabbit horseradish peroxidase conjugated secondary antibody only.

Western analysis, using an anti-human monoclonal antibody, of samples of mammary gland and lung tissues excised from a lactating rat 5.5 hours following intraperitoneal infusion of LPS suggests the presence of an eNOS immunoreactive band at 140 kDa in the particulate fractions (Figure 3.6). The antibody also detected many other smaller molecular weight products, as well as a small molecular weight product in the eNOS positive control. Subsequent attempts to reproduce this blot were unsuccessful.

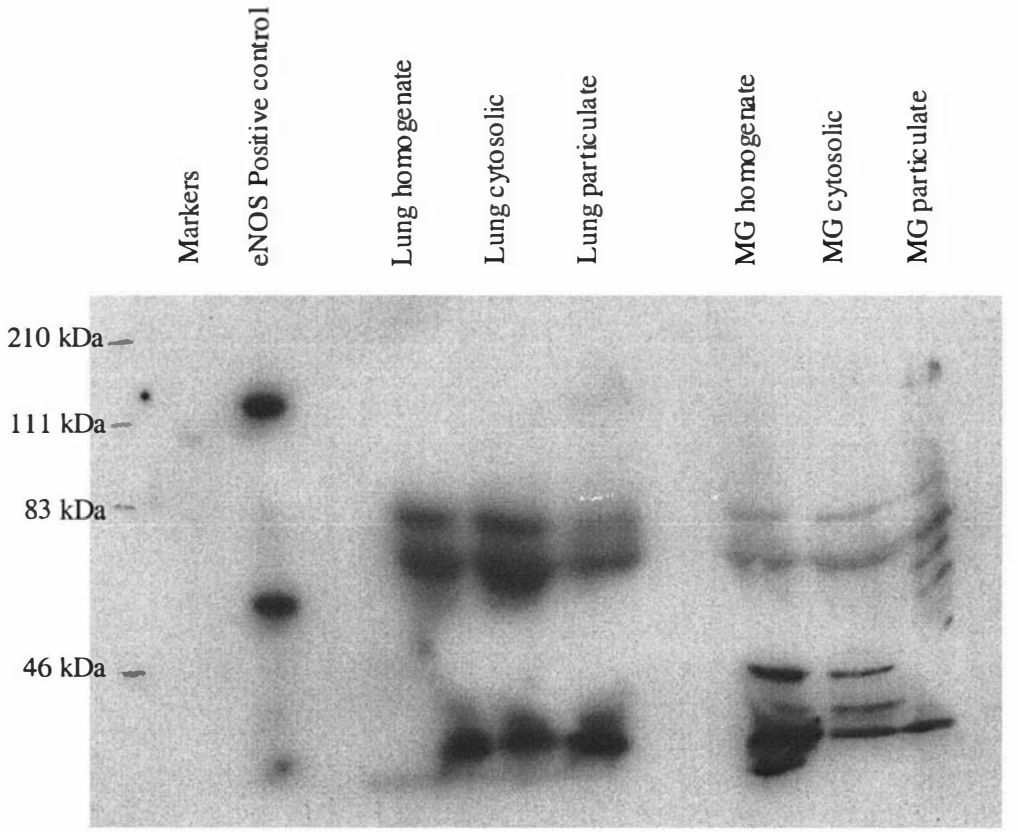

Figure 3.6: Western blot analysis of rat tissues from a rat treated with LPS using an anti-human eNOS monoclonal antibody. Samples of the mammary gland (MG) and lung were collected from a lactating (D 12 postpartum) rat which had been treated with 4 mg/kg LPS and euthanased 5.5 hours later. The samples were homogenised then ultra centrifuged to obtain the cytosolic and particulate fractions. Samples (50 μg protein per lane) were electrophoretically separated on a 7.5 % SDS-PAGE gel. A commercial eNOS positive control (human endothelial cell lysate) was also run. Proteins were transferred to a nitrocellulose membrane, which was probed with a mouse anti-human eNOS antibody.

No iNOS protein was detected in the lung or mammary gland of a female rat 5.5 hours following infusion of LPS using an anti-mouse iNOS antibody. The antibody did, however, detect iNOS immunoreactive protein in the positive control (Figure 3.7). The antibody also detected proteins smaller than 83 kDa in all the samples.

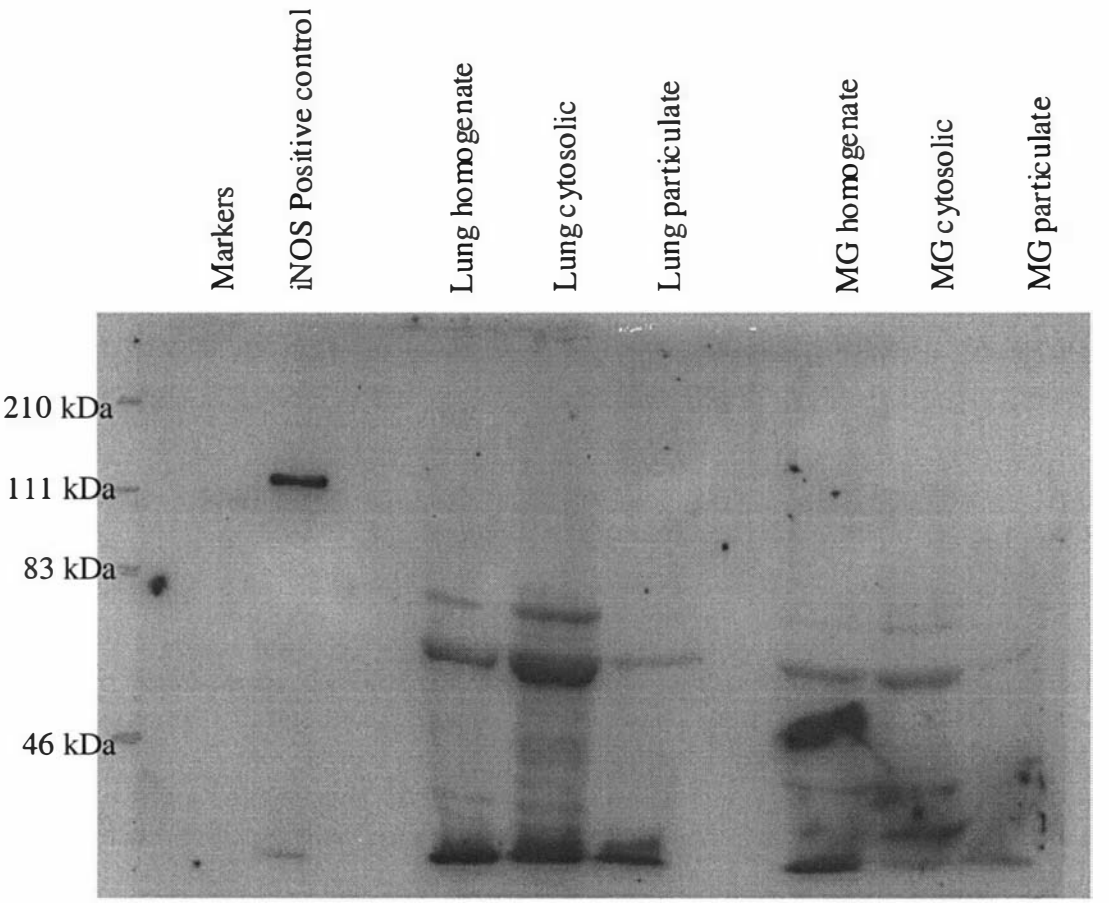

Figure 3.7: Western blot analysis of rat tissues from a rat treated with LPS using a rabbit anti-mouse iNOS antibody. Samples of the mammary gland (MG) and lung were collected from a lactating (D 12 postpartum) rat which had been treated with 4 mg/kg LPS and euthanased 5.5 hours later. The samples were homogenised then ultra centrifuged to obtain the cytosolic and particulate fractions. Samples (50 μg protein per lane) were electrophoretically separated on a 7.5 % SDS-PAGE gel. A commercial iNOS positive control (from induced mouse macrophages) was also run. Proteins were transferred to a nitrocellulose membrane, which was probed with a rabbit anti-mouse iNOS antibody.

3.4.2 *Nitric Oxide Synthase Activity Assay*

Product formation by NOS was linear over time as determined by the conversion of ^{3}H-arginine to ^{3}H-citrulline by homogenates of rat brain taken from a lactating rat. Very little activity was measured in samples of mammary tissue and lung (Figure 3.8).

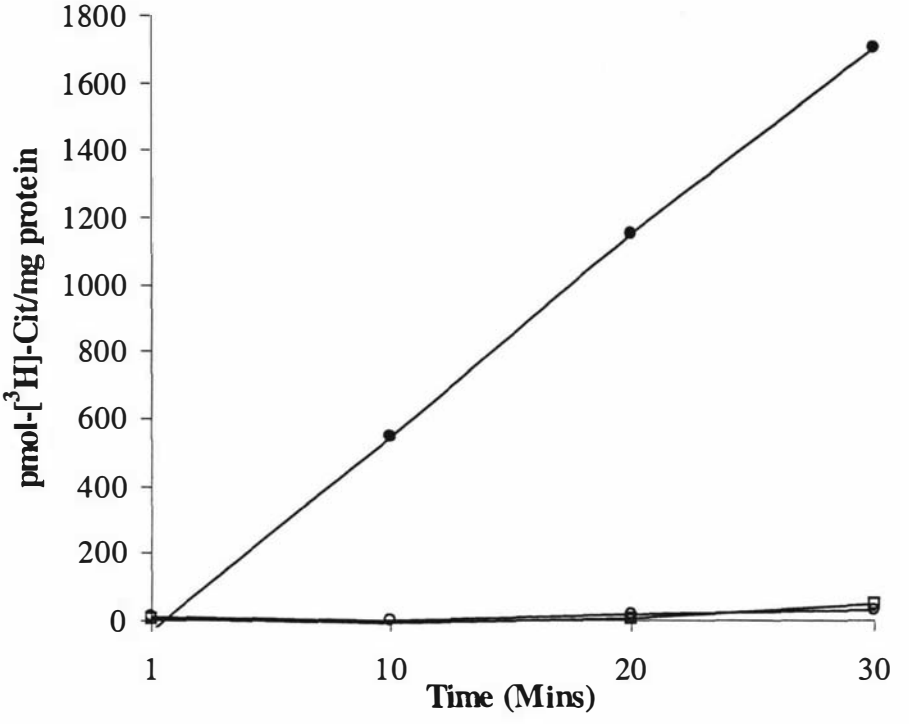

Figure 3.8: NOS activity in homogenates of brain, mammary tissue and lung of a lactating rat. Samples of the mammary gland (-o-), brain (-•-) and lung (-□-) were collected from a lactating (D 14 postpartum) rat and homogenised. The samples were stored overnight at -80 °C and the NOS activity was measured by determining the amount of conversion of ^{3}H-arginine to ^{3}H-citrulline. Activity of NOS is expressed as the pmol-[^{3}H]-citrulline produced/mg protein at the times indicated.

Further purification of the mammary gland and lung samples to obtain the particulate fractions did not increase the activity substantially (data not shown). The activity that was measured in the samples did not increase linearly with time.

The activity of NOS in homogenates of murine brains (Figure 3.9) following seven days at -80 °C was similar to that of the fresh sample, however, when the sample was stored at -80 °C for six days then -20 °C overnight, the activity was about 10-fold lower than that exhibited by the fresh sample. The activity exhibited by the murine brain homogenate was approximately half that measured in the rat brain homogenate (approximately 50 pmol [^{3}H]-cit/min/mg protein; Figure 3.8)

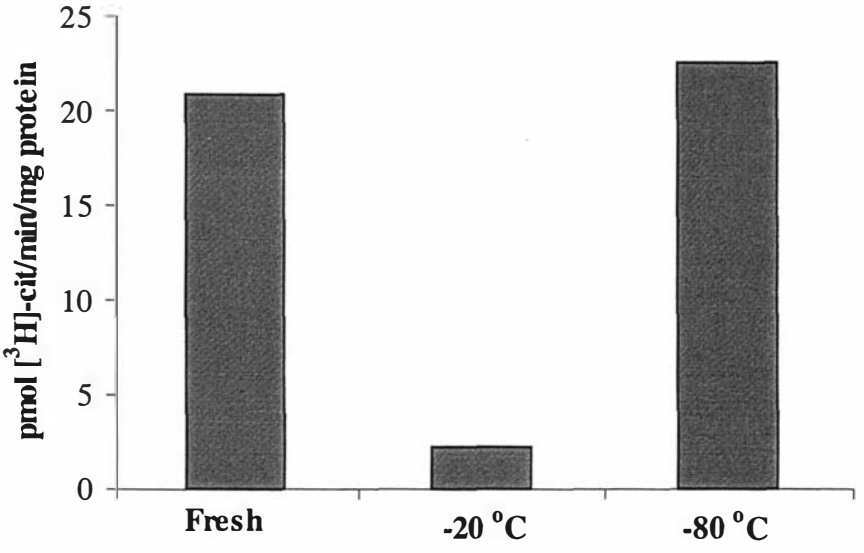

Figure 3.9: Effect of storage temperature on the NOS activity in the brains of female mice. The brains were excised from 4 female mice and homogenised. Sub samples were taken and treated as follows. One sub sample was analysed for activity immediately (Fresh). One sub sample was stored for 7 days at -80 °C (-80 °C), and another was stored at -80 °C for 6 days, then overnight at -20 °C (-20 °C). The NOS activity was measured in all samples by determining the amount of conversion of ^{3}H-arginine to ^{3}H-citrulline following a 10 minute incubation period. Activity of NOS is expressed as the pmol-[^{3}H]-citrulline produced/min/mg protein.

3.4.3 *Nitrate and Nitrite Assay*

Initial experiments, not presented in this Thesis, indicated that the amount of nitrite and nitrate in samples was variable and often the concentration of nitrate was greater than that of nitrite.

The concentration of nitrite in the samples were measured by the method described by Misko *et al.* (1993) in which the samples were incubated for 10 minutes with 2,3-diaminonapthalene. To determine if the timing of this incubation was crucial, the samples were incubated with 2,3-diaminonapthalene for 20 minutes (Figure 3.10). Comparison of the two curves indicates that there is no difference in the amount of nitrite detected by the 2,3-diaminonapthalene over this extra incubation period.

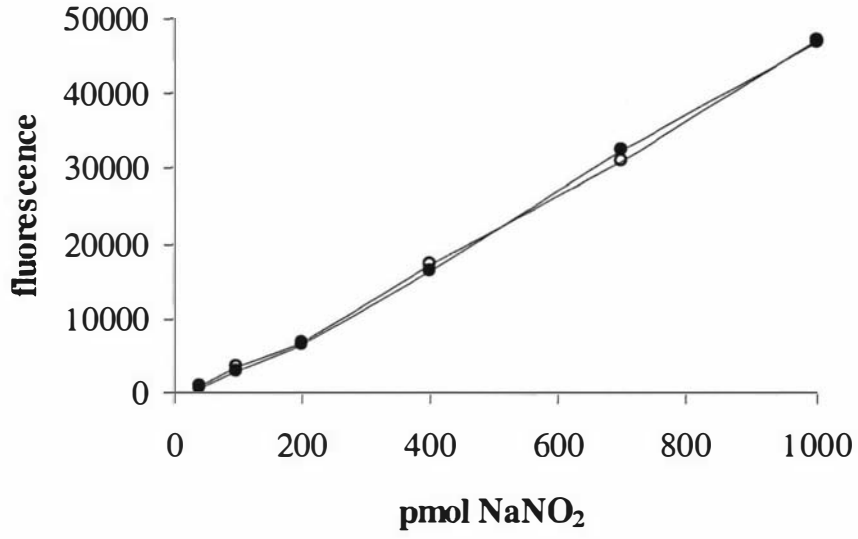

Figure 3.10: Effect of length of incubation time on the measurement of nitrite using 2,3-diaminonapthalene. $NaNO_2$ standards were prepared in tissue culture quiescent medium (DMEM:F12 containing 100 U/ml penicillin, 100 µg/ml streptomycin and 3.7 g/l $NaHCO_3$, pH 7.4) and assayed for the presence of nitrite by incubation with 2,3-diaminonaphthalene for either 10 (-●-) or 20 minutes (-○-).

Nitrate in the samples was converted to nitrite by incubating the samples for 45 minutes with nitrate reductase (Verdon *et al.*, 1995). In initial experiments (data not

shown) there was often not full conversion of nitrate to nitrite as indicated by direct comparison of the nitrite and nitrate curves. Thus, samples were incubated with nitrate reductase for 1.5 hours and the results compared to that of the 45 minute incubation. Full conversion of nitrate to nitrite occurred after a 1.5 hour incubation period with nitrate reductase (Figure 3.11). In contrast, full conversion did not occur if the standards were incubated for 45 minutes.

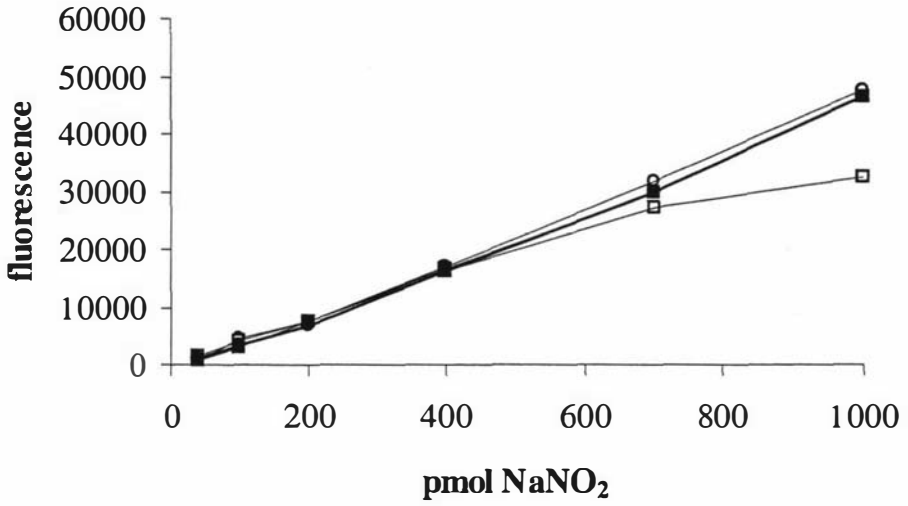

Figure 3.11: Length of incubation time with nitrate reductase on the conversion of nitrate to nitrite. $NaNO_3$ (-○-;-□-) and $NaNO_2$ (-●-;-■-) standards were prepared in tissue culture quiescent medium (DMEM:F12 containing 100 U/ml penicillin, 100 μg/ml streptomycin and 3.7 g/l $NaHCO_3$, pH 7.4) and assayed for the presence of nitrite by incubation with nitrate reductase for either 45 minutes (-□-;-■-) or 1.5 hours (-○-;-●-), followed by incubation for 10 minutes with 2,3-diaminonapthalene.

Experiments not presented in this Thesis indicate that the NADPH and glucose-6-phosphate dehydrogenase solutions must be made immediately prior to assay. Nitrate reductase solutions were stable when stored at -80 °C for approximately 1 month and the glucose-6-phosphate solution was stable at -20 °C. Both the 20 mM $NaNO_2$ and 20 mM $NaNO_3$ stocks (prepared in Milli-Q water) and stored at 4 °C were stable for a period of approximately 2 years and 5 months as determined by the comparison of

standard curves generated using fresh stocks, and the standard curves produced using the same stocks after nearly 2 ½ years of storage.

Experiments reported in subsequent chapters of this Thesis utilise this assay for determining the production of NO by both cultured Comma-D cells and explants of mammary tissue excised from both pregnant and lactating rats.

3.5 Discussion

3.5.1 *Western Analysis of Nitric Oxide Synthase*

NOS produces NO when it converts arginine to citrulline. Therefore, detection of the NOS enzyme can provide indirect evidence of the ability of the mammary gland to produce NO. The presence of two of the NOS isoforms, iNOS and eNOS were examined using commercially available antibodies.

Given that many different cells and tissue types respond to challenge with cytokines and cytotoxins with the increased production of NO, and the up-regulation of iNOS protein and mRNA (Section 1.2.5), this isoform was sought in the mammary gland of lactating rats. Western analysis of rat tissues collected from a lactating rat using an iNOS antibody did not indicate the presence of iNOS protein at the expected molecular weight of 130 kDa in any of the tissues examined (Figure 3.1). The antibody used did detect iNOS immunoreactive protein in the iNOS positive control, and it also detected many small molecular weight proteins (below 83 kDa) in the heart and mammary gland samples. Similarly, iNOS was not apparent at the expected molecular weight of 130 kDa in tissues excised from a rat 5.5 hours following injection of LPS (Figure 3.7). However, the antibody reacted with a number of proteins smaller than 83 kDa (Figure 3.1). The presence of iNOS immunoreactive protein following incubation of explants of mammary tissue with LPS (48 hours) has been reported (Onoda & Inano, 1998). No iNOS was detected in non-treated explants. As the maximum iNOS mRNA transcripts are not apparent until 6-8 hours following induction (Asano *et al.*, 1994; Adler *et al.*, 1995; Menegazzi *et al.*, 1996;

Low *et al.*, 1997), the failure to detect iNOS protein in the present experiments could be due to an inadequate length of time between treatment and euthanasia for the protein to have become apparent. In a subsequent experiment (data not shown), a lactating rat was injected with 3.6 mg/kg LPS into the teat canal of the right abdominal mammary gland and sacrificed 15 hours later. There was iNOS immunoreactive protein apparent in the liver and lung, but not in the mammary gland. LPS was infused at less than 4 mg/kg as it is reported as being the lowest dose that promotes maximal iNOS induction with minimal cardiovascular changes (Salvemini & Masferrer, 1996). Given the inconclusive results reported to date, to repeat this experiment with a higher dose of LPS would have been unethical.

The source of the small molecular weight proteins detected by the iNOS antibody in the Western blots presented in this study is not known (Figure 3.1 and Figure 3.7). One possibility is that they are proteolytic fragments of iNOS, which were produced either within the tissues examined, or as a product of the purification process. If these are proteolytic fragments then this may explain the absence of iNOS protein in the tissue samples and the abundance of the small molecular weight bands. The production of NO following stimulation with cytokines or cytotoxins had been previously reported in the mammary gland (Low *et al.*, 1997; Onoda & Inano, 1998) and therefore as the Western analyses presented in this chapter did not support this, other methods to investigate the presence of iNOS and/or production of NOS from this isoform were sought.

Immunocytochemistry has detected eNOS within the epithelium of the bovine mammary gland (Lacasse *et al.*, 1996). The aim of these experiments was to determine if eNOS was present in the mammary gland of lactating rats. Using either an anti-human eNOS (Figure 3.2) or an anti-bovine eNOS antibody (Figure 3.3), neither the mammary gland or spleen of a lactating rat appear to contain eNOS at the expected molecular weight of 140 kDa, although the antibodies did detect many smaller (below 83 kDa) molecular weight proteins. However, the anti-bovine eNOS antibody did not detect eNOS in the eNOS positive control (human endothelial cell lysate; Figure 3.3) suggesting a lack of specificity toward the human sample. However, this contradicts the information on the manufacturers' specification sheets, which indicate that the antibody shows specificity towards eNOS from rat, mouse and

human. This therefore places doubt on the results for the rat tissues using the anti-bovine eNOS antibody, as a positive result using this antibody was not achieved. Given that the antibody manufacturers do not indicate whether the antibody recognises bovine eNOS (one would assume it did), it is not known if analysis of bovine tissue using this antibody would have yielded any further information regarding the specificity of the antibody and further clues as to why it did not detect eNOS in the rat tissue samples.

Analysis of tissues from the LPS treated rat using the anti-human eNOS antibody suggested that there was some immunoreactivity in the particulate samples at ~140 kDa (Figure 3.6). However, subsequent attempts to repeat this blot, utilising the same samples were unsuccessful. It is not known why this blot could not be repeated although it may be possible that the storage of the samples resulted in the degradation of the eNOS protein.

The detection of the small molecular weight bands by the anti-human, and more so by the anti-bovine eNOS antibodies was a concern. The use of an eNOS blocking peptide, which was included with the anti-eNOS antibody from the manufacturer, did not stop this detection of the smaller molecular weight bands (Figure 3.4), suggesting that the secondary antibody could be binding directly to the lower molecular weight bands. This was subsequently shown not to be the case as the use of the secondary antibody alone only resulted in very few bands becoming apparent (Figure 3.5). Not long after this work was completed, the detection of small molecular weight proteins using an eNOS antibody, following extraction from rat mammary explants was reported (Onoda & Inano, 1998), with the authors suggesting that the small molecular weight bands were degradation products of eNOS. However, as the data were not shown, no direct comparison could be made.

The iNOS antibody and eNOS antibodies used in this study detected many small molecular weight products. There was also an apparent lack of eNOS or iNOS protein in the mammary gland, a result which directly contrasts previous reports which found that the mammary gland is capable of NO production and therefore suggests that NOS must be present for this to occur (Lacasse & Prosser, 1995b; Lacasse *et al.*, 1996; Low *et al.*, 1997). Thus, a different method for examining the

presence of the NOS enzyme and the production of NO within the mammary gland was sought.

3.5.2 *Nitric Oxide Synthase Activity Assay*

As the use of antibodies for the detection of NOS was not particularly successful (Section 3.5.1), an assay to measure the activity of the enzyme was investigated. Although the advantage of an activity assay over Western analysis is that it does more than just confirm if the enzyme is present, a disadvantage is that there is no real means of distinguishing between the isoforms. At this stage the goal was to confirm the presence of NOS or the production of NO in the mammary gland and therefore all avenues were being investigated. Minimal NOS activity was detected in homogenates of mammary gland and lung from a lactating rat (Figure 3.8), whereas the brain homogenate showed considerable activity. Further purification of the mammary gland and lung samples to obtain the particulate fractions did not increase the observed activity to any great extent. Although storage temperature affected the activity of the samples, it was determined that samples could be stored up to a week at -80 °C without a loss of activity (Figure 3.9). However, as the further purification of the NOS enzyme from mammary tissue did not improve the ability of the assay to accurately measure the NOS activity, a different method was sought to use to measure the presence of NOS or the production of NO from the mammary gland.

Thus, as the Western analysis provided inconclusive results and the activity assay appeared not to adequately measure the activity of NOS in the mammary gland, even following partial purification of the enzyme, another method of detection was sought.

3.5.3 *Nitrate and Nitrite Assay*

Although providing an indirect method, NO production is often detected by measuring the stable metabolites nitrite and nitrate (Stuehr & Marletta, 1987; Bouchard *et al.*, 1999; Blum *et al.*, 2000). The assay for the measurement of nitrate and nitrite (NOx) in samples is described in Section 2.4 and occurs in two steps. In the first step nitrate is reduced to nitrite by nitrate reductase (Verdon *et al.*, 1995). In the second step the concentration of nitrite was measured using 2,3-

diaminonapthalene based on the method of Misko *et al.* (1993). To improve the efficiency of the assay methods, the length of time of incubation of the samples with 2,3-diaminonapthalene was investigated. This showed that there was no difference in the amount of nitrite detected following either a 10 or 20 minute incubation period (Figure 3.10). As initial experiments were variable in the amount of nitrate converted using nitrate reductase, the incubation period was doubled and proved effective in ensuring full conversion occurred (Figure 3.11). The assay method described in Section 2.4 reflects these modifications.

The production of nitrite and nitrate from cultured macrophages is constant and occurs in a ration of 3:2 (Stuehr & Marletta, 1987). However, results not presented in this Thesis, show that the ratio of the two anions produced by Comma-D cells differs from this ratio and is often quite variable between experiments (in three independent experiments, ratios of 1:0.6, 1:1.6 and 1:1.5 were found for nitrite and nitrate). Therefore, the use of nitrate reductase for the conversion of nitrate to nitrite allows the measurement of all nitrite and nitrate produced by the cells, ensuring that all NO production is accounted for. This also suggests that in previous reports that solely measure nitrite as an indicator of NO production are underestimating the actual NO production by the cells and tissues (Low *et al.*, 1997; Onoda & Inano, 1998).

3.5.4 *General Comments*

Results described in this chapter provide evidence that the combination of the two methods for the detection of nitrite and nitrate provide an excellent assay for the indirect measurement of NO production and that the methods are capable of providing information that is more reliable than either the use of Western analysis or the arginine-citrulline activity assay.

CHAPTER FOUR

CHARACTERISATION OF NITRIC OXIDE PRODUCTION IN THE MOUSE MAMMARY EPITHELIAL CELL LINE, COMMA-D

4.1 Abstract

The production of nitric oxide (NO) by the murine mammary epithelial cell line, Comma-D was investigated to assess its suitability as a model system to understand the controls of NO production by the mammary gland. When grown to confluence then made quiescent, Comma-D cells respond to both rat and mouse recombinant IFN-γ by releasing increasing amounts of nitrite and nitrate (NOx) into the media. These increases were inhibited using aminoguanidine (AG) and N^{ω}-nitro-L-arginine (L-NNA), both known nitric oxide synthase (NOS) inhibitors. Following stimulation, the first significant increase in the amount of NOx in the media, when compared to non-stimulated cells, is apparent after 12 hours in culture. Lipopolysaccharide (LPS, a component of the bacterial cell wall) was also shown to increase the production of NOx although there was no apparent dose response. These results show that murine mammary epithelial cells can produce NO and that they respond to stimulators and inhibitors of NOS in a well-defined manner. Thus, it is considered that Comma-D cells provide a valid model with which to investigate further the production of NO by the mammary gland.

4.2 Introduction

The Comma-D cell line is often used as a model system for the investigation of mammary function and has been shown to exhibit many characteristics of normal mammary epithelial cells (Danielson *et al.*, 1984). Although NO production has been characterised in various tissue and cell types (Section 1.2.3), there is little information about the production of NO in the mammary gland. However, the studies published show that the mammary gland is capable of NO production and is responsive to known stimulators and inhibitors of the NO system (Lacasse *et al.*, 1995; Lacasse & Prosser, 1995a; Lacasse & Prosser, 1995b; Low *et al.*, 1997; Onoda & Inano, 1998). In this chapter the production of NO is investigated in Comma-D cells in a series of experiments in an attempt to determine if the cells provide a valid model system for future experiments examining the regulation of NO production. Each experiment is described independently and an introduction and discussion of the results are also included. A general discussion follows.

4.3 General Materials and Methods

4.3.1 *Comma-D Culture*

The Comma-D cells used for experiments described in this chapter were between passage numbers 17 and 27. Unless otherwise stated, Comma-D cells were seeded at 2 x 10^5 cells/well into 24 well tissue culture plates and grown for 4 days in growth medium (Section 2.3.1). The cells were then made quiescent by the removal of serum and growth factors for 48 hours. During this time, the cells were given fresh medium at 24 hours, thus allowing a 24 hour period of NOx accumulation at the end of the 48 hours. These media were collected and stored prior to analysis of NOx. Treatments were applied to the plates in triplicate in quiescent media. Following 24 hours in culture, the media were collected and stored for future analysis of NOx. Cells were harvested by sonicating in 500 µl of PBS, which both detaches and lyses the cells. Cell solutions were stored at -20 °C prior to being analysed for DNA content as described in Section 2.6.

4.3.2 *Statistical Analyses*

Unless otherwise stated analyses of difference in DNA content between treatments and experiments was done by ANOVA using SAS (SAS System for Windows, Release 6.12, 1996; SAS Institute Inc; Cary, NC, USA). Differences in the production of NOx were also analysed by ANOVA although in experiments when basal media were collected (24 hours pre-treatment), the basal production of NOx was used as a covariate and the experiment analysed by ANCOVA. Statistical significance was accepted when $P < 0.05$. Statistical differences between means presented in bar graphs are shown by the use of superscript letters. Commonality of letters indicates no significant difference, where as treatments with different letters are significantly different at $P < 0.05$. Statistical differences between means presented in line graphs are shown by the use of an asterisk (*) where means with an asterisk are significantly different at $P < 0.05$.

All analyses included the use of residual plots to determine if transformation of the data were required. In those experiments that were analysed using transformed data, either a log or square root transformation was used. The data presented are the means following back-transformation.

4.4 Growth of Comma-D Cells in Interior vs. Exterior Wells

4.4.1 *Introduction*

The culture of Comma-D cells in the interior eight wells of a 24 well tissue culture plate consistently results in greater cell numbers (DNA content) when the cells are grown to confluence (Bennett, 1995). Such a difference in the rate of proliferation of the cells in inner and outer wells may also lead to a difference in NOx production. For example, NOx production increases in bovine aortic endothelial cells (BAEC) during proliferation (Arnal *et al.*, 1994).

4.4.2 *Experimental Design*

In five independent experiments, Comma-D cells were seeded into 24 well tissue culture plates and grown to confluence (5 days following seeding) in the presence of serum and growth factors. Media and cells were collected and subsequently analysed for NOx and DNA respectively.

4.4.3 *Results*

Whether the cells were cultured in the interior eight wells or the exterior 16 wells did not alter either the DNA content of the wells (cell number) or the production of NOx by the cells within those wells (Table 4.1).

Table 4.1: Production of NOx and DNA in interior vs. exterior wells. Comma-D cells were seeded into 24 well tissue culture dishes and cultured in growth medium until confluent. Media and cells were collected and analysed for NOx and DNA respectively. Both the NOx and DNA concentrations of the external (n = 16) and internal (n = 8) wells are presented as means ± SEM of 5 independent experiments.

	External Wells	Internal Wells
DNA (μg/ml)	29.0 ± 0.4	28.2 ± 0.6
NOx (pmol/μg DNA)	90.5 ± 3.4	91.1 ± 4.9

4.4.4 *Discussion*

Bennett (1995) showed that the interior wells contained greater cell numbers when compared to the exterior wells. Thus treatments were allocated to different wells in the plate, so that this bias was removed. In these experiments no apparent difference existed in the rate of growth of the cells in different parts of the plate. However, treatments applied to tissue culture plates throughout this Thesis (Figure 2.1) were grouped in the same manner as that used by Bennett (1995) as it was convenient to do so. The production of NOx is not altered by the position on the plate.

The differences between the results presented here and those of Bennett (1995) may lie in the degree of confluence of the cultures. Once confluence is reached cells stop proliferating, therefore it is possible that if the cells were grown to near confluence, differences may be apparent. In the experiments presented above, the cells were confluent by 5 days following seeding. It may be that Bennett (1995) grew the cells to a near confluent stage in contrast to the fully confluent state of cells cultured in these experiments.

In BAEC the production of NOx/μg protein/hr decreases once confluence is reached (Arnal *et al.*, 1994). If Comma-D cells respond to a confluent state as the BAEC do, then there would be a decreased production of NOx by the cells in the wells that had reached confluence earlier. The NOx production by the cells cultured in the internal wells was not different from the production by the cells in the exterior wells suggesting that both were at the same stage of confluence.

Thus, in conclusion, neither the growth of Comma-D cells, nor the production of NOx by the cells are affected by the position on the tissue culture plate.

4.5 Induction of Quiescence

4.5.1 *Introduction*

The role of cytokines in the induction of NO production has been studied by examining the effects on Comma-D cells which were cultured for a period of 48 hours without serum and growth factors to induce quiescence prior to treatment with various cytokines (Low *et al.*, 1997). However, the basis for using this period of time was not reported. Thus, this experiment examines whether 48 hours is an adequate period of time in which to induce quiescence.

4.5.2 *Experimental Design*

Comma-D cells were grown for 4 days in 24 well culture plates (four independent experiments). The cells were then either cultured in growth medium, or cultured without serum and growth factors (quiescent medium) for 72 hours. During this time, media were collected on eight occasions (0, 6, 12, 24, 30, 48, 54 and 72 hours) from a 'pair' of treatment groups (triplicate wells of either growth medium or quiescent medium) and replaced with PBS. After removal of medium from the last pair of treatment groups (72 hours), the cells were collected from all wells. Total NOx in the media and DNA content of the cultures were measured as described in Sections 2.5 and 2.6.

4.5.3 *Statistical Analyses*

Differences in the production of NOx and the DNA content of the wells between the cells in growth medium and those in quiescent medium were analysed at each time point by ANOVA using SAS. Differences in the amount of DNA at each time point for those cells in either quiescent medium or growth medium were also analysed by ANOVA using SAS.

4.5.4 *Results*

To determine if the cells in the cultures were still growing, the changes in the amount of DNA over time were examined for cultures in both quiescent and growth medium. There was no change in the amount of DNA in the cultures in quiescent medium over the experimental period (Figure 4.1). In contrast, there was a significant change ($P < 0.05$) in the DNA content of the cultures in growth medium during the first 6 hours. There was no change in DNA content of the cultures in growth medium after 6 hours.

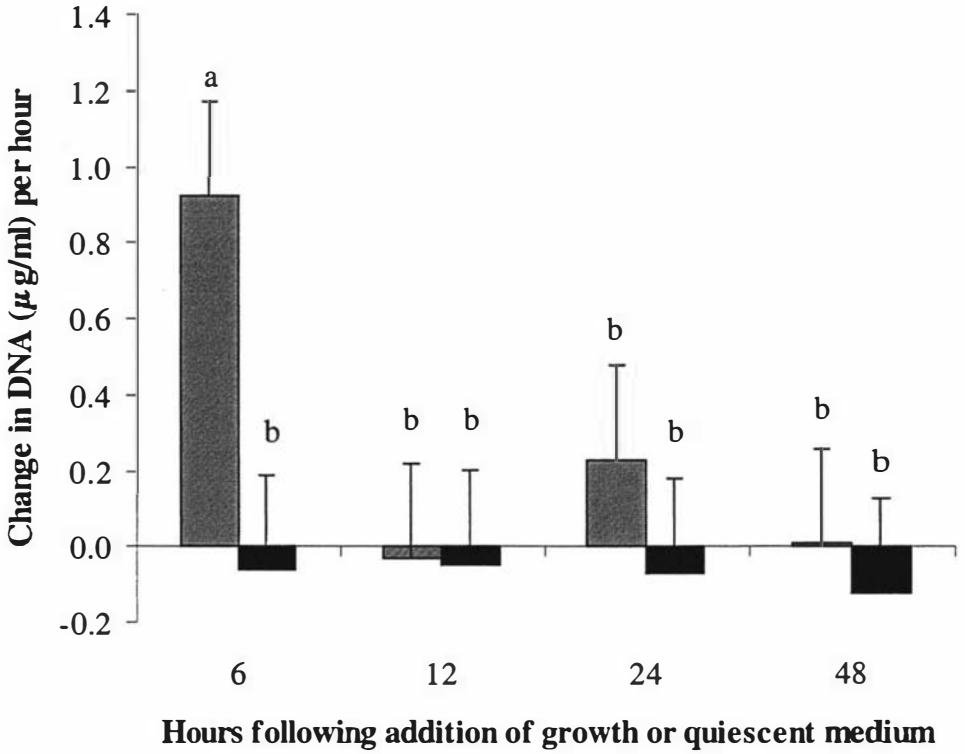

Figure 4.1: Induction of quiescence in Comma-D cells. Comma-D cells, were grown for 4 days in growth medium, then either cultured without serum and growth factors (quiescent medium; black bars) or left in growth medium (grey bars) for various lengths of time. Cells were collected and the cultures were analysed for DNA content. Data are expressed as the mean ± SEM of 4 independent experiments. Differences between the means are indicated by superscript letters, where means not sharing common letters are significantly different ($P < 0.05$).

There were significant differences ($P < 0.05$) between cells in quiescent and growth media in their production of NOx at 6 and 12 hours, however, by 24 hours, both the cells in quiescent and those in growth media were releasing similar amounts of NOx into the media (Figure 4.2).

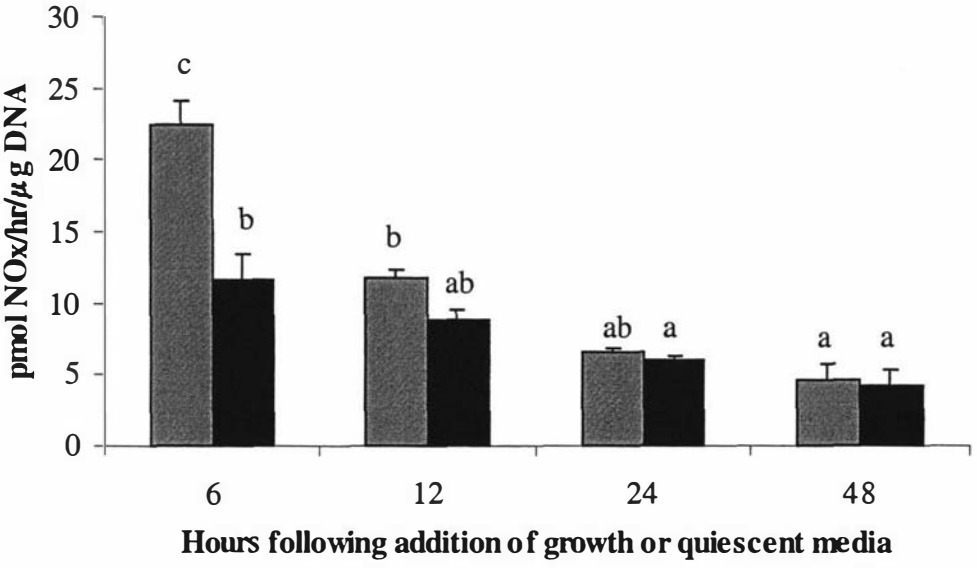

Figure 4.2: Nitrite plus nitrate (NOx) production of Comma-D cells in growth and quiescent media over time. Comma-D cells, grown for 4 days in growth medium, were then either cultured without serum and growth factors (quiescent medium; black bars), or left in growth medium (grey bars) for various lengths of time. Media were collected and analysed for NOx concentration. Data are expressed as the mean ± SEM of 4 independent experiments. Differences between the means are indicated by superscript letters, where means not sharing common letters are significantly different ($P < 0.05$).

4.5.5 *Discussion*

To examine whether quiescence had been induced in the cells cultured in quiescent medium, the growth rate (change in DNA/hr) and NOx production of the cells were compared to those cells cultured in growth medium. There was no significant

difference in the change in DNA content of the cultures in quiescent medium between the times measured (Figure 4.1). Although there was a negative change in DNA content of these cultures, the change was not significantly different from 0. This indicates that the cells in quiescent medium were not actively growing. In contrast, the change in DNA over the first 6 hours for those cultures in growth medium was significantly higher ($P < 0.05$) than those in the quiescent medium, and clearly indicates that over the first 6 hours in culture, these cells were still actively growing (Figure 4.2). No differences in the change in DNA of the cultures in growth medium were apparent after 6 hours indicating that the cells had stopped growing. Cells in these experiments were routinely grown for 4 days following seeding as this was the time at which most cultures exhibited confluence in the 24 well culture dishes. The lack of an increase in DNA of the cultures in growth medium after 6 hours is most likely related to the attainment of a fully confluent state.

Both the cells in quiescent and growth medium decreased their NOx production over time. At 6 and 12 hours, NOx production was higher in those cells cultured in growth medium than in those cells cultured in quiescent medium. However, by 48 hours the production from the cells in the growth medium was the same as that from those cells in quiescent medium. Confluent bovine aortic endothelial cells also produce less NOx than growing cells and the levels of NOx produced decreases in the days following confluence (Arnal *et al.*, 1994).

Although the rate of growth for the cells cultured in growth medium was not significantly different from that of the cells in quiescent medium from 12 hours onward, it was not until after 48 hours in culture that the cells were producing significantly less NOx than at previous times. In contrast the production of NOx by the cells in quiescent medium was more consistent with no significant differences apparent between 6 and 12 hours, or between 12, 24 and 48 hours. Thus, these results support a period of 48 hours as being an adequate length of time in which to induce quiescence in Comma-D cells.

4.6 Effect of Human Interferon-γ

4.6.1 *Introduction*

Many cells types respond to cytokines with an increased production of NOx (See Moncada *et al.*, 1991 and Stuehr & Griffith, 1992 for reviews), including Comma-D cells which respond to treatment with hIFN-γ with dose dependent increases in the concentration of NOx in the media (Low *et al.*, 1997). This experiment was designed and conducted to investigate the production of NOx from Comma-D cells and to validate the use of Comma-D cells as a model for induction of NO production in the mammary gland.

4.6.2 *Experimental Design*

In two preliminary experiments, Comma-D cells grown to confluence then made quiescent for 48 hours as previously described (Section 2.3.2), were treated with 0, 2.5, 25 or 250 U/ml hIFN-γ for 24 hours. Media and cells were collected and the amount of DNA in each well and the NOx concentration in the media were measured.

4.6.3 *Results*

There was no significant effect of treatment on the DNA content (results not shown) and there was no apparent effect of hIFN-γ on NOx concentration in the media (Figure 4.3).

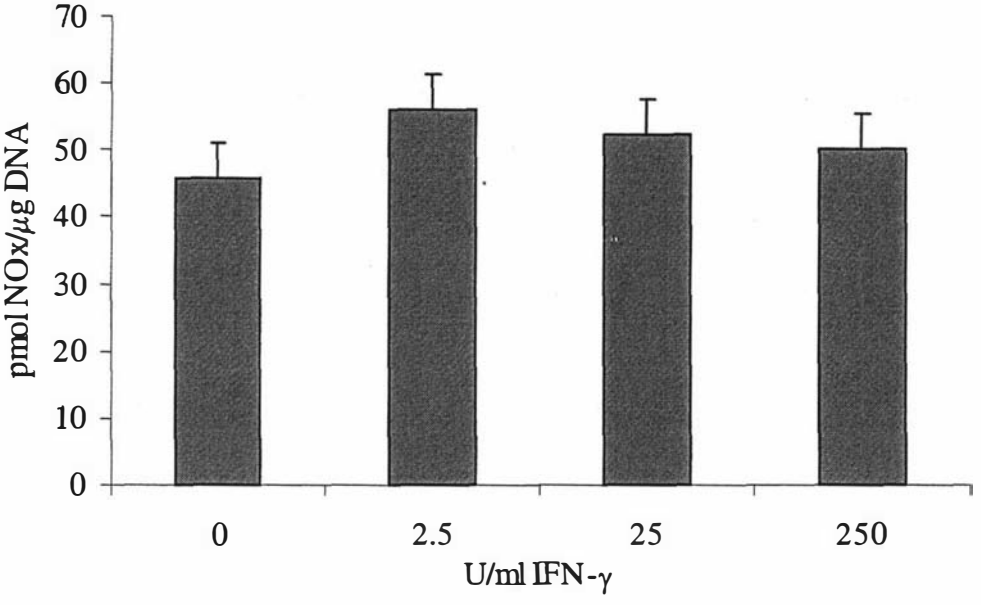

Figure 4.3: Effect of hIFN-γ on NOx production by Comma-D cells. Comma-D cells, grown for 4 days, were made quiescent by the removal of serum and growth factors for 48 hours. The cells were then treated with hIFN-γ (at 0, 2.5, 25 and 250 U/ml) for 24 hours. The media were collected and analysed for NOx. Data are expressed as the mean value from two independent experiments ± SEM.

4.6.4 *Discussion*

The lack of response to hIFN-γ contradicts previous reports in which Comma-D cells responded to treatment with hIFN-γ with a dose dependent increase in NOx (Low *et al.*, 1997). As sequence alignment of human and mouse IFN-γ (SWISS-PROT accession numbers P01579 and P01580) reveal that the two sequences share a 41 % identity it may have been more suitable to use a mouse IFN-γ for the murine derived Comma-D cells. The source of the cytokine used between this experiment and that of Low *et al.* (1997) was different (Boehringer versus Sigma respectively), however, they would not be expected to evoke different responses. Therefore the reason for the disparity in these results with those of Low *et al.* (1997) remains unknown. As no effect of hIFN-γ was found in this experiment, IFN-γ from a different origin was investigated (Section 4.7).

4.7 Response to Rat and Mouse Interferon-γ

4.7.1 *Introduction*

The response of Comma-D cells to hIFN-γ is variable and both a dose response increase in nitrite production (Low *et al.*, 1997) and no response (Section 4.6.3) have been reported. As the hIFN-γ and mIFN-γ sequences only share a 41 % identity it was suggested that this lack of homology could be the possible reason for the lack of response from the murine derived Comma-D cells (Section 4.6.4). Thus two rodent recombinant IFN-γs (rat and mouse) were used in the following experiments to examine their ability to induce production of NOx.

4.7.2 *Experimental Design*

In three independent experiments, Comma-D cells grown and made quiescent as previously described (Section 2.3.2) were treated with either mouse recombinant IFN-γ or rat recombinant IFN-γ at 0, 25, 250 or 500 U/ml.

The effect of treatments on the NOx concentrations in the media were analysed using ANCOVA with basal NOx levels used as the covariate.

4.7.3 *Results*

Treatment with rat or mouse IFN-γ at various doses did not affect the DNA content of the cultures. All treatments except 25 U/ml mIFN-γ caused a significant increase in NOx compared to those cells cultured without treatment. There were also significant differences in the production between 25 and 250 U/ml IFN-γ for both the mouse and rat IFN-γs. However, there were no significant differences between the rat and mouse IFN-γs at any treatment level (Figure 4.4).

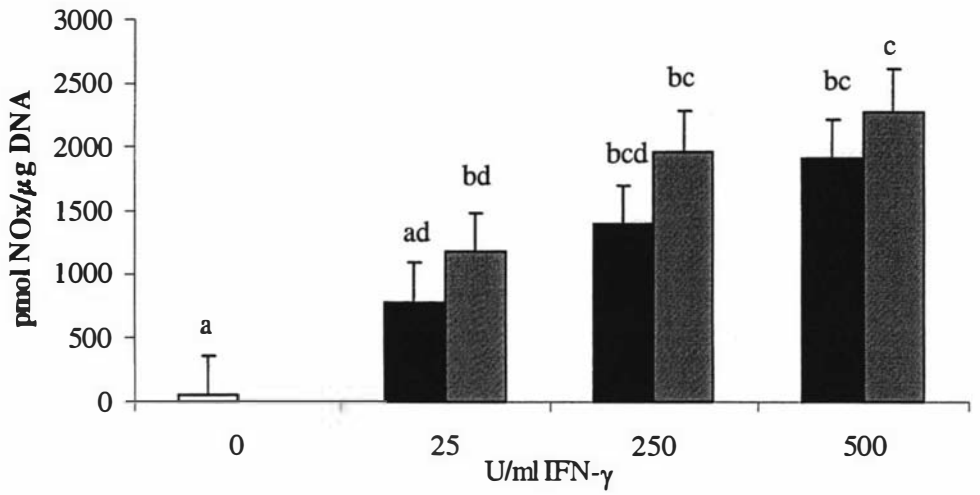

Figure 4.4: Effect of rat and mouse recombinant IFN-γ on NOx production in Comma-D cells. Comma-D cells, grown for 4 days, were made quiescent by the removal of serum and growth factors for 48 hours. The cells were then either left untreated (0) or were treated with mIFN-γ (black bars) or rIFN-γ (grey bars) at 25, 250 and 500 U/ml for 24 hours. The media were collected and analysed for NOx. Data are expressed as the mean ± SEM of 3 independent experiments. Differences between the means are indicated by superscript letters, where means not sharing common letters are significantly different ($P < 0.05$).

4.7.4 *Discussion*

This experiment examined the production of NOx following stimulation with mouse and rat recombinant IFN-γ and the results indicate that both rat and mouse IFN-γ cause what appears to be a dose dependent increase in the amount of NOx in the media of cultured cells. Sequence alignment shows that the mouse and rat IFN-γ sequences (SWISS-PROT accession numbers P01580 and P01581 respectively) share an 83 % identity. The Comma-D cells used in this chapter did not respond to human IFN-γ with increases of NOx in the media (Figure 4.3), however, they did respond to the rat and mouse IFN-γ (Figure 4.4). There was no significant difference in the amount of NOx produced by cells treated with either type of cytokine at any of the doses examined. Thus subsequent experiments described in this chapter utilise the

mouse recombinant IFN-γ as the differences between the NOx produced by the mIFN-γ and rIFN-γ treated cells were minor and the use of a homologous system was deemed more appropriate.

4.8 Response to Mouse Interferon-γ

4.8.1 *Introduction*

As the previous experiment indicated that Comma-D cells respond to mIFN-γ with what appears to be a dose-dependant response, the following experiment was conducted to further characterise this response over a greater number of doses.

4.8.2 *Experimental Design*

In five independent experiments, Comma-D cells grown and made quiescent as described earlier were treated with 0, 1, 2.5, 10, 25, 100, 250 or 500 U/ml mIFN-γ.

4.8.3 *Results*

There was no significant difference between the DNA content of cultures between different treatment groups. Mouse recombinant IFN-γ produced a dose dependent increase in the concentration of NOx in the media (Figure 4.5). There was no statistical difference between 0, 1, 2.5 and 10 U/ml of IFN-γ. However, 100 U/ml was significantly different ($P < 0.05$) from all other treatment levels except 25 and 250 U/ml. At approximately 1600 pmol/μg DNA, 500 U/ml produced the biggest increase in NOx into the media. This was significantly larger ($P < 0.0001$) than any other treatment level.

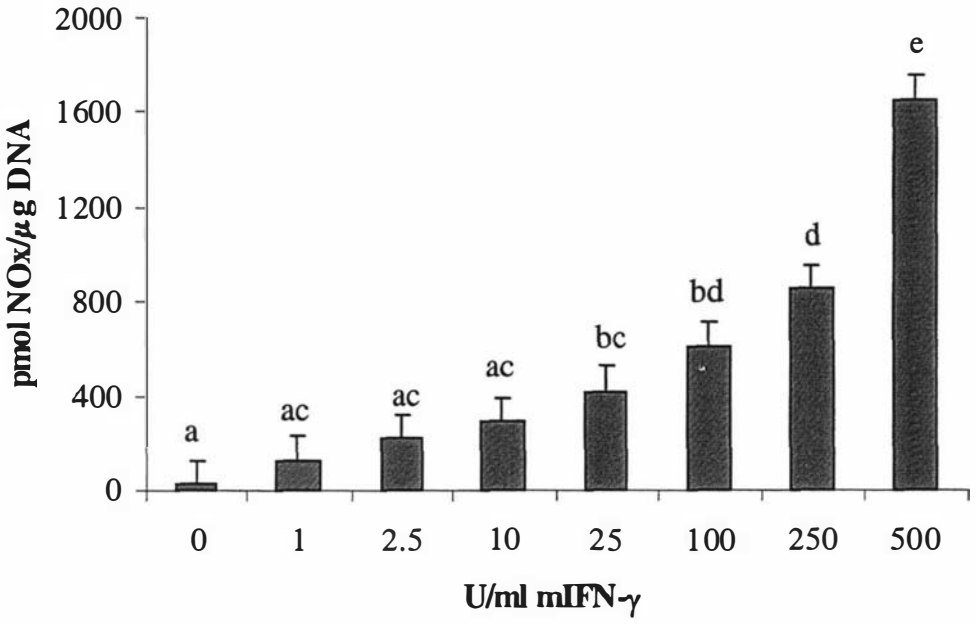

Figure 4.5: Production of NOx by Comma-D cells in response to treatment with mIFN-γ. Comma-D cells, grown for 4 days, were made quiescent by the removal of serum and growth factors for 48 hours. The cells were then either left untreated (0) or were treated with mIFN-γ at 1, 2.5, 10, 25, 100, 250 and 500 U/ml for 24 hours. The media were collected and analysed for NOx. Data are expressed as the mean ± SEM of 5 independent experiments. Differences between the means are indicated by superscript letters, where means not sharing common letters are significantly different ($P < 0.05$).

4.8.4 *Discussion*

Comma-D cells respond to treatment with mIFN-γ with a dose dependent increase in the production of NOx into the media (Figure 4.5). However, as the production by those cells treated with 500 U/ml is significantly greater than that produced by those cells treated with 250 U/ml, further analysis of the data such as the use of Michaelis-Menten enzyme kinetics is not possible as a maximal induction was not reached. Although there were differences in the response of Comma-D cells to hIFN-γ (Section 4.6.3; Low *et al.*, 1997), the use of 500 U/ml mIFN-γ as a maximum dose

was considered more than suitable. This was based on the finding of Low *et al.* (1997) who reported that the response of Comma-D cells to hIFN-γ had an EC_{50} of 10 U/ml and a maximal dose response of between 4-6-fold using 25 and 250 U/ml. Why the cells did not reach maximal induction with a dose of less than 500 U/ml mIFN-γ is not known. However, when the cells were stimulated with 250 U/ml mIFN-γ there was an approximate 35-fold increase in NOx in the media when compared to untreated cells (Figure 4.5). In contrast, although Low *et al.* (1997) presented their results in nmol/mg protein and thus direct comparison is not possible, their results show a 6-fold increase in nitrite using 250 U/ml hIFN-γ when compared to the untreated cells. This difference in response to 250 U/ml may help explain why the use of less than 500 U/ml mIFN-γ was unable to elicit a maximal response from the cells. However, Low *et al.* (1997) report nitrite concentrations only and even if the values reported are doubled to account for the reported ratios of the anions (Section 3.5.3) then this would still only indicate an approximate 10-fold increase. These results suggest that the use of mouse IFN-γ to stimulate the murine cell line, Comma-D results in a higher effectiveness with respect to the dose used and the amount of NOx produced by the cells than that reported previously for human IFN-γ (Low *et al.*, 1997).

4.9 Induction of NO Production

4.9.1 *Introduction*

In the experiments done to this point, the conditioned media were collected for the analysis of NOx either 24 hours after changing the media, or 24 hours after the application of the treatments. However, following stimulation, maximum iNOS transcripts are not apparent until 6-8 hours later, (Asano *et al.*, 1994; Adler *et al.*, 1995; Menegazzi *et al.*, 1996; Low *et al.*, 1997) and the maximum amount of nitrate in the media is not measured until 24-48 hours following treatment (Adler *et al.*, 1995). Other studies allow the accumulation of NOx for various lengths of time following treatment, for example Corbacho *et al.*, (2000) collected the media 72

hours after treatment whereas Low *et al.*, (1997) collected media after only 24 hours. Thus the object of this experiment was to determine the optimum incubation period following application of the treatments for accurate measurement of NOx in the medium thereby ensuring an accurate representation of NO production.

4.9.2 *Experimental Design*

In three independent experiments, Comma-D cells were grown to confluence in 24 well tissue culture plates then made quiescent by the removal of serum and growth factors for 48 hours. The cells were then either given fresh quiescent medium (quiescent), or treated with 25 U/ml mIFN-γ (treated) for 27 hours. During this time, media were collected from a 'pair' of treatment groups (triplicate wells of either quiescent medium or quiescent medium containing mIFN-γ; eight pairs of treatment groups) at the following times; 2, 4, 6, 8, 10, 12, 25 and 27 hours, and the media were replaced with PBS. After removal of medium from the last pair of treatment groups (27 hours), the cells were collected. Total NOx in the media and DNA content of the cells were measured as described in Sections 2.5 and 2.6.

4.9.3 *Statistical Analyses*

Differences in the production of NOx and the DNA content of the wells between the cells in quiescent and treatment media were analysed at each time point by ANOVA. Differences in the amount of DNA at each time point for those cells in either quiescent or treated media were also analysed by ANOVA.

4.9.4 *Results*

There was no difference between the amount of DNA in the wells containing quiescent cells and those that were treated with mIFN-γ, nor was there a change in the amount of DNA over time for either treatment.

There was a continual decline in the rate of NOx produced by cells cultured in the quiescent medium over the 27 hour period (difference between NOx concentration at 2 hours and 27 hours $P = 0.0001$).

The rate of accumulation of NOx in the medium of cells treated with mIFN-γ also declined during the first 10 hours in culture, however, there was a significant increase ($P < 0.05$) in the rate of accumulation of NOx in the medium of the mIFN-γ treated cells between 10 and 12 hours (Figure 4.6). By 12 hours, the production by the cells treated with 25 U/ml mIFN-γ was significantly greater ($P < 0.05$) than that produced by cells cultured in quiescent media. The production of NOx by the cells treated with mIFN-γ continued to increase, however, the amounts produced at 25 and 27 hours are not statistically different.

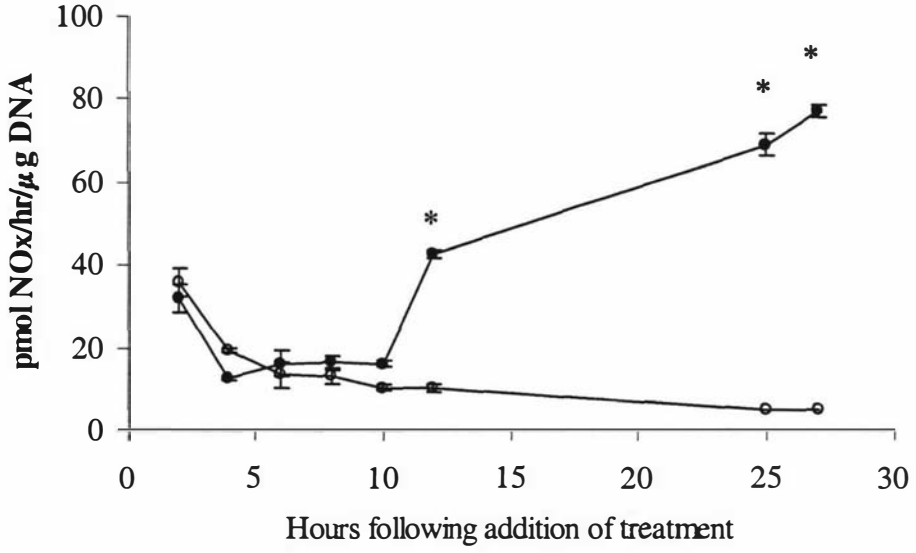

Figure 4.6: Time course of the induction of NO using 25 U/ml mIFN-γ. Comma-D cells, grown to confluence then made quiescent for 48 hours were either treated with 25 U/ml mIFN-γ (-●-) or given fresh quiescent media (-o-). Media were collected at the times shown and analysed for NOx. Data are expressed as the mean ± SEM of 3 independent experiments. Statistical differences between the means are indicated by an asterisk (*; $P < 0.05$).

4.9.5 *Discussion*

The rate of NOx accumulation was significantly lower ($P < 0.05$) in the medium of the cells cultured for 4 hours with mIFN-γ than in the medium of those cells cultured for 2 hours. This decline in NOx production has been previously reported in cells cultured post-confluence (Figure 4.2; Arnal *et al.*, 1994) and was also apparent in the cells cultured in quiescent media for the duration of this experiment (Figure 4.6). The rate of accumulation of NOx in the medium of the mIFN-γ treated cells at 12 hours is not significantly different from that at 2 hours, however, there was a significant increase ($P < 0.05$) in the rate of accumulation of NOx in the medium of the cells cultured for 12 hours with mIFN-γ and those cultured for 10 hours. Following treatment with 25 U/ml mIFN-γ the cells required 12 hours before a significant increase ($P < 0.05$) was apparent in the rate of accumulation of NOx in the medium compared to the untreated cells (those in quiescent medium). Following stimulation maximum iNOS transcripts are apparent at 6-8 hours (Asano *et al.*, 1994; Adler *et al.*, 1995; Menegazzi *et al.*, 1996; Low *et al.*, 1997). There was no significant difference in the rate of accumulation of NOx by the treated cells between 25 and 27 hours, suggesting that maximal rate (pmol/hr/μg DNA) had been reached. The results indicated that 24 hours is an adequate period in which to allow accumulation of NOx in the media.

4.10 Inhibition of Nitric Oxide Production

4.10.1 *Introduction*

The enzyme nitric oxide synthase (NOS) produces NO and the concentrations of nitrite and nitrate (NOx) in the medium of cultured cells are used to demonstrate this. If inhibition of the NOS enzyme leads to decreases in the amount of NOx in the medium this provides confirmation that the increases in NOx in the medium are a result of the activity of the enzyme. Various inhibitors of NOS including aminoguanidine (AG) and N^{ω}-nitro-L-arginine (L-NNA) inhibit the production of NO. AG is an inhibitor with specificity for iNOS (Corbett & McDaniel, 1996; Moore

& Handy, 1997). L-NNA is an effective inhibitor of nNOS and eNOS, however, it is a freely reversible inhibitor of iNOS making it less effective for this isoform (Griffith & Kilbourn, 1996; Moore & Handy, 1997).

Thus, to confirm that the increases in NOx in the medium following treatment of Comma-D cells with IFN-γ is due to stimulation of the NOS enzyme, AG and L-NNA were tested. Also, to determine if the basal amount of NOx in the media of cultured cells is from basal NOS activity, unstimulated (i.e. not treated with cytokines or cytotoxins) cells were also treated with these inhibitors.

4.10.2 *Experimental Design*

In two separate sets of experiments, inhibitors (AG or L-NNA) were added to the media either with (five independent experiments), or without, the addition of mIFN-γ (four independent experiments). The concentrations of inhibitors used to treat unstimulated cells are given in Table 4.2. The concentrations of inhibitors and cytokines used in the experiments, with cells in which NO production was stimulated with cytokines, along with the abbreviation used for the treatments are given in Table 4.3.

Table 4.2: Treatments used to inhibit the production of NOx in unstimulated Comma-D cells. Cells were treated with either L-NNA or AG at the concentrations given. The abbreviations for the treatments are also given.

Treatment Abbreviation	μM AG	mM L-NNA
10	10	-
50	50	-
100	100	-
0.1	-	0.1
0.5	-	0.5
1	-	1

Table 4.3: Treatments used to inhibit the production of NOx in stimulated Comma-D cells. Cells were treated with mIFN-γ and either L-NNA or AG at the concentrations given. The abbreviations for the treatments are also given.

Treatment Abbreviation	U/ml mIFN-γ	μM AG	mM L-NNA
25	25	-	-
25 + 10	25	10	-
25 + 50	25	50	-
25 + 100	25	100	-
25 + 0.1	25	-	0.1
25 + 0.5	25	-	0.5
25 + 1	25	-	1
250	250	-	-
250 + 10	250	10	-
250 + 50	250	50	-
250 + 100	250	100	-
250 + 0.1	250	-	0.1
250 + 0.5	250	-	0.5
250 + 1	250	-	1

4.10.3 *Results*

Inhibition of NOx Production from Unstimulated Cells

There was no significant difference in the DNA content of cultures between treatments. NOx was not detected in the media of untreated cells or those treated with AG at any concentration used (data not shown). Nor was there any NOx detected in the media of cells treated with 0.1 mM L-NNA (Figure 4.7), however, when unstimulated cells were treated with either 0.5 and 1 mM L-NNA, there was an increase in the amount of NOx in the medium. Treating the cells with 1 mM L-NNA resulted in significantly more ($P < 0.05$) NOx in the medium of those treated with 0.5 mM L-NNA.

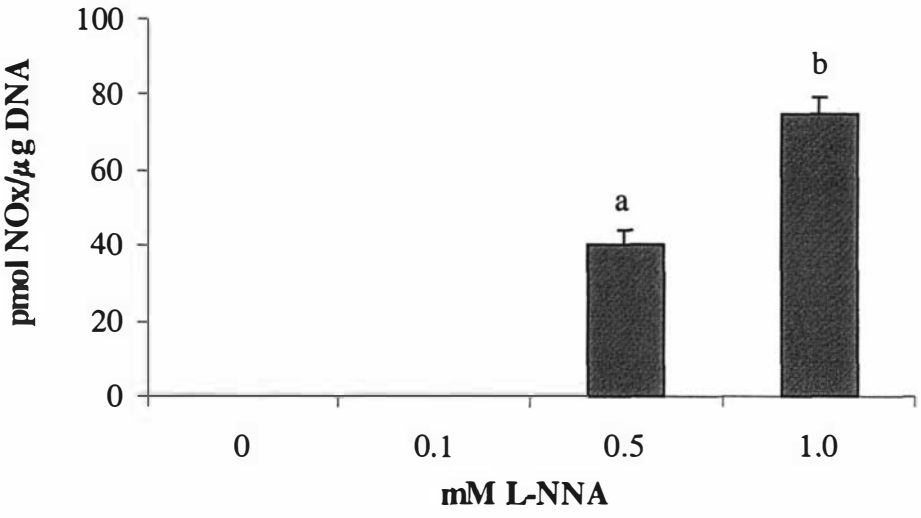

Figure 4.7: Effect of L-NNA on unstimulated NOx production in Comma-D cells. Comma-D cells, grown for 4 days, were made quiescent by the removal of serum and growth factors for 48 hours. The cells were then either left untreated (0) or were treated with L-NNA at 0.1, 0.5 or 1 mM for 24 hours. Media were collected and analysed for NOx. Data are expressed as the mean ± SEM of 4 independent experiments, no bar indicates the sample contained less NOx than could be detected by the assay. Differences between the means are indicated by superscript letters, where means not sharing common letters are significantly different ($P < 0.05$).

Inhibition of NOx Production from mIFN-γ Treated Cells

There was no significant difference in the DNA content of cultures between treatments. Treatment of cells with 25 or 250 U/ml mIFN-γ resulted in an increase in concentration of NOx in the media (Figure 4.8). The use of inhibitors decreased the amount of NOx in the media in a dose dependent manner, when compared to the stimulated production alone. The co-treatment of cells with 25 U/ml mIFN-γ and either AG at 50 or 100 μM, or L-NNA at 0.5 and 1 mM resulted in a significant decrease ($P < 0.05$) in NOx production when compared to cells treated with 25 U/ml mIFN-γ alone (Figure 4.8 A and B). This was also the result when cells were stimulated with 250 U/ml mIFN-γ and inhibited with AG (Figure 4.8 C). However, when cells were stimulated with 250 U/ml mIFN-γ and inhibited with L-NNA, all concentrations of inhibitor significantly decreased ($P < 0.05$) the production (Figure

4.8 D). At 1 mM L-NNA the decrease was such that it was not significantly different from the non-treated cells.

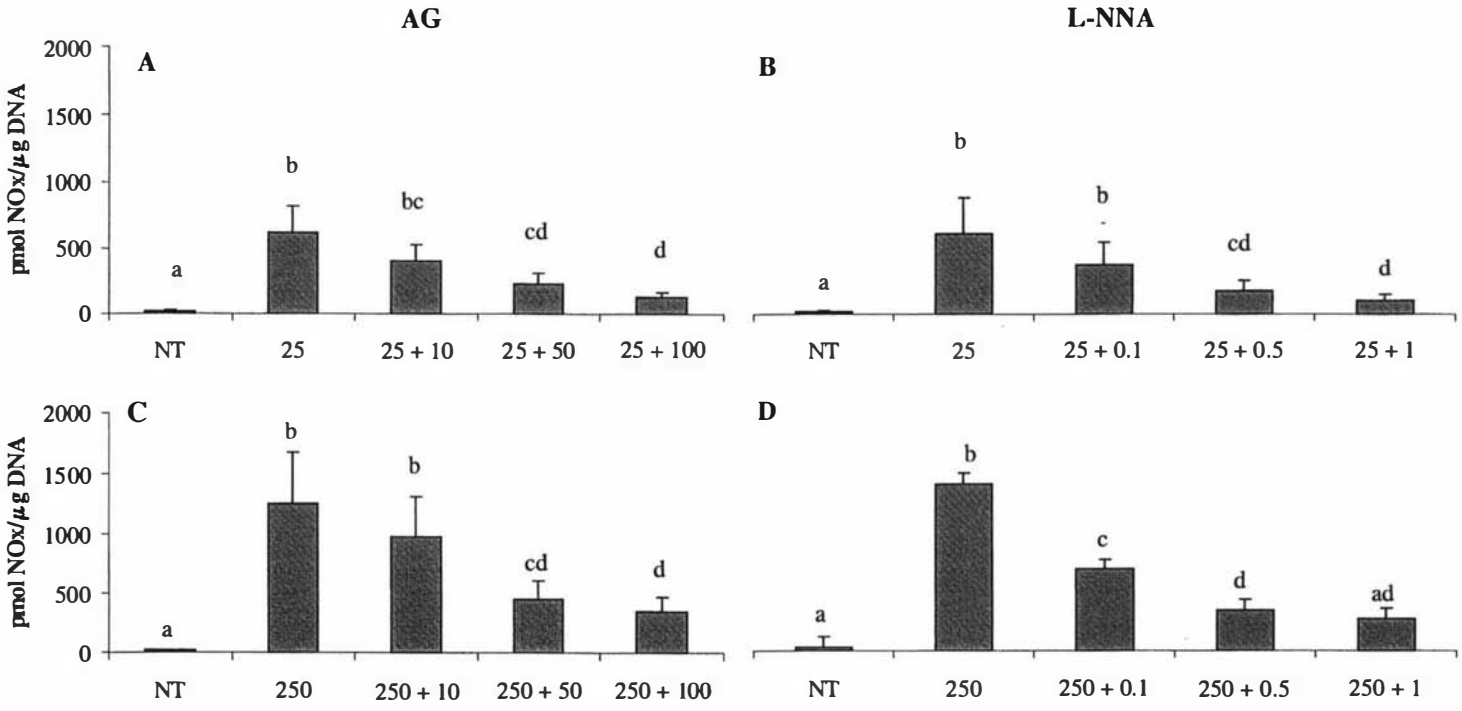

Figure 4.8: Stimulation and inhibition of NOx production in Comma-D cells. Comma-D cells, grown for 4 days, were made quiescent by the removal of serum and growth factors for 48 hours. The cells were then either left untreated (NT) or were treated with **A:** mIFN-γ (25 U/ml; 25), mIFN-γ (25) + aminoguanidine (AG) at 10, 50 or 100 μM, **B:** mIFN-γ (25), mIFN-γ + L-NNA at 0.1, 0.5 or 1 mM, **C:** mIFN-γ (250 U/ml; 250), mIFN-γ (250) + AG at 10, 50 or 100 μM, **D:** mIFN-γ (250), mIFN-γ (250) +L-NNA at 0.1, 0.5 or 1 mM for 24 hours. Media were collected and analysed for NOx. Data are expressed as the mean ± SEM of 5 independent experiments. Differences between the means are indicated by superscript letters, where means not sharing common letters are significantly different ($P < 0.05$).

4.10.4 *Discussion*

Inhibition of NOx Production from Unstimulated Cells

NOx was not detected in the medium of untreated cells nor those treated with AG. However, there was a significant increase in the NOx production when the cells were treated with 0.5 and 1 mM L-NNA, with the greater increase being with the higher concentration of inhibitor. This increase is opposite to that which occurs when cells are stimulated and exposed to inhibitors (Figure 4.8). Although there is a significant increase in the production of NOx when the cells are treated with L-NNA, it is very small relative to that released when the cells are treated with cytokines such as IFN-γ (Figure 4.5). Why an inhibitor such as L-NNA causes an increase in the NOx production from unstimulated cells and does not inhibit the activity of NOS is not known. It is also not known as to why, in these experiments, the untreated cells and those treated with AG did not release detectable amounts of NOx into the media.

It is possible that arginase may play a role in the increased NOx in the medium of L-NNA treated Comma-D cells (Figure 4.7). Arginine is catabolised by many different pathways, of which NOS and arginase are the enzymes for just two. Whereas the action of NOS on arginine produces citrulline and NO, arginase is responsible for the production of ornithine and urea from arginine (Wu & Morris, 1998). Thus, if arginase is utilising arginine, it therefore would result in less arginine being available to be used by NOS for the production of NO. The increase in NOx in the medium may be due to the effect of the L-NNA on arginase, thus allowing more arginine in the medium to be metabolised by NOS. There are inconsistent reports as to whether inhibitors such as AG and L-NNA are direct inhibitors of arginase (Robertson *et al.*, 1993; Hrabak *et al.*, 1994; Wu & Morris, 1998). However, Robertson *et al.* (1993) report that L-NNA inhibits rat liver arginase (AI) with an IC_{50} of 27.2 mM, much higher than the 1 mM L-NNA used throughout the experiments in this Thesis to inhibit NOS. The predominant form of arginase in the mammary gland is the extrahepatic form (AII), although a small amount of the AI hepatic form is found (Jenkinson & Grigor, 1994). Whether NOS inhibitors such as L-NNA also inhibit AII is not known, however, as both rat AII and AI forms share similar Km values for L-arginine (Jenkinson & Grigor, 1994) then this is probable. Future experiments

could examine this in more detail by investigating whether L-NNA or AG actually inhibit the activity of arginase. Experiments could also determine if the inhibition of arginase using specific arginase inhibitors has any effect on the production of NO by cells treated with L-NNA. Future experiments are needed to determine why L-NNA fails to inhibit the basal production of NOx from Comma-D cells.

Inhibition of NOx Production from mIFN-γ Treated Cells

The use of both AG and L-NNA resulted in dose dependent decreases in the amount of NOx produced in the media when compared to the stimulated production alone (Figure 4.8). However, the production stimulated by either concentration of mIFN-γ was not completely inhibited by even the highest concentration of AG (100 μM), there was still NOx production, which was significantly greater ($P < 0.05$) that that produced by non-treated cells (Figure 4.8 A and C). The effect of L-NNA on those cells treated with 25 U/ml mIFN-γ was similar to that described for AG (Figure 4.8 B). In contrast, the production of NOx by cells treated with 250 U/ml IFN-γ + 1 mM L-NNA was not significantly different from that produced by the non-treated cells (Figure 4.8 D) suggesting that there had been full inhibition of the NOx produced by the stimulated cells. It is not known why the 1 mM L-NNA was unable to fully inhibit the NOx produced when the cells were stimulated with 10-fold less (25 U/ml) mIFN-γ. However, the increased production of NOx by cells treated with 1 mM L-NNA only (Figure 4.7) may play a role in this. As full inhibition of the NOx production following stimulation was not achieved in all experiments, further analysis using Michaelis-Menten enzyme kinetics was not considered to be appropriate.

4.11 Effect of Lipopolysaccharide

4.11.1 *Introduction*

LPS was one of the first cytotoxins used to induce NOS (Stuehr & Marletta, 1985; Stuehr & Marletta, 1987), although at the time it was the induction of nitrate biosynthesis that was being studied as NO and NOS were only just being identified. Many subsequent researchers have also used LPS *in vitro* (Pendino *et al.*, 1993; Robert & Spitzer, 1997; Onoda & Inano, 1998) and *in vivo* (Billiar *et al.*, 1990; Kauser *et al.*, 1997; Robert & Spitzer, 1997) to induce the production of NO.

Mastitis is one of the most frequent diseases occurring within the dairy industry and can be a major cause of economic loss. The various strains of *Staphylococcus aureus* are major pathogens causing bacterial mastitis (Shoshani *et al.*, 2000; Wedlock *et al.*, 2000). The gram-negative pathogen, *Escherichia coli*, is also responsible for infections of the udder (Harmon, 1994). LPS, a component of gram-negative bacterial cell walls is often used to induce sterile mastitis in dairy cattle (Shuster *et al.*, 1993; Shuster & Kehrli, 1995; Bouchard *et al.*, 1999). Recently, the production of NO during induced mastitis was reported, with the concentrations of NOx in the milk peaking within 3 hours following infusion of LPS (Bouchard *et al.*, 1999). The relationship between mastitis and NO is reviewed in greater detail in Chapter 8.

Therefore, this experiment investigated whether LPS could stimulate the production of NOx from mammary epithelial cells (Comma-D cells). This would then provide information as to whether increases in the concentration of NOx in milk following infusion of LPS could be due to stimulation of the mammary epithelial cells.

4.11.2 *Experimental Design*

Following seeding, cells were grown for 4 days then made quiescent by the removal of serum and growth factors for 48 hours (four independent experiments). The cells were then treated with LPS at the following concentrations: 1, 5 and 10 μg/ml. Media and cells were collected and analysed for NOx and DNA content as described in Sections 2.5 and 2.6.

4.11.3 *Results*

No effect of treatment on the amount of DNA in the wells was detected. The production of NOx induced by all concentrations of LPS was significantly greater ($P < 0.05$) than that produced in non-treated wells, although there was no statistical difference within the production by the different treatments (Figure 4.9).

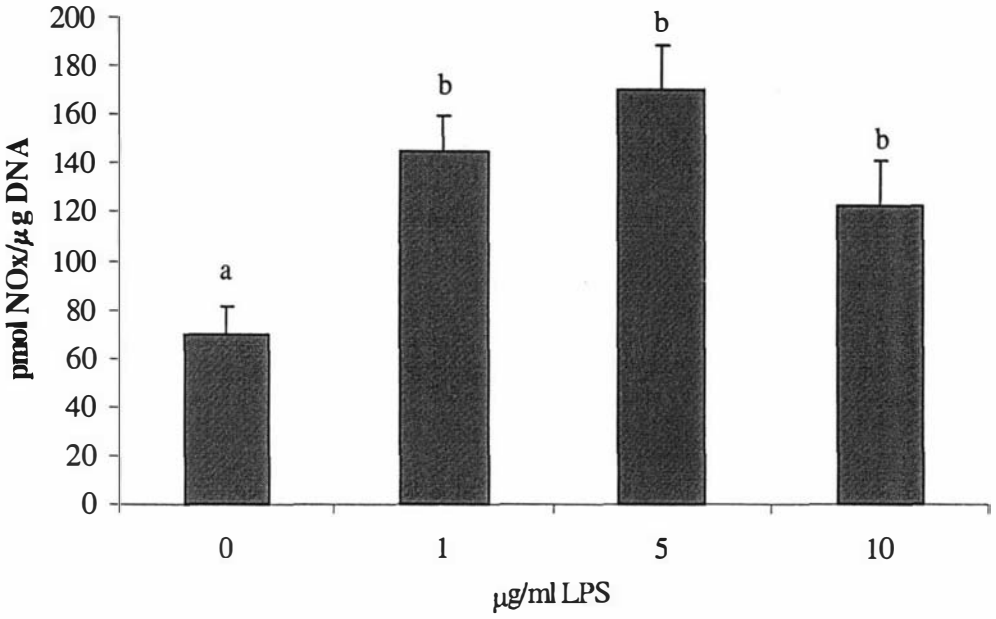

Figure 4.9: Production of NOx by Comma-D cells following treatment with LPS. Comma-D cells, grown to confluence then made quiescent, were treated with LPS at various concentrations for 24 hours. Media were collected and analysed for NOx. Data are expressed as the mean ± SEM of 4 independent experiments. Differences between the means are indicated by superscript letters, where means not sharing common letters are significantly ($P < 0.05$) different.

4.11.4 *Discussion*

Although LPS significantly increased ($P < 0.05$) the production of NOx by Comma-D cells, the increase did not appear to be influenced by the concentration used. The production of NOx following treatment with LPS is much less than that measured

following stimulation with mIFN-γ. For example, the response to LPS was approximately 2-fold whereas there was up to a 65-fold increase in response to mIFN-γ (Figure 4.5).

The reported effects of LPS on NO production are varied between tissue/cell types. For example, in the macrophage cell line RAW 264.7, LPS alone stimulates mac-NOS expression (iNOS) and IFN-γ is only effective in the presence of LPS (Lorsbach *et al.*, 1993; Lowenstein *et al.*, 1993). In contrast, in A549 (human alveolar type II epithelium like) cells, IFN-γ is the most important synergist (of IFN-γ, IL-1β, TNF-α and LPS), and LPS alone does not modify the response (Asano *et al.*, 1994). However, in cultured rat hepatocytes treated with a range of cytokines and LPS, LPS alone was the weakest stimulator of iNOS mRNA accumulation and NOx into the media and was in fact lower than the control cells (Geller *et al.*, 1993b).

The lack of response of LPS on the mammary epithelial cells (Comma-D) may be explained by the finding that there are differences in the response of tissues and the cells that they are comprised of to LPS. For example, LPS had a significant effect on the barrier function of tissue samples from the human distal colon where it stimulated electrogenic secretion, however, there was no effect on the colorectal cell line HT-29/B6 (Buhner *et al.*, 2000). This suggests that LPS does not act directly on the enterocytes and that other cells within the tissue may moderate the response. Nor does LPS have an effect on the transepithelial resistance of cultured monolayers of the canine kidney epithelial cell line, MDCK (Stelwagen, personal communication, 2001) or Comma-D cells (Turner, personal observation). Thus, the lack of dose dependent response of the Comma-D cells to LPS could be due to the lack of a direct stimulation of the epithelial cells and suggests that the lack of other cells types commonly found associated with mammary epithelial cells may be the cause of this.

Because of the reported variability of the effects of LPS on NO production in various cell types, and the apparent lack of a concentration dependent effect on the Comma-D cells, the effect of LPS was also investigated in mammary explants and is described in Chapter 5.

4.12 Effect of Passage Number on the Production of NOx by Unstimulated Comma-D Cells

4.12.1 *Introduction*

The previous results presented in this chapter indicated that there is much variability in the amount of NOx produced by Comma-D cells during unstimulated conditions (collected from the 24 hour period prior to treatment). When experimental data were analysed, the amount of NOx produced by the cells during this basal period was used as a covariate, thus avoiding any bias on the treatment effect. Culture of the cells occurred under standardised conditions, however, cells of differing passage numbers were used. Thus the basal production of NOx by the cells was correlated with the passage number to determine if the variability was related to the different passage numbers.

4.12.2 *Method*

Using data from previously reported experiments (n=23 independent experiments), the average DNA content of cultures and the basal production of NOx were both correlated with the passage number of the cultures.

4.12.3 *Results*

No significant correlation was found between the average DNA content of cultures from 23 independent experiments and the passage number of the cells used in the experiment (R^2=0.1437; Figure 4.10). In addition, there was no significant correlation between the basal production of NOx by the cells and the passage number (pmol NOx/well, R^2=0.1512; pmol NOx/μg DNA, R^2=0.002; Figure 4.10).

4.12.4 *Discussion*

As analysis of the basal production of NOx had indicated that there is no correlation between the passage number of the cells and the amount of NOx produced (Figure 4.10), the reason for the variability in NOx production by the cells in the different

experiments is not known. However, as the analysis of treatment effects on NOx production, are usually carried out by ANCOVA using the basal values as the covariate, any bias due to the variability of the basal values should be avoided.

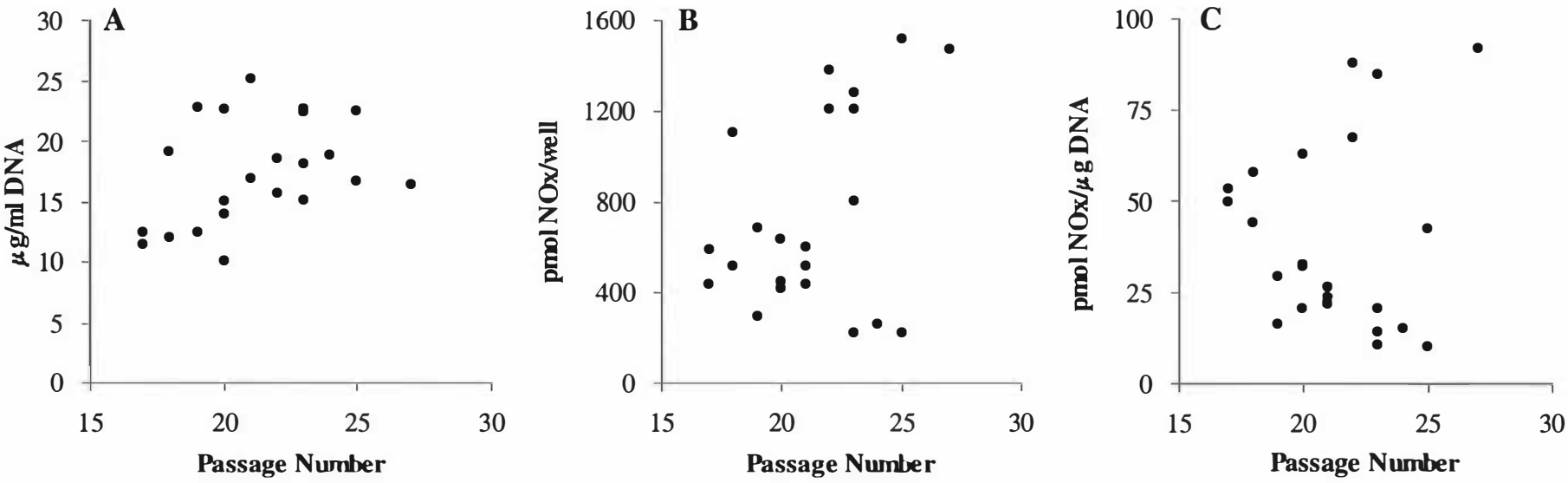

Figure 4.10: Correlation between passage number and basal NOx production by Comma-D cells. In 23 independent experiments, Comma-D cells, grown for 4 days in growth medium were then cultured for 24 hours in quiescent medium (serum and growth factor free). Media were discarded and replaced with fresh quiescent medium and following a 2nd 24 hour period the media were collected and analysed for NOx. The passage number of the cells was then correlated with; **A:** DNA content, **B:** pmol NOx produced/well, **C:** pmol NOx/μg DNA.

4.13 General Discussion

The experiments presented in this chapter were undertaken to characterise the production of NO from the murine mammary epithelial cell line, Comma-D. The results confirm the results of previous studies, which report the production of NO by mammary cells (Lacasse *et al.*, 1995; Lacasse & Prosser, 1995a; Lacasse & Prosser, 1995b; Low *et al.*, 1997; Onoda & Inano, 1998). In this chapter, Comma-D cells were shown to respond to IFN-γ in a well-defined manner and the increase in NOx in the medium can be inhibited, although not fully, with known NOS inhibitors. As discussed earlier (Sections 4.6.4 and 4.8.4), the lack of response of the cells to hIFN-γ as was previously described (Low *et al.*, 1997) is inexplicable. However, on the basis of the results presented in Section 4.7.3, the use of mouse recombinant IFN-γ to stimulate the production of NOx was considered more appropriate and was therefore used in subsequent experiments. The variability between cultures in the basal production of NOx was examined and was not found to be due to the passage number of the cells (Section 4.12.3). As most experiments use a pre-treatment NOx concentration as the covariate for statistical analyses, this variability was minimised.

A difficulty found throughout these studies was the inconsistency between experiments as to whether the NOx produced by the Comma-D cells during the basal state was above the detection limit of the assay. All Comma-D experiments were conducted such that NOx was measured following a period of 24 hours accumulation. This occurred whether the cells were stimulated with cytokines and cytotoxins or not. Further investigation ruled out the possible linkage to the passage number of the cells (Figure 4.10). The lack of detectable NOx precluded firm conclusions being drawn from some experiments. For example, in the four independent experiments examining the unstimulated production of NOx from Comma-D cells (Section 4.10.3), NOx was not detected in the medium of those cells either untreated or treated with AG, whereas in direct contrast, in the five independent experiments examining the production of NOx from stimulated cells, NOx was detectable in the non-treated cells (albeit just above the detection limit of the assay). In this instance, this meant that the effect of AG on unstimulated cells was undetermined as no comparison to untreated cells was possible. It may be that future experiments would have to allow a

period of 48 hours for NOx to accumulate in the medium prior to its collection in order to increase the levels such that they are consistently above the detection limit of the assay.

Comma-D cells provide a valuable tool to study many basic aspects of mammary gland function, as many of their characteristics are distinctive of normal mammary epithelial cells (Danielson *et al.*, 1984). However, they were derived from the mammary tissue of BALB/c mice in mid pregnancy (Danielson *et al.*, 1984) and this undifferentiated state can place limitations on their usefulness. Although further differentiation of the Comma-D cell line, such as the expression of milk protein, is possible with the use of both prolactin and Matrigel (an extracellular matrix preparation; Bennett, 1995), the use of explants of mammary tissue was considered to be more suitable as explants can be cultured from glands collected from rats at different stages of pregnancy or lactation. The use of explants will allow the direct comparison of NO production during different stages of pregnancy and lactation and will also allow expansion of the scope of the experiments.

CHAPTER FIVE

COMPARISON BETWEEN PREGNANCY AND LACTATION

5.1 Abstract

In the uterus, the reproductive status of the animal effects the production of NO. Although there have been studies published that have examined NO production by the mammary gland, there has not been any work reported which examines the effect of reproductive status on the mammary gland production of NO. Northern blots show the presence of two transcript sizes (2 kb and 4 kb) for eNOS in pregnant (D 12-14 gestation) and lactating (D 12-18 postpartum [pp]) mammary tissue, however, the signal at the 2 kb site tends to be increased in lactating tissue. Northern analysis also indicates low levels of iNOS mRNA in both pregnant and lactating tissues. Explants of mammary tissue taken from rats euthanased by the use of sodium pentabarbitone, during pregnancy, mid-lactation (D 12-14 pp) and late-lactation (D 17-18 pp) show significant differences in the amount of NOx produced during the first 24 hours in culture. Euthanasia using CO_2 results in significantly decreased levels of NOx in the media of explants taken from pregnant rats. Treatment of mammary explants from pregnant and mid-lactating rats with rIFN-γ or LPS results in significant increases in the amount of NOx in the media. In explants from pregnant rats, the method of euthanasia results in differences in the response to LPS but has no effect on the response to rIFN-γ. In explants from both pregnant and lactating rats, this stimulated production can be inhibited by either aminoguanidine (AG) or N^{ω}-nitro-L-arginine (L-NNA). These results show that the mammary gland responds similarly to stimulation at different stages of pregnancy and lactation, but that there are differences in the basal production of NOx in sodium pentabarbitone euthanased animals. It is speculated that differences between explants from pregnant and

lactating rats, in the basal production of NOx may be due to the decrease in the 4kb transcript for eNOS mRNA in the lactating tissues. Because of the differences in basal NOx production between euthanasia methods, consistency in the method of euthanasia is required.

5.2 Introduction

The mammary gland produces nitric oxide (NO) under a variety of conditions and in different physiological states. Early studies examined the role of NO in the control of mammary blood flow in lactating goats (Fleet *et al.*, 1993; Lacasse *et al.*, 1995; Lacasse *et al.*, 1996). Subsequent studies have used cultured mammary cells (Lacasse & Prosser, 1995b; Low *et al.*, 1997), explants of mammary tissue from virgin mice (Bolander, 2001) or explants of mammary glands from rats primed with 17β-estradiol and progesterone (Onoda & Inano, 1998) to investigate the production of NO within the mammary gland. The presence of nitric oxide synthase (NOS), the enzyme responsible for the conversion of arginine to citrulline and the production of NO, has also been detected immunohistochemically in the mammary gland (Lacasse *et al.*, 1996; Iizuka *et al.*, 1998; Onoda & Inano, 1998). These studies all show that the mammary gland responds to a variety of stimuli with the production of NO. For example, increases in iNOS mRNA are apparent when either Comma-D cells (mammary epithelial cell-line derived from a mid-pregnant mouse) are treated with either IFN-γ or IFN-γ plus tumor necrosis factor-α (TNF-α; Low *et al.*, 1997), or C3-L5 cells (murine mammary adenocarcinoma) are treated with IFN-γ plus LPS (Orucevic *et al.*, 1999). LPS treatment of mammary explants from 17β-estradiol and progesterone primed rats results in increases in iNOS protein and decreases eNOS protein. Increases in nitrite concentration in the media are also apparent (Onoda & Inano, 1998). Comma-D cells also respond with an increase in nitrite concentration in the media following stimulation with LPS (Section 4.11.3). Thus the production of NO by the mammary gland appears to be important in various physiological states. However, given the vast changes that occur in the mammary gland from pregnancy, through lactation and into involution, these studies do not provide an overall picture of the function of NO in the mammary gland.

The uterus also undergoes vast changes in structure and function under different reproductive states, and the activity of NOS has been shown to vary across these. For example, the activity of eNOS in the guinea pig uterine artery increases approximately 4-fold in pregnant animals when compared to the non-pregnant controls (Weiner *et al.*, 1994). Cultured uterine tissues from pregnant rats (D 18 gestation) release approximately 5-fold more NOx into the media than tissue from non-pregnant rats, or that taken from rats 1 day postpartum (Yallampalli *et al.*, 1994). Differences are also apparent in the concentrations of the different NOS isoforms. For example, in the rat uterus, iNOS protein concentrations were increased during pregnancy and decreased during delivery. In contrast, nNOS protein was present only in non-pregnant animals and was not apparent during pregnancy or delivery, whereas eNOS protein concentrations did not alter across the stages examined (Dong *et al.*, 1996).

The mammary gland is capable of producing NO and studies in the uterus show that the reproductive status of the animal may effect the production of NO. There have not been any comparative studies done, however, on the mammary gland production of NO at different stages of pregnancy and/or lactation. Thus the objectives of this chapter were to examine the production of NO by the mammary gland during pregnancy and lactation and to determine if the mammary gland responses are different between the different stages. This would give a further insight into the control of NO production within the mammary gland.

5.3 Materials and Methods

5.3.1 *Animals*

Seven pregnant (approximately 12-14 days gestation) and 11 lactating (between 12-18 days postpartum) female rats of the Sprague-Dawley strain were used in these experiments. Animals were cared for as described in Section 2.1.

5.3.2 *Method of Euthanasia*

Rats used in the following experiments were euthanased either using CO_2, or by the intraperitoneal infusion of an overdose of sodium pentabarbitone as described in Section 2.5.2.

5.3.3 *Tissue Samples*

Immediately following euthanasia, tissue was excised from the left abdominal mammary gland, placed into cryovials and snap frozen in liquid nitrogen. DNA, RNA and protein were subsequently measured as described in Sections 2.6, 2.7 and 2.12.

The effect of developmental state on each of these measurements (DNA/RNA/protein) were analysed by ANOVA.

5.3.4 *Explant Culture*

Tissue Samples for Explant Culture

Immediately following euthanasia, the right abdominal mammary glands were collected, placed into a petri dish containing a small amount of culture medium and then processed for explant culture as described in Section 2.5.

Explant Culture, Experimental Design

Explants were prepared and cultured as described in Section 2.5.2. Following 24 hours in culture, the media were collected for subsequent analysis of NOx ('basal') and replaced with fresh media containing the treatment. Treatments consisted of various concentrations of IFN-γ, LPS and inhibitors and are given in Table 5.1 along with the abbreviations used for the treatments. Following 24 hours in culture with the treatment, the media were collected and subsequently analysed for NOx. Explants were collected and weighed as described in Section 2.5.2.

Effect of stage of development on basal NOx production, and the effect of euthanasia method and length of time in culture on NOx production by pregnant rats were analysed by ANOVA. All other analyses of treatment effects were analysed by ANCOVA using basal NOx values as the covariate.

Table 5.1: Treatments used to inhibit the production of NOx in stimulated mammary explants. Explants were incubated in media containing, LPS or rIFN-γ and either L-NNA or AG at the concentrations given below. The abbreviations for the treatments are also given.

Treatment Abbreviation	μg/ml LPS	U/ml rIFN-γ	mM L-NNA	μM AG
LPS	10	-		-
LPS+0.1	10	-	0.1	-
LPS+0.5	10	-	0.5	-
LPS+1	10	-	1	-
LPS+10	10	-	-	10
LPS+50	10	-	-	50
LPS+100	10	-	-	100
IFN	-	250	-	-
IFN+0.1	-	250	0.1	-
IFN+0.5	-	250	0.5	-
IFN+1	-	250	1	-
IFN+10	-	250	-	10
IFN+50	-	250	-	50
IFN+100	-	250	-	100

5.3.5 *Comma-D Culture*

Comma-D cells were grown under various conditions to provide a source of total RNA, which were used as controls for Northern analysis.

Northern Control eNOS

Following protocols described in Section 2.3, a 75 cm^2 flask was seeded with Comma-D cells and grown to confluence. The cells were then passaged into two flasks and cultured overnight at 37 °C. The cells were harvested and RNA was extracted using TRIzol® as described in Section 2.11.2.

Northern Control iNOS

Following protocols described in Section 2.3, Comma D cells were seeded into four 75 cm^2 flasks and grown for 4 days. All flasks were then made quiescent by culture in quiescent medium for 3 days. The medium was discarded and two flasks each received 16 ml of quiescent medium containing 400 U (25 U/ml) mIFN-γ. The remaining two flasks each received 16 ml fresh quiescent medium. Following 6 hours in culture the cells were harvested and RNA was extracted from the treated and non-treated cells using TRIzol® as described in Section 2.11.2.

5.3.6 *Northern Analysis*

Total RNA (20 μg), extracted from mammary tissues (Section 5.3.3) and Comma-D cells (Section 5.3.5) was subjected to electrophoresis on formaldehyde/agarose gels, transferred onto nitrocellulose membranes then probed with a cDNA probe for either iNOS or eNOS as described in Sections 2.13, 2.14 and 2.16.

5.4 Results

Preliminary data from some of the following experiments were presented at the 1st International Conference, Biology, Chemistry and Therapeutic Applications of Nitric Oxide. San Francisco, CA, June 2000 (Turner *et al.*, 2000).

5.4.1 *Tissues DNA Protein and RNA*

There were significantly greater concentrations of DNA, RNA and protein in mammary gland tissue from rats during mid-lactation than in tissue from pregnant rats (Figure 5.1). The concentration of protein in tissue from pregnant rats was also significantly lower than that in late lactation ($P < 0.0001$). Concentrations of both DNA and RNA were similar in pregnancy and late lactation. Stage of lactation had no significant effect on any of the parameters.

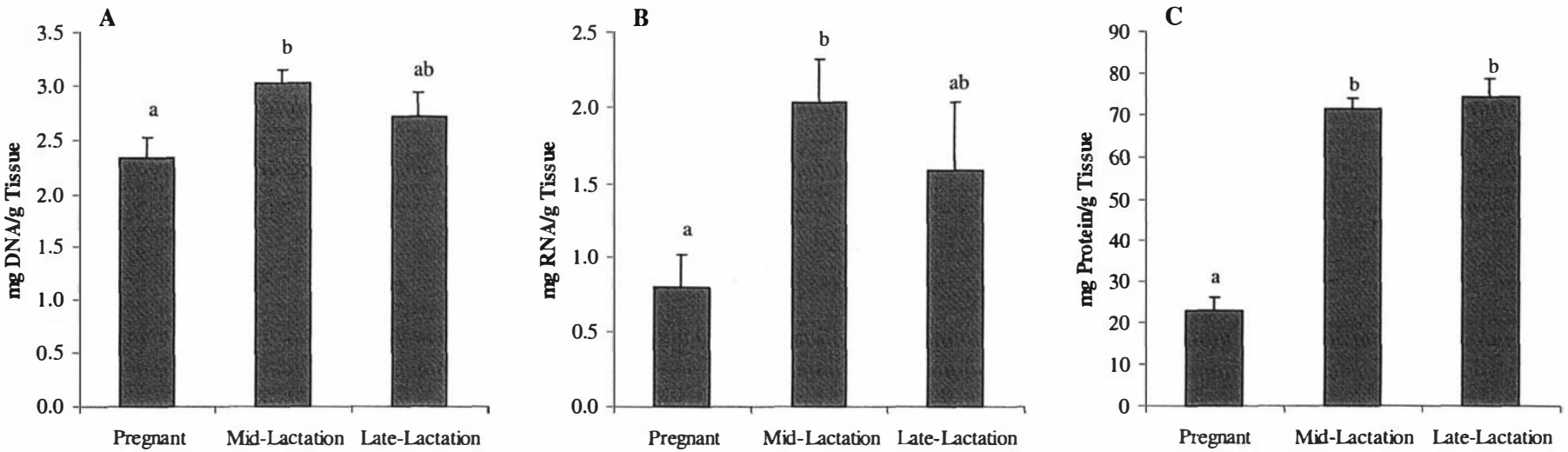

Figure 5.1: Concentration (mg/g tissue) of DNA, RNA and protein in mammary gland tissue of rats at different stages of development. Mammary tissue was collected from rats during mid-pregnancy (n = 6; D 12-14 gestation), mid-lactation (n = 9; D 12-14 postpartum) and late-lactation (n = 3; D 17-18 postpartum). Data are expressed as the mean ± SEM. Differences between the means are indicated by superscript letters, where means not sharing common letters are significantly different ($P < 0.05$). **A:** Tissue DNA. **B:** Tissue RNA. **C:** Tissue protein.

5.4.2 *Northern Analysis of Pregnant and Lactating Mammary Tissue*

Northern analysis of mammary tissue of pregnant rats between 12 and 14 days of gestation showed a faint signal when hybridised with the murine iNOS probe (Figure 5.2). A very strong signal is apparent in the control Comma-D cells following induction with 25 U/ml mIFN-γ for 24 hours.

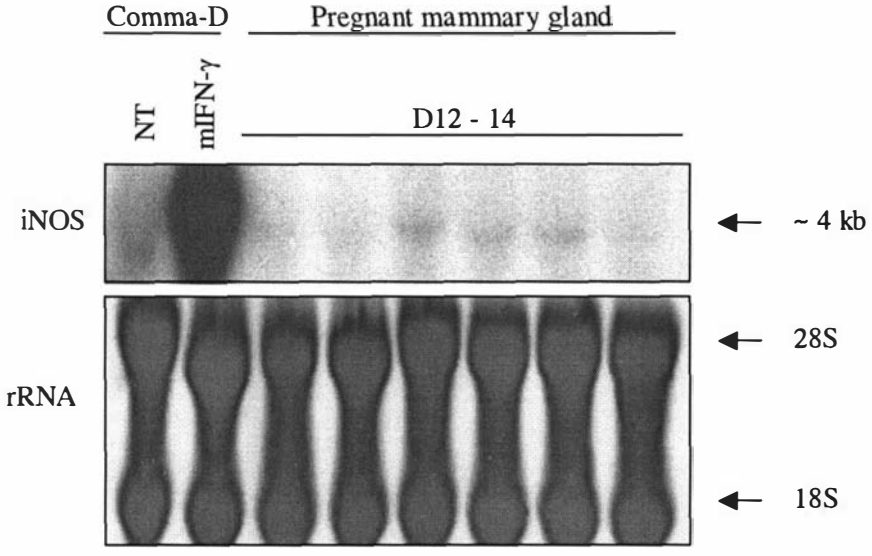

Figure 5.2: Expression of iNOS mRNA in mammary tissue from pregnant rats. Total RNA (20 μg/lane) from Comma-D cell negative controls (NT: Comma-D cells grown to confluence then made quiescent), Comma-D cell positive controls (mIFN-γ: Comma-D cells grown to confluence, made quiescent then treated with 25 U/ml mIFN-γ for 6 hours) and mammary tissue collected from 6 pregnant rats was prepared then analysed for the expression of iNOS by Northern analysis (top panel). Following the transfer of the RNA from the gel to the membrane, total RNA was visualised using methylene blue (bottom panel).

A similar signal was apparent in the analysis of the lactating tissue. There was no apparent change in the amount of iNOS across lactation, nor between the pregnant and lactating tissues (Figure 5.3).

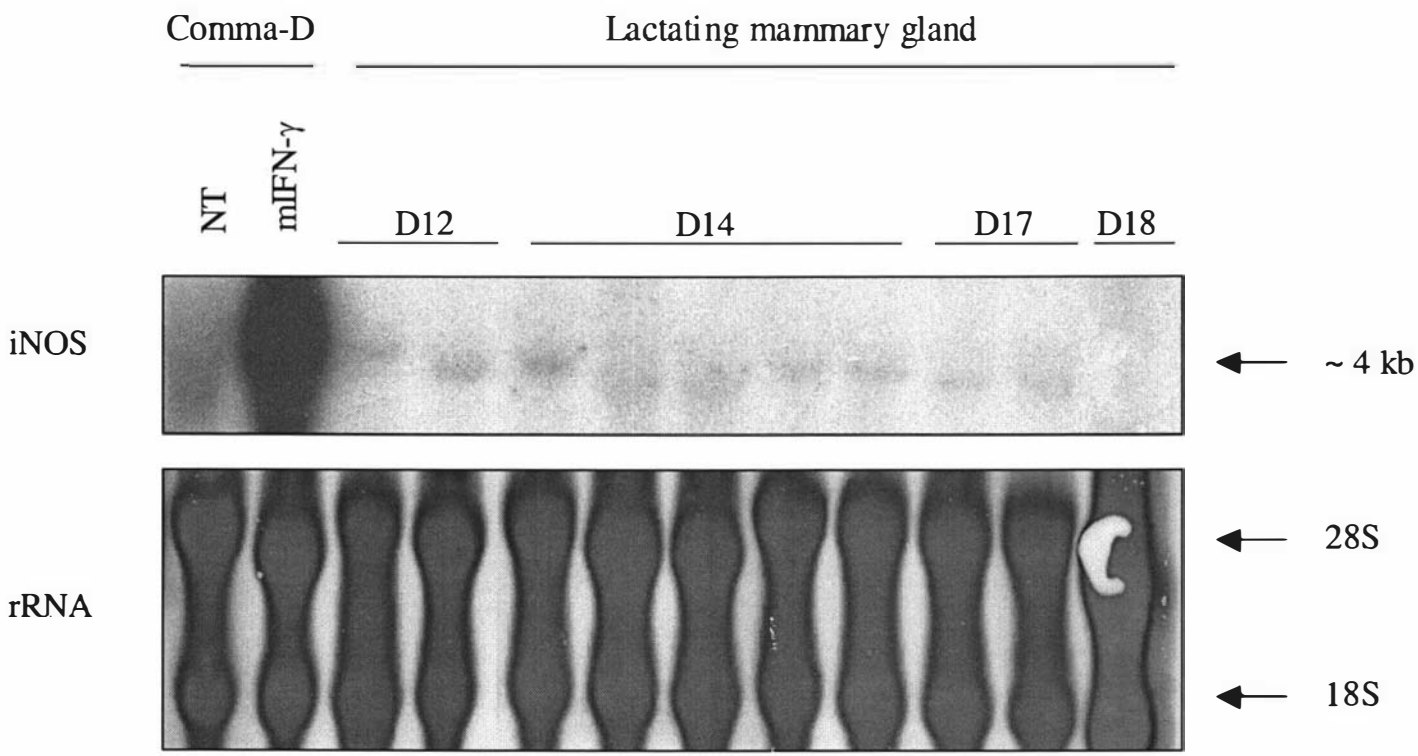

Figure 5.3: Expression of iNOS mRNA in mammary tissue from lactating rats. Total RNA (20 μg/lane) from Comma-D cell negative controls (NT: Comma-D cells grown to confluence then made quiescent), Comma-D cell positive controls (mIFN-γ: Comma-D cells grown to confluence, made quiescent then treated with 25 U/ml mIFN-γ for 6 hours) and mammary tissue collected from 10 lactating rats at days 12, 14, 17 and 18 following parturition was prepared then analysed for the expression of iNOS by Northern analysis (top panel). Following the transfer of the RNA from the gel to the membrane, total RNA was visualised using methylene blue (bottom panel).

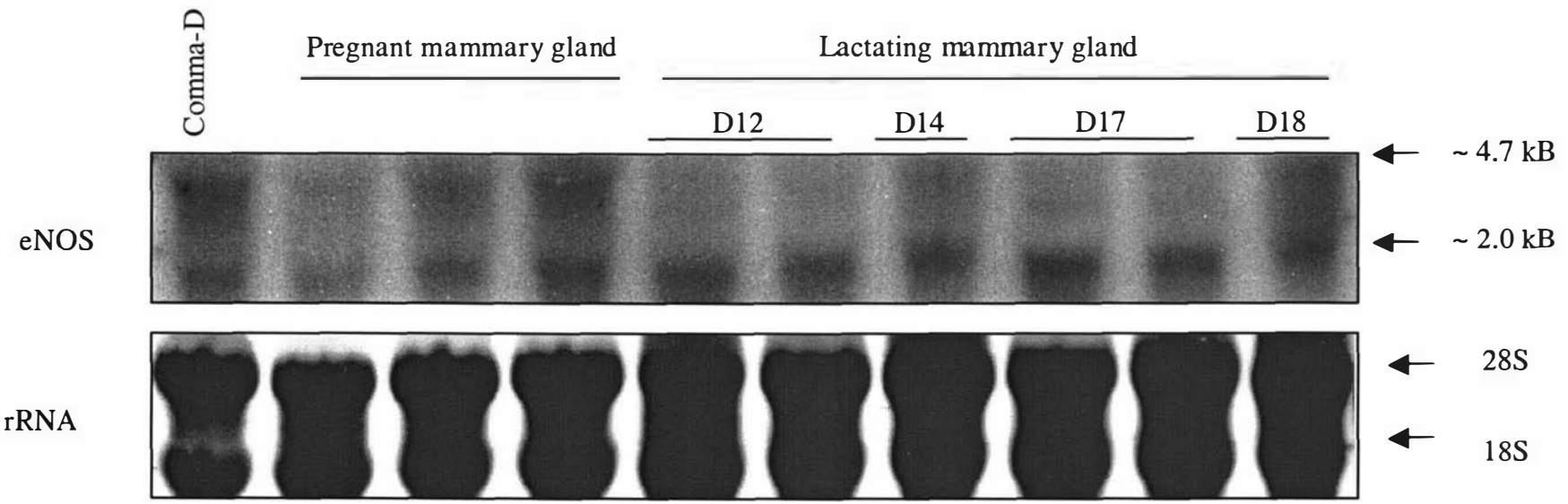

Figure 5.4: Expression of eNOS mRNA in mammary tissue from pregnant and lactating rats. Total RNA (20 μg/lane) from Comma-D cell controls (collected during the growth phase of the cells), mammary tissue collected from 3 pregnant rats (D 12-14 of gestation), and mammary tissue collected from 6 lactating rats on days 12, 14, 17 and 18 following parturition was prepared then analysed for the expression of eNOS by Northern analysis (top panel). Following the transfer of the RNA from the gel to the membrane, total RNA was visualised using methylene blue (bottom panel).

When probed with a bovine eNOS probe there were two bands apparent, one at approximately 4 kb and the other slightly below 2 kb (Figure 5.4). Although both bands were apparent in both pregnant and lactating tissues, the 2 kb band tended to be much stronger and the 4 kb band more variable in signal in the samples from lactating rats collected on days 12 and 17 postpartum. Methylene blue staining of the membrane prior to Northern analysis confirmed that the lanes were loaded with very similar amounts of RNA (Figure 5.4, bottom panel).

5.4.3 *Unstimulated (Basal) Production of NOx by Mammary Explants*

An examination of the production of NOx by mammary explants from rats euthanased using sodium pentabarbitone indicates that the production during the first 24 hours in culture is affected by the development stage of the gland from which the explants were harvested (Figure 5.5). The production of NOx by explants from pregnant rats was significantly greater than that produced by explants from rats during either mid ($P < 0.001$) or late ($P < 0.0001$) lactation. The production of NOx by explants taken from rats during mid-lactation was significantly greater than that produced by explants taken from rats during late lactation ($P < 0.0001$).

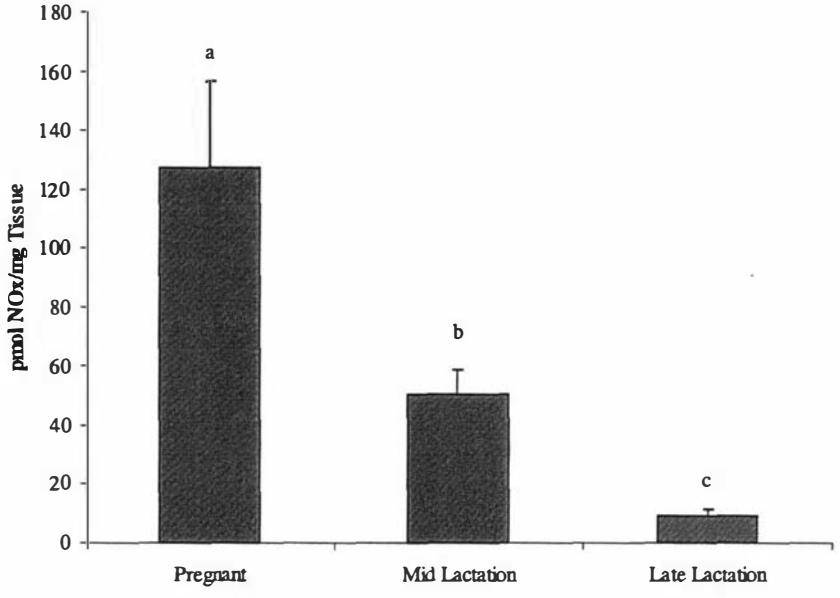

Figure 5.5: The effect of stage of development on basal production of NOx by mammary explants from rats. Pregnant (n=4; D 12-14 gestation) or lactating (Mid: n=7, D 12-14; Late: n=3, D 17-18 postpartum) rats were euthanased by administration of sodium pentabarbitone. Explants were cultured for 24 hours. The medium was then collected and subsequently analysed for NOx. Data are expressed as the mean ± SEM. Differences between the means are indicated by superscript letters, where means not sharing common letters are significantly different ($P < 0.001$).

There was a significant difference ($P < 0.0001$) in the production of NOx by explants from pregnant rats when comparing the two methods of euthanasia. Explants from rats euthanased using CO_2 produced significantly less NOx into the medium than explants from rats euthanased by an overdose of sodium pentabarbitone (Figure 5.6). Differences in the amount of NOx produced by mammary explants from pregnant rats euthanased by different methods were still apparent after 48 hours in culture (Figure 5.6). However, the production of NOx over the first and second 24 hour periods of incubation was similar for explants from rats euthanased by either method (Figure 5.6). NOx production over the two incubation periods was also similar when explants were prepared from rats euthanased with sodium pentabarbitone at either mid (n=7) or late lactation (n=3; data not shown).

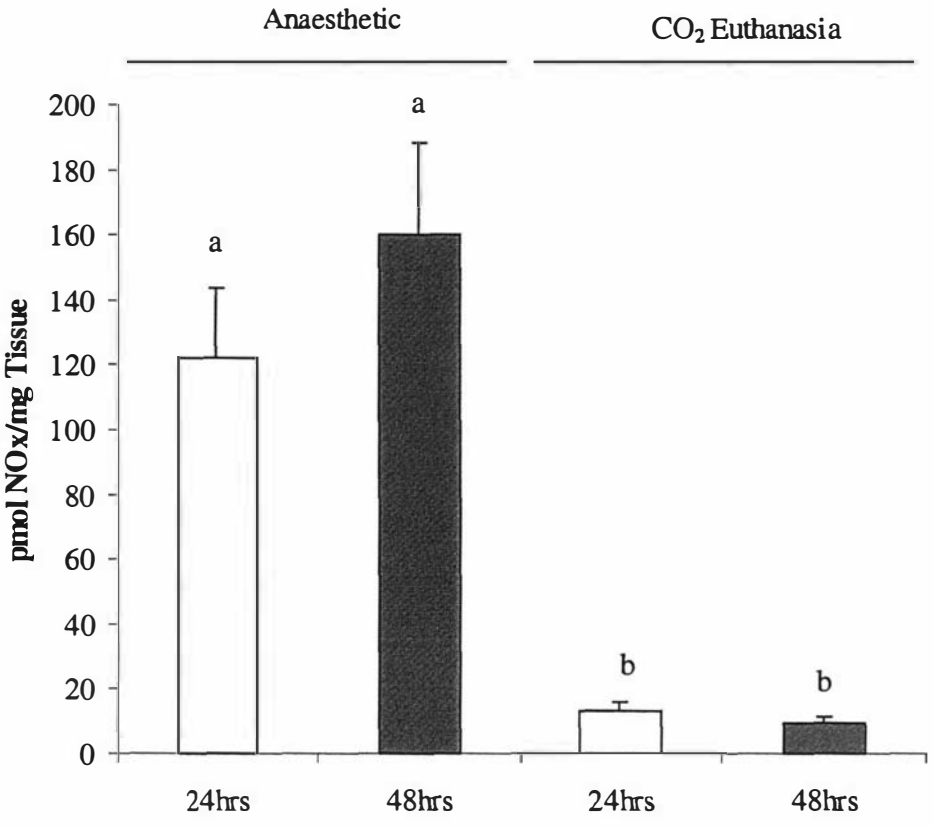

Figure 5.6: Effect of euthanasia method and length of culture on the production of NOx by mammary explants from pregnant rats. Pregnant (D 12-14 gestation) rats were euthanased either by administration of sodium pentabarbitone (anaesthetic; n=4), or by CO_2 euthanasia (CO_2; n=3). Explants were cultured for 48 hours. The media were replaced with fresh medium at 24 hours. Medium collected at 24 and 48 hours was subsequently analysed for NOx. Data are expressed as the mean ± SEM. Differences between the means are indicated by superscript letters, where means not sharing common letters are significantly different ($P < 0.0001$).

The production of NOx by mammary explants, taken from rats that were euthanased using an overdose of sodium pentabarbitone, during mid-lactation was 50.3 ± 8.6 pmol NOx/mg tissue (Figure 5.5). Only one rat in mid-lactation was euthanased using CO_2. The production of NOx by the explants from this rat was 14.7 pmol NOx/mg tissue. It therefore appears that explants collected during mid-lactation from rats euthanased using CO_2 might also be producing less NOx. However, without further data this finding remains tentative.

5.4.4 *Stimulation of NOx Production in Mammary Explants*

Explants cultured from rats euthanased using CO_2 released significantly less ($P < 0.005$) NOx into the medium in response to treatment with LPS than those explants cultured from rats euthanased using an overdose of sodium pentabarbitone (Figure 5.7).

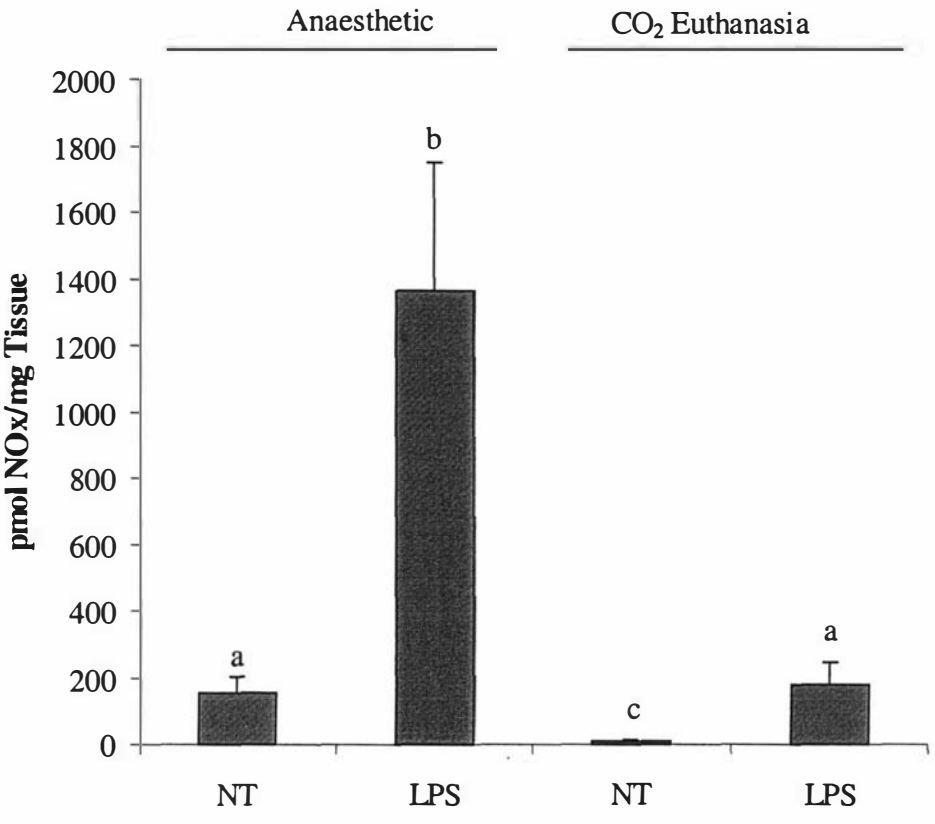

Figure 5.7: Effect of euthanasia method on the response of mammary explants from pregnant rats to LPS. Explants were prepared from pregnant (D 12-14 gestation) rats that were euthanased either by administration of sodium pentabarbitone (anaesthetic; n=4), or by CO_2 euthanasia (CO_2; n=3). After 24 hours in culture, the explants were either left untreated (NT) or were treated with 10 μg/ml LPS for 24 hours (LPS). Media were collected and analysed for NOx. Data are expressed as the mean ± SEM. Differences between the means are indicated by superscript letters, where means not sharing common letters are significantly different ($P < 0.05$).

As the method of euthanasia significantly affected the production of NOx by explants from pregnant rats in response to LPS (Figure 5.7), and no information is available regarding the effect of euthanasia method on the production by explants from lactating rats, the comparison between production by explants from pregnant and lactating rats was carried out on the data from rats euthanased using sodium pentabarbitone only. There was no difference in the production of NOx in response to LPS by mammary explants from pregnant rats compared with those from lactating rats (Figure 5.8).

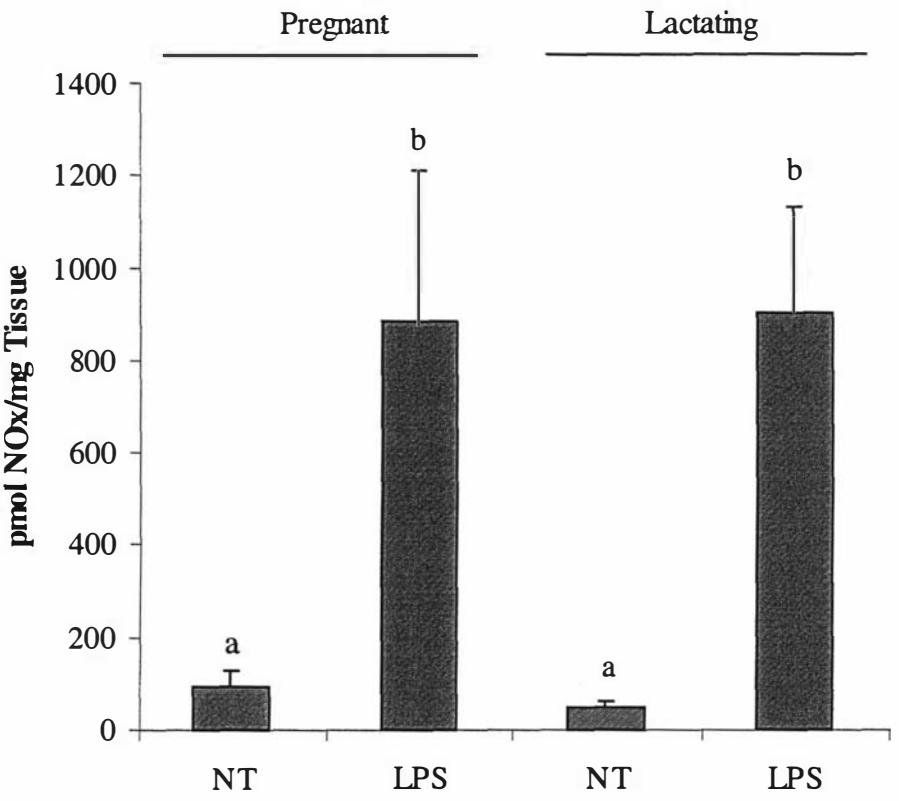

Figure 5.8: The response of explants of mammary tissue to LPS. Mammary explants, excised from pregnant (D 12–14 gestation; n=4) or lactating (D 12-14 postpartum; n=7) rats, euthanased using sodium pentabarbitone were cultured for 24 hours then either left untreated (NT) or were treated with LPS (10 μg/ml) for 24 hours (LPS). Media were collected and analysed for NOx. Data are expressed as the mean ± SEM. Differences between the means are indicated by superscript letters, where means not sharing common letters are significantly different ($P < 0.0001$).

There was no significant difference in the production of NOx by mammary explants from pregnant rats in response to stimulation with rIFN-γ between the two methods of euthanasia (Figure 5.9).

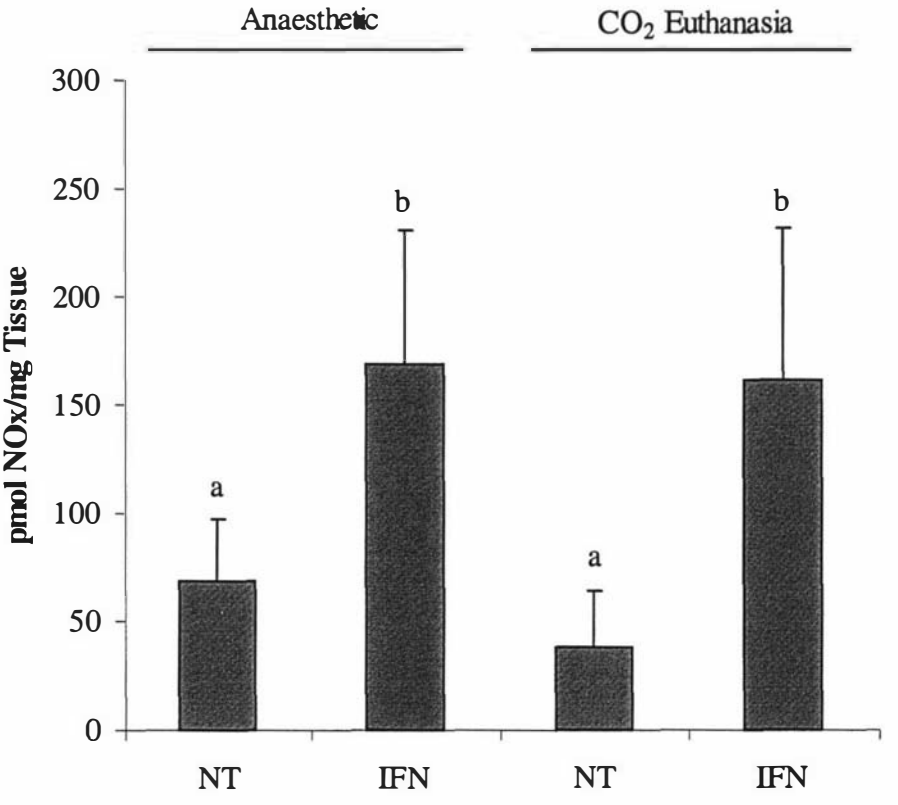

Figure 5.9: Effect of euthanasia method on the response of mammary explants from pregnant rats to rIFN-γ. Explants were prepared from pregnant (D 12-14 gestation) rats that were euthanased either by administration of sodium pentabarbitone (anaesthetic; n=4), or by CO_2 euthanasia (CO_2; n=3). After 24 hours in culture, the explants were either left untreated (NT) or were treated with 250 U/ml rIFN-γ for 24 hours (IFN). Media were collected and analysed for NOx. Data are expressed as the mean ± SEM. Differences between the means are indicated by superscript letters, where means not sharing common letters are significantly different ($P < 0.05$).

As the method of euthanasia had no significant effect on the response of mammary explants from pregnant rats to rIFN-γ (Figure 5.9), comparisons between the response of explants from pregnant and lactating rats was made using data from both methods of euthanasia. Treatment of mammary explants from either pregnant or lactating rats with 250 U/ml rIFN-γ for 24 hours resulted in a significant increase ($P < 0.05$) in the concentration of NOx in the medium when compared to the production of non-treated explants (Figure 5.10). There was no significant difference in the amount of NOx produced following induction of NOx with rIFN-γ between explants from pregnant and lactating rats (Figure 5.10).

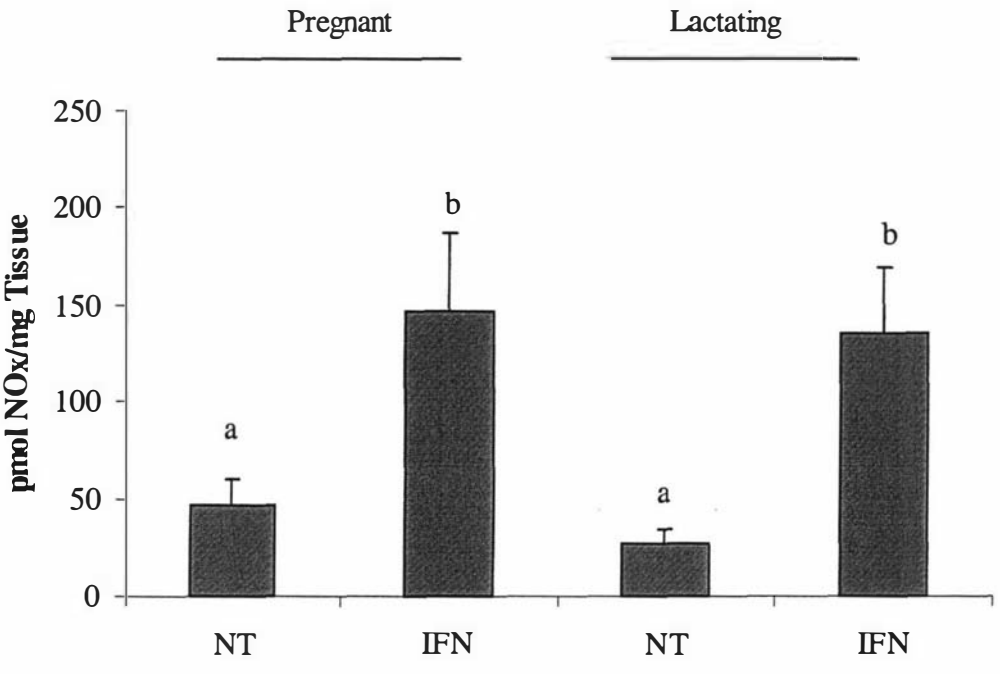

Figure 5.10: The response of explants of mammary tissue to rIFN-γ. Mammary explants, excised from pregnant (D 12–14 gestation; n=7) or lactating (D 12-14 postpartum; n=8) rats were cultured for 24 hours then either left untreated (NT) or were treated with rIFN-γ (250 U/ml) for 24 hours. Media were collected and analysed for NOx. Data are expressed as the mean ± SEM. Differences between the means are indicated by superscript letters, where means not sharing common letters are significantly different ($P < 0.05$).

5.4.5 *Inhibition of Stimulated NOx by Mammary Explants*

The previous experiments (Section 5.4.3 and 5.4.4) showed that the method of euthanasia affects the response of mammary explants from pregnant rats to LPS but has no significant effect on the response of the explants to rIFN-γ. This anaesthetic effect did not become apparent until data from all experiments were analysed at the conclusion of the trials. Due to the availability of rats, often tissue was taken from animals euthanased by other colleagues thus leading to the lack of consistency in the method of euthanasia between experiments. In the following experiments described in this section the pregnant rats were euthanased using CO_2 and the lactating rats were euthanased using an overdose of sodium pentabarbitone.

Mammary explants from pregnant rats treated for 24 hours with 10 μg/ml LPS significantly increased the NOx in the media when compared to non-treated explants (Figure 5.11 A and B). When AG, a known NOS inhibitor was also included at either 50 or 100 μM, there was a significant decrease ($P < 0.05$) in the LPS stimulated production of NOx when compared to explants treated with LPS alone (Figure 5.11 A). However, 10 μM AG had no effect on the production. The inclusion of 0.1, 0.5 or 1 mM L-NNA also resulted in a significant decrease ($P < 0.05$) in LPS stimulated NOx production, at 1 mM this production was not significantly different from the amount of NOx produced by the untreated (NT) explants (Figure 5.11 B).

Treatment of explants of mammary tissue from pregnant rats with rIFN-γ (250 U/ml) significantly increased ($P < 0.05$) the amount of NOx in the media (Figure 5.11 C and D). When explants were stimulated with rIFN-γ, NOx production was similar in the presence of 0, 10, 50 and 100 μM AG (Figure 5.11 C). In contrast, treatment with L-NNA at either 0.5 or 1 mM decreased significantly ($P < 0.05$) the NOx production that was stimulated by rIFN-γ, while 0.1 mM L-NNA was without effect (Figure 5.11 D).

When mammary explants from lactating rats were cultured with 10 μg/ml LPS for 24 hours, there was a significant increase ($P < 0.05$) in the NOx measured in the media (Figure 5.12 A and B). The inclusion of either 50 or 100 μM AG resulted in a significant decrease ($P < 0.05$) in the LPS stimulated NOx production when

compared to the production by explants treated with LPS alone (Figure 5.12 A). At 100 μM AG, the decreased production was not different from that of non-treated (NT) explants. There was no significant difference between NOx production stimulated by LPS in the presence of 10 μM AG compared to that stimulated by LPS alone. There was a significant decrease ($P < 0.05$) in LPS stimulated NOx production with 0.1, 0.5 or 1 mM L-NNA (Figure 5.12 B). The production of NOx by explants treated with both LPS and 1 mM L-NNA was not significantly different from that of non-treated explants.

Mammary explants taken from lactating rats and cultured with rIFN-γ (250 U/ml) for 24 hours produce significantly more ($P < 0.05$) NOx than non-treated explants (Figure 5.12 C and D). The inclusion of the NOS inhibitor AG at 10, 50 or 100 μM had no effect on the rIFN-γ stimulated NOx production. L-NNA at either 0.1 or 1 mM had no effect on rIFN-γ stimulated NOx production, however, L-NNA at 0.5 mM significantly decreased ($P < 0.05$) the rIFN-γ stimulated production to such an extent that it was not different from the production of NOx by non-treated cells.

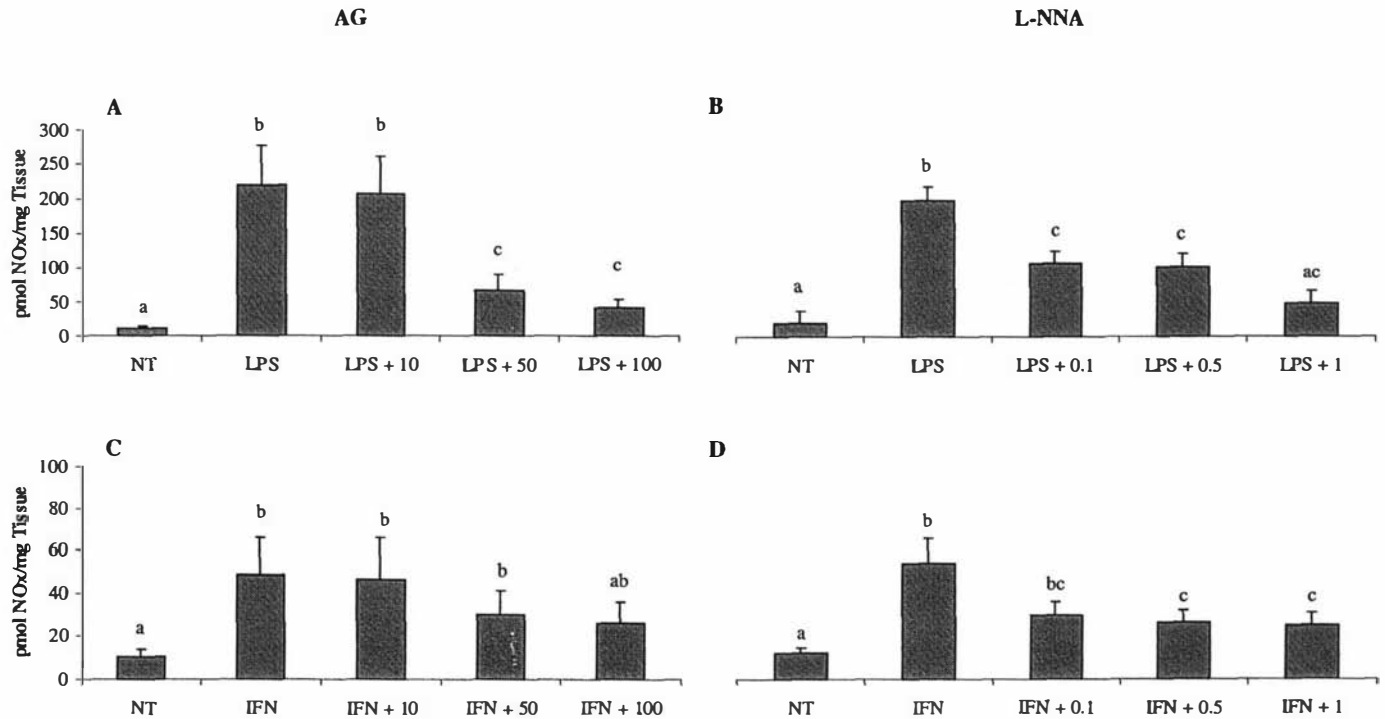

Figure 5.11: Stimulation and inhibition of NOx production in explants of mammary tissue from pregnant rats. Mammary explants collected from 3 rats between D 12 and 14 of gestation were cultured for 24 hours then either left untreated (NT) or were treated with either **A:** LPS (10 μg/ml), LPS + AG at 10, 50 or 100 μM, **B:** LPS + LNNA at 0.1, 0.5 or 1 mM, **C:** rIFN-γ (250 U/ml) + AG at 10, 50 or 100 μM or **D:** rIFN-γ + LNNA at 0.1, 0.5 or 1 mM. Following 24 hours exposure to the treatments, the media were collected and subsequently analysed for NOx. Note the difference in scale between the rIFN-γ and LPS treated explants. Data are expressed as the mean ± SEM. Differences between the means are indicated by superscript letters, where means not sharing common letters are significantly different ($P < 0.05$).

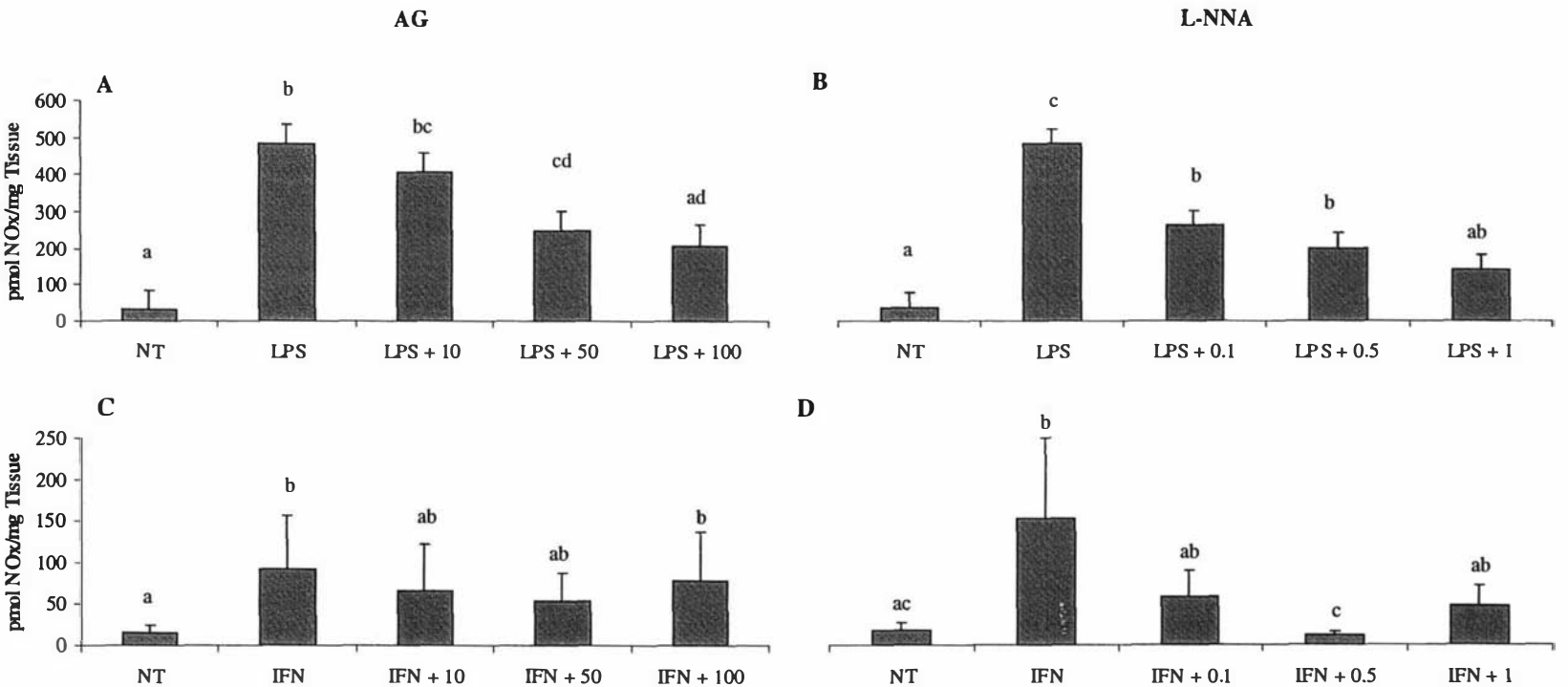

Figure 5.12: Stimulation and inhibition of NOx production in explants of mammary tissue from lactating rats. Mammary explants collected from 3 rats between D 12 and 14 postpartum were cultured for 24 hours then either left untreated (NT) or were treated with either **A:** LPS (10 μg/ml), LPS + AG at 10, 50 or 100 μM, **B:** LPS + LNNA at 0.1, 0.5 or 1 mM, **C:** rIFN-γ (250 U/ml) + AG at 10, 50 or 100 μM or **D:** rIFN-γ + LNNA at 0.1, 0.5 or 1 mM. Following 24 hours exposure to the treatments, the media were collected and subsequently analysed for NOx. Note the difference in scale between the rIFN-γ and LPS treated explants. Data are expressed as the mean ± SEM. Differences between the means are indicated by superscript letters, where means not sharing common letters are significantly different ($P < 0.05$).

5.5 Discussion

5.5.1 *Tissue DNA, RNA and Protein Concentrations*

The results from the experiments examining the production and release of NOx into the media presented in this chapter are expressed per gram of mammary tissue. However, the protein and nucleic acid concentrations of the tissues were also determined to characterise the composition of the mammary glands more accurately and to allow comparison with results reported in the literature.

Total mg protein/g mammary tissue of both pregnant and lactating rats were in agreement with those reported by both Baldwin & Milligan (1966) and Bennett (1995), as were the RNA concentration in the glands from pregnant rats at a similar stage of pregnancy. However, the RNA concentration of the glands from lactating rats (2.0 mg RNA/g tissue at D 14 and 1.6 mg/g tissue at D 18 of lactation) was about a third of that reported by Tucker (1964) and Bennett (1995) and much lower than the 22.3 mg/g tissue found by Baldwin & Milligan (1966). It is possible that these differences could be as a result of the method of extraction or quantification of the RNA. The method of RNA extraction used in each case was different (the commercially available TRIzol® was used in this experiment), and the methods of quantification also varied. Both Bennett (1995) and Tucker (1964) quantified RNA by measuring the absorbance of the sample at 260 nm and assuming that 1 absorbance unit is equal to 40 μg/ml of single stranded RNA. This method of measurement is often used, however, it is reliant upon the purity of the sample as proteins and free nucleotides can contribute to the absorbance. The RiboGreen™ method used in this Thesis (Section 2.12), although it does not distinguish RNA from DNA, is a much more sensitive assay than standard ultraviolet absorbance determination. A comparison of concentrations from samples measured by both the RiboGreen™ and ultraviolet absorbance assays indicated that the values obtained by the ultraviolet absorbance assay were on average about 2.5-fold higher than that of the RiboGreen™ assay (results not shown). The differences in RNA concentration of the lactating glands between the results presented here and those of Tucker (1964), Baldwin & Milligan (1966) and Bennett (1995) could therefore be due to differences in the

sensitivity of the assays used and to interference by other components which are in higher abundance during lactation, for example, proteins.

The concentration of DNA in mammary glands during pregnancy was higher than that reported by Bennett (1995) and Rosso *et al.,* (1981) while the concentration during mid lactation was lower than that reported by Knight *et al.*, (1984) and Bennett (1995). These small differences could be due to the effect of the stage of lactation. Stage of lactation influences the concentration of DNA in mammary glands as milk content contributes to the weight of the gland but not the cell number, and therefore has a dilution effect. In the present study, mammary glands collected 10 days prior to parturition would contain negligible milk and therefore the DNA concentration would not be diluted. This is in contrast to DNA measured in glands collected by Bennett (1995) and Rosso *et al.*, (1981), at peri-parturition, when milk secretions appear (Cowie *et al.*, 1980). Similarly, mammary glands collected around the period of peak milk production would contain greater but more variable amounts of milk and therefore dilute the DNA concentration to differing degrees. This could thus account for the small differences between the DNA concentrations reported in this study and those of Knight *et al.,* (1984) and Bennett (1995).

5.5.2 *Northern Analyses*

Although both Low *et al.,* (1997) and Orucevic *et al.,* (1999) have used Northern analysis to show the induction of iNOS in mammary cell lines with cytokines or LPS, this study appears to be the first time Northern analysis has been used to examine the production of unstimulated iNOS mRNA in mammary tissue. In these experiments no differences were found in the iNOS mRNA content of unstimulated glands during either pregnancy or lactation, or between pregnancy and lactation. This is not the case in other tissues associated with reproduction. For example, in the uterus of pregnant rats, there is an approximate 8-fold increase in iNOS mRNA concentration when compared to non-pregnant controls with mRNA levels returning back to approximate non-pregnant levels during labour and 1 day postpartum (Dong *et al.*, 1998).

No data are available in the literature on the content of eNOS mRNA in the mammary gland although there have been studies in which eNOS immunoreactive protein was measured in the secretory epithelium during lactation (Lacasse *et al.*, 1996; Iizuka *et al.*, 1998) and in myoepithelial cells of estrodiol/progesterone primed rats (Onoda & Inano, 1998). Pregnancy results in an increase in the eNOS mRNA of skeletal muscles of pregnant guinea pigs when compared to non-pregnant controls (Weiner *et al.*, 1994). The Northern blot presented in this Thesis (Figure 5.4) shows eNOS mRNA transcripts of approximately 4 and 2 kb within the mammary tissue of both pregnant and lactating rats with the 2 kb band tending to have a stronger signal in the samples from lactating rats. Alternate transcript sizes for eNOS mRNA (4 kb and 2.6 kb) have been reported previously in the suprachiasmatic nuclei of the hypothalamus (Caillol *et al.*, 2000). The different transcripts could be due to alternative splicing of the eNOS gene. It is interesting that the samples from the lactating rats, which tended to show a weaker signal for the 4 kb eNOS transcript, were also shown in culture to release less NOx into the media when compared to explants cultured from pregnant tissues (Figure 5.5).

To my knowledge, this is the first report of eNOS mRNA within the mammary gland of pregnant and lactating rats although previous studies had confirmed the presence of eNOS immunoreactive protein. The experiments presented in this chapter confirm that the mammary gland is capable of the expression of both iNOS and eNOS mRNA. This finding leads to an exciting array of further experiments aimed at the elucidation of the physiological controls of this expression.

5.5.3 *CO_2 Euthanasia and Anaesthetic Effects*

Initially the aims of this study were to directly compare NO production by mammary gland tissue from pregnant and lactating rats and its response to common stimulators of NOS. However, the results obtained in this section have suggested an apparent effect of the method of euthanasia on NOx production by mammary explants. The comparisons made between the effect of euthanasia method on NOx production by pregnant rats provide some interesting information. However, due to a lack of animals there is not enough information to draw conclusions of the effects of euthanasia method on NO production by explants cultured from lactating rats.

The effect of anaesthetic on NO production appears to be dependent upon the anaesthetic and tissue under study. For example, isoflurane and propofol increase the production of hippocampal NO and increased the release of NO from cultured porcine aortic endothelial cells respectively (Petros *et al.*, 1993; Matsuoka *et al.*, 1999). And while halothane increases NO production in the rat cortex and cerebellum, it attenuates endothelium-dependent relaxation of isolated denuded rabbit aortic rings (Blaise *et al.*, 1994; Sjakste *et al.*, 1999). Sodium pentabarbitone also has conflicting effects depending on the tissue type, for example, it has no effect on NO release in the rat cortex and cerebellum but enhances LPS induced cardiovascular dysfunction in rats (Hoque *et al.*, 1996; Sjakste *et al.*, 1999). In this chapter, when sodium pentabarbitone was used to euthanase pregnant rats, the cultured explants produced significantly more NOx during the first 24 hours in culture, and there was also an enhanced response to LPS when compared to the response from the explants from rats euthanased using CO_2.

Subjecting either human umbilical vein endothelial cells or bovine pulmonary artery endothelial cells to hypoxia (0 % O_2) results in the decrease of eNOS mRNA to about one third of that apparent in cells cultured in a normoxic (21 % O_2) environment. However, this inhibitory effect occurs only after 24 hours of hypoxia (McQuillan *et al.*, 1994). Production of nitrite and nitrate is enhanced in type II alveolar epithelial cells exposed to 15 % CO_2, when compared to cells grown in 5 % CO_2. When grown in this hypercapnic environment, the cells also respond to LPS and cytokine with a greater production of NO (Lang *et al.*, 2000). Once again this effect is only apparent after 24 hours in culture. These studies suggest that either a lack of O_2, or an increase in CO_2, results in greater NO production. In this chapter, the explants excised from pregnant rats euthanased using 100 % CO_2 produced significantly less NO than those euthanased using sodium pentabarbitone. Whether this difference between the euthanasia methods is due to an inhibition by the exposure to CO_2 is speculative. Euthanasia using CO_2 exposes the rats to 100 % CO_2 for approximately 1-2 minutes, far short of the 24 hours required by McQuillan *et al.*, (1994) and Lang *et al.*, (2000) to detect any effect.

The method of CO_2 euthanasia can result in significant differences in various immunologic and hematologic variables and Pecaut *et al.*, (2000) suggest that

consistency in euthanasia procedures is important for the accurate interpretation of data. In the results described in this Thesis it can be only speculated as to whether the levels of NOx in the media of rats euthanased using CO_2 are a result of inhibition or whether the use of sodium pentabarbitone has resulted in increased production. However, if the latter is the case then it indicates that there are differences in the response of the pregnant and lactating tissues to sodium pentabarbitone. At this stage and without further information (especially regarding whether there is an effect of euthanasia method on the production of NO by explants from lactating rats), the only conclusion that can be drawn from this discrepancy is to echo the sentiments of Pecaut *et al.,* (2000) in that consistency in methods is required.

5.5.4 *NOx Production in Mammary Explants*

As reported previously for mammary epithelial cells (Figure 4.5 and 4.9), mammary explants from both pregnant and lactating rats responded to the stimulation with LPS (Figure 5.8) and IFN-γ (Figure 5.10) with increased production of NOx. There were no differences in the amount of NOx produced between the two states. There were, however, differences in the responses to the inhibitors, AG and L-NNA between the explants collected from pregnant and lactating rats. The effect was also dependent upon the stimulation (LPS or IFN-γ) used. L-NNA (1 mM) inhibited the LPS stimulation of NOx production from explants from both pregnant and lactating rats to such an extent that the production was not different from that of the non-treated explants (Figure 5.11 B and 5.12 B). In contrast, AG (100 μM) did not fully inhibit the LPS stimulated NOx production from explants from pregnant rats (Figure 5.11 A), but did inhibit the production from explants from lactating rats to such an extent that the production was no different from that of non-treated explants (Figure 5.12 A). Differences were also apparent in the effects that AG and L-NNA had on the inhibition of IFN-γ stimulated NOx production from explants from pregnant (Figure 5.11 C and D) and lactating (Figure 5.12 C and D) rats. Full inhibition of IFN-γ stimulated NOx production by explants from pregnant rats (Figure 5.11 C) was achieved with 100 μM AG, but not in explants from lactating rats (Figure 5.12 C). The opposite was apparent with 1mM L-NNA where full inhibition of IFN-γ stimulated production was apparent in explants from lactating rats (Figure 5.12 D)

and not in explants from pregnant rats (Figure 5.11 D). Why there are these differences in the effects of inhibitors between the different states of the mammary gland (pregnant versus lactating) is not known. It is unlikely that it is related to the differences in basal production of NOx (Figure 5.5) as the analysis of the effects of inhibitors is carried out using a covariate analysis, which takes these differences into account. It may be possible that the differences are due to the different euthanasia methods. In these experiments the pregnant rats were euthanased using CO_2 and the lactating rats were euthanased using an overdose of sodium pentabarbitone. Where the euthanasia method affected the response of explants from pregnant rats to LPS (Figure 5.7), it did not affect the response to IFN-γ (Figure 5.9). Without further experiments it is not possible to determine whether euthanasia method is responsible for the different responses to the inhibitors apparent.

5.5.5 *General Comments*

Although there are still many questions remaining from this section of work, it does provide a base to build on the current knowledge of NO production within the mammary gland. The results presented in this chapter confirm the presence of eNOS and iNOS mRNA within the mammary gland during both pregnancy and lactation and show that NO production can be stimulated during both developmental states using either rIFN-γ or LPS. Further experiments are required to determine the biological significance of these results.

The effect of the method of euthanasia has wide-ranging implications for not only mammary gland NO research, but for other systems in which mammalian tissue is being studied. Many researchers often fail to mention the method of euthanasia and thus prevent the accurate comparison of data between studies

CHAPTER SIX

EFFECT OF PROLACTIN ON NITRIC OXIDE PRODUCTION BY THE MAMMARY GLAND

6.1 Abstract

The mammary gland is capable of both the production of prolactin (PRL) and proteolytic processing into a 16 kDa fragment (16K PRL). Both PRL and 16K PRL appear to influence the production of NO in various cells and tissues. Thus, the effect of PRL on the production of NO in the mammary gland was investigated. Treatment of Comma-D cells in culture with 5 μg/ml of PRL stimulated the production of NOx during both the growth and quiescent phases by 118 % and 229 % respectively. PRL treatment also increased the production of NOx by explants of mammary tissue from pregnant (495 % increase) and lactating (223 % increase) rats. Comma-D cells cultured in the presence of PRL during the treatment phase and treated with mIFN-γ produced significantly more ($P < 0.05$) NOx than those cells cultured without PRL. In contrast, PRL did not significantly alter the IFN-γ induced increase ($P < 0.05$) in NOx in mammary explants from pregnant and lactating rats. The production of NOx by explants was more variable and the lack of significant effect of PRL could be due to this variability. Comma-D cells, treated with LPS, produced significantly more ($P < 0.05$) NOx into the medium than non-treated cells, except when the cells were exposed to PRL for the whole of the experiment. Whether PRL was included or not made no difference to the response of mammary explants from pregnant and lactating rats to treatment with LPS. Western analysis showed no evidence of proteolytic processing of PRL to 16K PRL. These results show that PRL has a stimulatory effect on the basal NO production by both mammary epithelial cells and explants of mammary tissue although given the pharmacological doses of PRL used in this study, and the differences in responsiveness between the explants from pregnant and

lactating rats and the Comma-D cells, the biological implications of these need further investigation.

6.2 Introduction

Pituitary prolactin (PRL), first described for its role in milk secretion has now been shown to have varied functions and as such is often classified as both a hormone and a cytokine. For reviews on PRL and its many varied functions see Ben-Jonathan *et al.* (1996) and Bole-Feysot *et al.* (1998). Given that the mammary gland is responsive to cytokines (Sections 4 & 5), the concept of PRL having cytokine-like roles raises exciting new questions for its function in the mammary gland.

Many extrapitutiary tissues, including secretory epithelial cells of the lactating mammary gland have been shown to synthesise and secrete PRL (Ben-Jonathan *et al.*, 1996; Lkhider *et al.*, 1997; Bole-Feysot *et al.*, 1998). Thus the mammary gland would not only be exposed to PRL from other tissues including the pituitary gland, but to locally synthesised PRL as well. The mammary gland is also capable of the processing of PRL into proteolytic fragments. Cultured mammary tissue from pregnant rats (D 10) cleave rat PRL to yield a 14 kDa fragment (14K PRL), whereas tissue from lactating rats yields fragments of 16, 14 and 11 kDa (Clapp, 1987; Baldocchi *et al.*, 1992; Baldocchi *et al.*, 1994). There are opposing views as to whether tissue from pregnant or lactating rats is more efficient at this cleavage. For example, Baldocchi *et al.* (1992) report that the cleavage of full-length PRL to 14K PRL occurs more rapidly when the incubation is done in the presence of tissue from pregnant rats rather than from lactating rats. In contrast, Clapp (1987) reported that tissue from lactating rats is more potent at cleaving the PRL than that from virgin or pregnant animals.

The processing in many tissues of full-length PRL into proteolytic fragments is well described and these fragments have been shown to be biologically active (Clapp *et al.*, 1988; Ferrara *et al.*, 1991; Clapp *et al.*, 1993; Corbacho *et al.*, 2000). These active PRL fragments play a role in the control of endothelial cell growth. The 16K

PRL (a 16 kDa proteolytic fragment from the N-terminal of full length PRL), generated from rat PRL, inhibits both the basal, and basic fibroblast growth factor (bFGF) stimulated, growth of bovine adrenal cortex capillary endothelial cells in a dose dependent manner. A similar effect is observed with human recombinant 16K PRL on the basal, and either bFGF or vascular endothelial growth factor-stimulated, growth of bovine brain capillary endothelial cells. Human 16K PRL also inhibits the stimulation of human umbilical vein endothelial cell proliferation by bFGF (Clapp *et al.*, 1988; Clapp *et al.*, 1993).

PRL and the proteolytic fragments generated from it may also play a role in the control of the production of NOx. For example, 16K PRL stimulates the production of NOx in fibroblasts and type II alveolar cells but not in smooth muscle cells (Corbacho *et al.*, 2000), whereas, dependent upon the concentration, full-length PRL inhibits the production of NOx (Corbacho, personal communication, 2000) or has no effect (Corbacho *et al.*, 2000). In direct contrast, 1 μg/ml PRL transiently increased intracellular nitrate and nitrite in mouse mammary epithelial cells in a dose dependant manner (Bolander, 2001).

Given the ability of the mammary gland to synthesise and process PRL and the reported contradictory effects of PRL on NO production, these experiments examine the effect PRL has on the mammary gland and whether the developmental state of the gland affects this.

6.3 Materials and Methods

6.3.1 *Experimental Design – Comma-D Culture*

Comma-D cells were seeded in 24 well tissue culture dishes in either growth medium (G; Section 2.3.1), or growth medium containing 5 μg/ml PRL (G-PRL). Cells were grown for 3 days then media were discarded and replaced with fresh media (G or G-PRL). Following a 24 hour incubation, the media were collected ("growing") and subsequently assayed for NOx. The media were replaced with either quiescent medium (Q; Section 2.3.1), or quiescent medium containing 5 μg/ml PRL (Q-PRL) and the cells were incubated for a further 24 hours. The media were then discarded and replaced with fresh Q or Q-PRL media. Following a further 24 hours in culture the media were collected ("quiescent") and replaced with fresh Q, or Q-PRL, media containing treatments. The treatments applied were as follows (Table 6.1; concentrations of the treatments given on the first mention); LPS (10 μg/ml), mIFN-γ (250 U/ml), mIFN-γ + AG (100 μM), mIFN-γ + L-NNA (1 mM), AG, L-NNA. Following 24 hours incubation in the treatment media, cells and media were collected and DNA content and NOx were measured.

Table 6.1: Culture media used in each experimental phase. For each treatment group (- PRL [no prolactin]; + PRL trt only [prolactin included during treatment phase only]; + PRL all [prolactin included throughout experiment]), the type of medium (Growth or Quiescent) used to culture the cells during each phase of the experiment are given. Prolactin (PRL) when included was added at a concentration of 5 μg/ml.

	- PRL	+ PRL trt only	+ PRL all
Growth Phase	Growth	Growth	Growth + PRL
Quiescent Phase	Quiescent	Quiescent	Quiescent + PRL
Treatment Phase	Quiescent	Quiescent + PRL	Quiescent + PRL

6.3.2 *Experimental Design – Explant Culture*

The culture media types and abbreviations for the quiescent and treatment phases given in Table 6.1 also apply to the explant culture experiments. Treatment groups were - PRL, + PRL trt only and + PRL all. Of the six rats used in these experiments, three pregnant (D 12-14 gestation) and one lactating (D 14 postpartum) rat were euthanased using CO_2, while two lactating (D 13-14 postpartum) rats were euthanased with sodium pentabarbitone (as described in Section 2.5.2). Explants of mammary tissue, prepared as described in Section 2.5.2, were cultured in 24 well tissue culture dishes in either quiescent media (Q), or quiescent media containing 5 μg/ml PRL (Q-PRL), for 24 hours. The media were collected ("basal") and replaced with fresh Q, or Q-PRL, media containing treatments. The treatments applied were as follows; LPS (10 μg/ml), mIFN-γ (250 U/ml), LPS + AG (100 μM), LPS + L-NNA (1 mM), AG, L-NNA. Following 24 hours in treatments, explants and media were collected as described in Section 2.5.2.

6.3.3 *Statistical Analyses*

Data were analysed with the GLM procedure of SAS (SAS System for Windows, Release 6.12, 1996; SAS Institute Inc; Cary, NC, USA).

Comma-D Culture

Differences in DNA between experiments and between treatments were examined by ANOVA. Differences in NOx production by Comma-D cells between G and G-PRL (growing production) were examined by ANOVA. Comparisons between Q and Q-PRL (quiescent production) were analysed by ANCOVA using the pre-experiment production as the covariate. Differences between treatments (LPS, IFN-γ and inhibitors) were analysed by ANCOVA using both pre-experiment and basal production values as covariates.

Explant Culture

Differences in explant weight between experiments and between treatments were examined by ANOVA. Comparisons between Q and Q-PRL basal production by explants were analysed by ANOVA. Differences between treatments (LPS, IFN-γ and inhibitors) were analysed by ANCOVA using the basal production values as a covariate.

6.3.4 *SDS-PAGE and Western Analysis*

Sample Preparation

Samples of conditioned media, collected from a representative Comma-D experiment (Section 6.3.1) and two representative explant culture experiments (using explants from pregnant and lactating rats; Section 6.3.2) were concentrated using Vivaspin 500 µl concentrators with a molecular weight cut off of 10 kDa (Vivascience Ltd, Lincoln, UK). Briefly, 500 µl of sample was put into the concentrators, which were centrifuged at 2800 x *g* (MSE Micro Centaur Benchtop Centrifuge) for approximately 6-12 minutes (until the sample had been concentrated 10-fold). Concentrated samples were stored at -20 °C.

Preparation of Media Controls

Controls were prepared as follows. Briefly, 500 µl each of quiescent media, quiescent media containing 5 µg/ml PRL, growth media, and growth media containing 5 µg/ml PRL were concentrated 10-fold as described for the samples.

SDS-Polyacrylamide Gel Electrophoresis and Electrophoretic Transfer

Concentrated media samples and controls (20 µl) were reduced by the addition of 2-mercaptoethanol and diluted with sample loading buffer (5µl; 2.5 ml 0.5 M Tris; pH 6.8, 1.5 ml Milli-Q water, 8 ml glycerol, 1.0 g SDS, 10 % 2-mercaptoethanol and 1 % bromophenol blue). Samples were vortexed, boiled for approximately 2 minutes, left to cool then electrophoretically separated on 15 %, 1.5 mm thick gels, which were prepared and run, as described in Section 2.8. The proteins were electrophoretically transferred to nitrocellulose membrane as described in Section 2.9.

Western Analysis

Membranes were blocked, probed with a rabbit anti-ovine PRL antibody (AFP-C3581016789; Dr AF Parlow, Harbor-UCLA Medical Centre, Torrance, CA, USA; 1:750 000) and a goat anti-rabbit horseradish peroxidase conjugated secondary antibody (1:10 000). The blots were developed with ECL and exposed to radiographic film as described in Section 2.10.

6.4 Results

6.4.1 *Effect of Prolactin on the Production of NOx by Comma-D Cells*

There were no significant differences in DNA content between the different treatments (data not shown).

Production of NOx during the growth phase was significantly higher ($P < 0.005$) in those cells cultured in the presence of PRL (Figure 6.1 A). This difference in production was even greater during the quiescent phase (analysed using the pre-experiment production as a covariate; $P < 0.0001$; Figure 6.1 B).

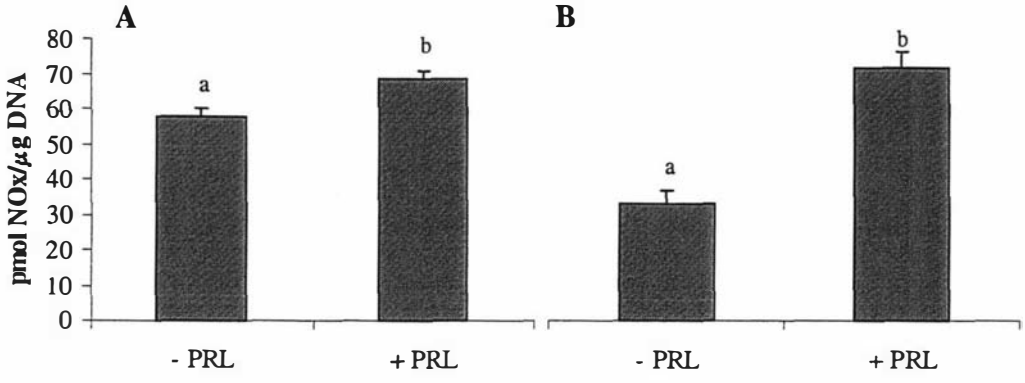

Figure 6.1: Effect of prolactin on the basal production of NOx by Comma-D cells. A: Cells were seeded into 24 well tissue culture dishes in either growth (G; - PRL), or growth media + PRL (5 μg/ml; G-PRL; + PRL) and grown for 3 days. Media were discarded and replaced with fresh G, or G-PRL, media. Following 24 hours in culture the media were collected and subsequently analysed for NOx. **B:** The media collected from 'A' were replaced with quiescent media (Q; - PRL), or quiescent media + PRL (5 μg/ml; Q-PRL; + PRL) and the cells cultured for 24 hours. Media were discarded and replaced with fresh Q, or Q-PRL, media and following a 2nd 24 hour period the media were collected and subsequently analysed for NOx. Data are presented as the mean ± SEM of 5 independent experiments. Differences between the means are indicated by superscript letters, where means not sharing common letters are significantly different ($P < 0.05$).

Comma-D cells grown to confluence then made quiescent respond to 250 U/ml mIFN-γ with a significant increase ($P < 0.05$) in NOx in the media. The inclusion of PRL during the whole of the experiment (+ PRL all) did not alter the extent of this production (Figure 6.2). However, when cells were treated with PRL during the treatment phase only (+ PRL trt only), the production of NOx was significantly higher ($P < 0.05$) than that produced by cells cultured without PRL (- PRL). There was no significant difference in the response to mIFN-γ between the cells cultured with PRL, either for the whole of the experiment, or just during the treatment phase.

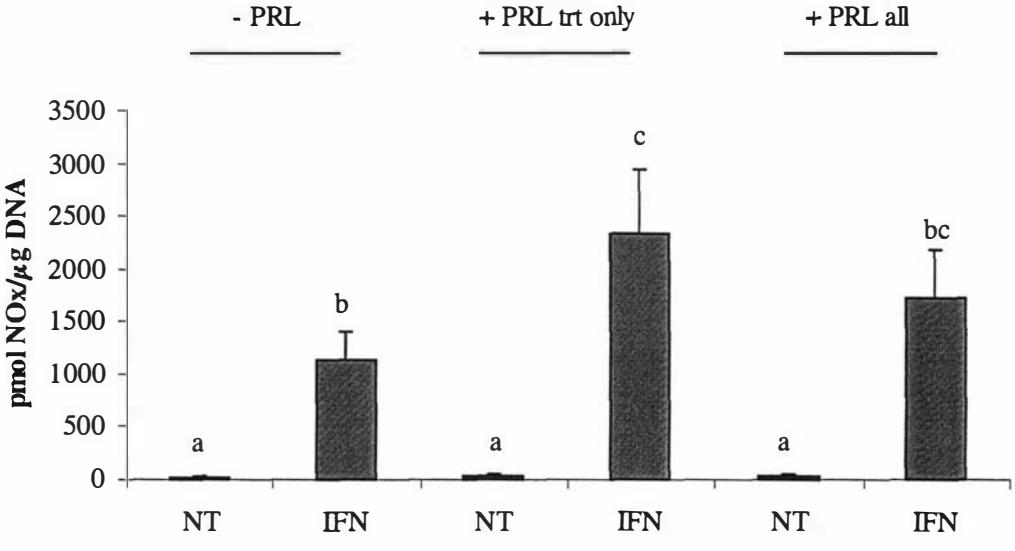

Figure 6.2: Effect of mIFN-γ and prolactin on the production of NOx by Comma-D cells. Comma-D cells, grown for 4 days in either the absence (- PRL or + PRL trt only), or presence (+ PRL all), of PRL (5 μg/ml) were then made quiescent by culture in quiescent medium (serum and growth factor free) in the absence (- PRL or + PRL trt only) or presence (+ PRL all), of PRL. Cells were then either left untreated (NT) or were treated with mIFN-γ (IFN; 250 U/ml) in either quiescent medium (- PRL) or quiescent medium containing PRL (+ PRL trt only or + PRL all), for 24 hours. The media were collected and analysed for NOx. Data are presented as the mean ± SEM of 5 independent experiments. Differences between the means are indicated by superscript letters, where means not sharing common letters are significantly different ($P < 0.05$).

Treatment of quiescent Comma-D cells with LPS significantly increased the production of NOx into the media by cells cultured without PRL (- PRL; $P = 0.0001$) or with PRL during the treatment phase (+ PRL trt only; $P = 0.006$). There was no difference in this production between the two media types (Figure 6.3). LPS did not affect the production of NOx by cells cultured in the presence of PRL (+ PRL all) throughout the experiment.

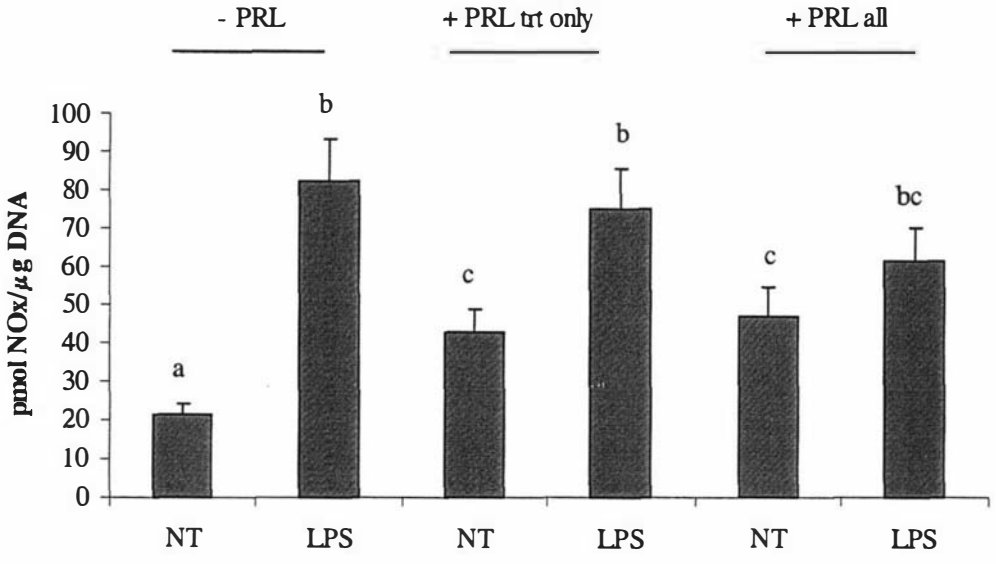

Figure 6.3: Effect of LPS and prolactin on the production of NOx by Comma-D cells. Comma-D cells, grown for 4 days in either the absence (- PRL or + PRL trt only), or presence (+ PRL all), of PRL (5 μg/ml) were then made quiescent by culture in quiescent medium (serum and growth factor free) in the absence (- PRL or + PRL trt only) or presence (+ PRL all), of PRL. Cells were then either left untreated (NT) or were treated with LPS (10 μg/ml) in either quiescent medium (- PRL) or quiescent medium containing PRL (+ PRL trt only or + PRL all), for 24 hours. The media were collected and analysed for NOx. Data are presented as the mean ± SEM of 5 independent experiments. Differences between the means are indicated by superscript letters, where means not sharing common letters are significantly different ($P < 0.05$).

The treatment of cells with L-NNA significantly increased ($P < 0.05$) the production of NOx when compared to non-treated cells (Figure 6.4). This occurred whether the cells were grown in the presence of PRL from the beginning of the experiment (+ PRL all) or in PRL during the treatment phase (+ PRL trt only). The inclusion of AG significantly decreased ($P < 0.001$) the production of NOx in cells cultured with PRL for the whole of the experiment, however, AG had no effect when cells were cultured with PRL for the treatment phase only.

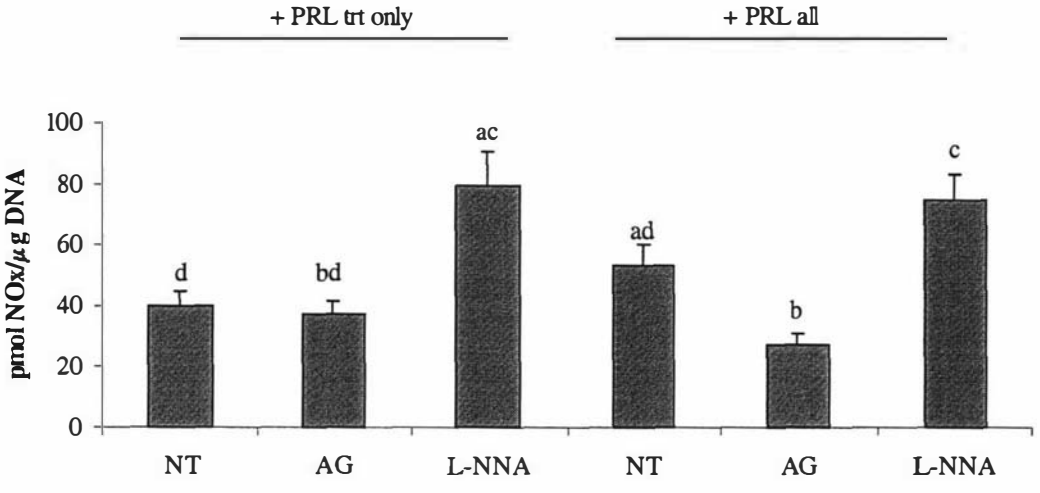

Figure 6.4: Effect of prolactin on the inhibition of unstimulated NOx production in Comma-D cells. Comma-D cells, grown for 4 days in either the absence (+ PRL trt only), or presence (+ PRL all), of PRL (5 μg/ml) were then made quiescent by culture in quiescent medium (serum and growth factor free) in the absence (+ PRL trt only) or presence (+ PRL all), of PRL. Cells were then either left untreated (NT) or were treated with AG (100 μM) or L-NNA (1 mM) in quiescent medium containing PRL (+ PRL trt only or + PRL all), for 24 hours. The media were collected and analysed for NOx. Data are presented as the mean ± SEM of 5 independent experiments. Differences between the means are indicated by superscript letters, where means not sharing common letters are significantly different ($P < 0.05$).

As the production of NOx by Comma-D cells was greater in mIFN-γ stimulated cells (Figure 6.2) when compared to LPS stimulated cells (Figure 6.3), the effect of PRL on the inhibition of NOx production by AG and L-NNA was examined in cells stimulated with mIFN-γ. The inclusion of PRL from either the start of the experiment (+ PRL all), or just during the treatment period (+ PRL trt only) did not effect the inhibition of NOS production in cells stimulated with mIFN-γ and co-treated with either AG (100 μM; Figure 6.5 A), or L-NNA (1mM; Figure 6.5 B). The release of NOx into the media from cells cultured with either inhibitor was significantly lower ($P < 0.05$) than the production of NOx from cells cultured without the inhibitors. However, the production of NOx from the cells cultured with inhibitors was still significantly greater ($P < 0.05$) than that produced by non-treated (NT) cells (Figure 6.5 A and B).

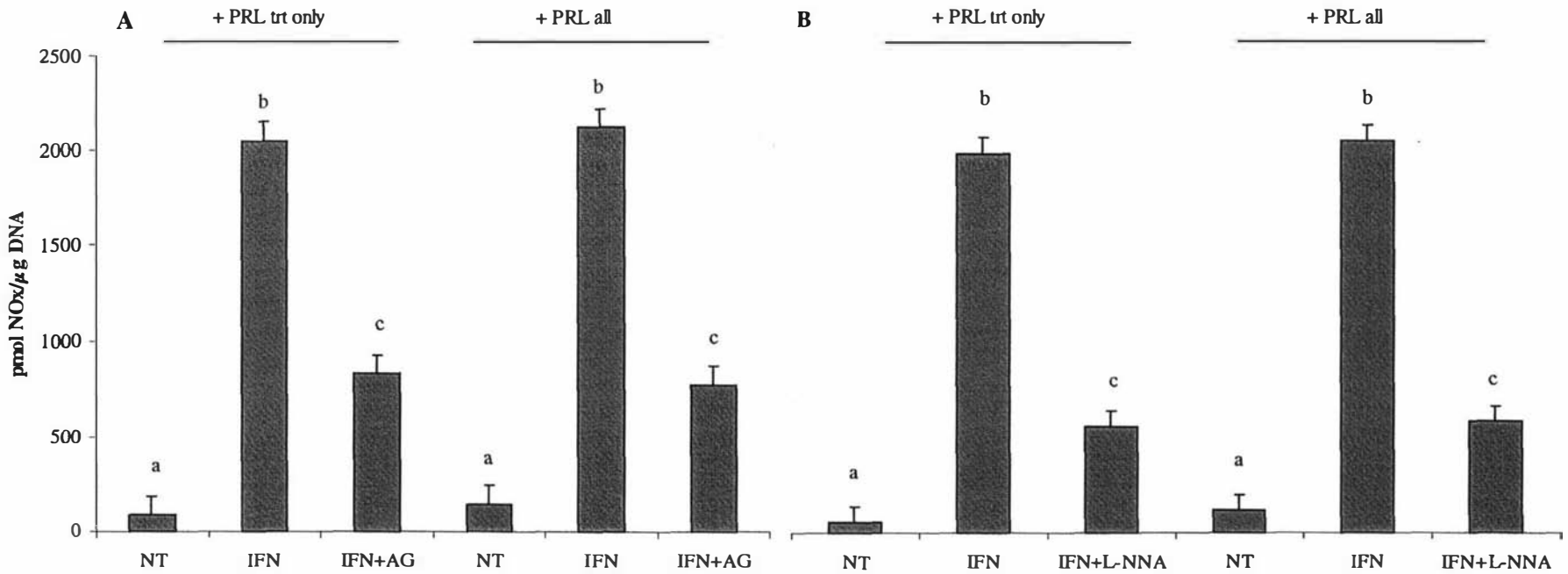

Figure 6.5: Effect of prolactin on the inhibition of stimulated NOx production in Comma-D cells. Comma-D cells, grown for 4 days in either the absence (+ PRL trt only), or presence (+ PRL all), of PRL (5 μg/ml) were then made quiescent by culture in quiescent medium (serum and growth factor free) in the absence (+ PRL trt only), or presence (+ PRL all), of PRL. Cells were then either left untreated (NT) or were treated with **A**: mIFN-γ (250 U/ml) or mIFN-γ + AG (100 μM), **B**: mIFN-γ or mIFN-γ + L-NNA (1 mM), in the presence (+ PRL trt only; + PRL all), of PRL for 24 hours. The media were collected and analysed for NOx. Data are presented as the mean ± SEM of 5 independent experiments. Differences between the means are indicated by superscript letters, where means not sharing common letters are significantly different ($P < 0.05$).

6.4.2 *Effect of Prolactin on NOx Production by Mammary Explants from Pregnant Rats*

Explant weight did not vary between treatments (data not shown).

Basal production of NOx by explants taken from pregnant rats was significantly increased ($P < 0.0001$) by the inclusion of PRL in the media (Figure 6.6).

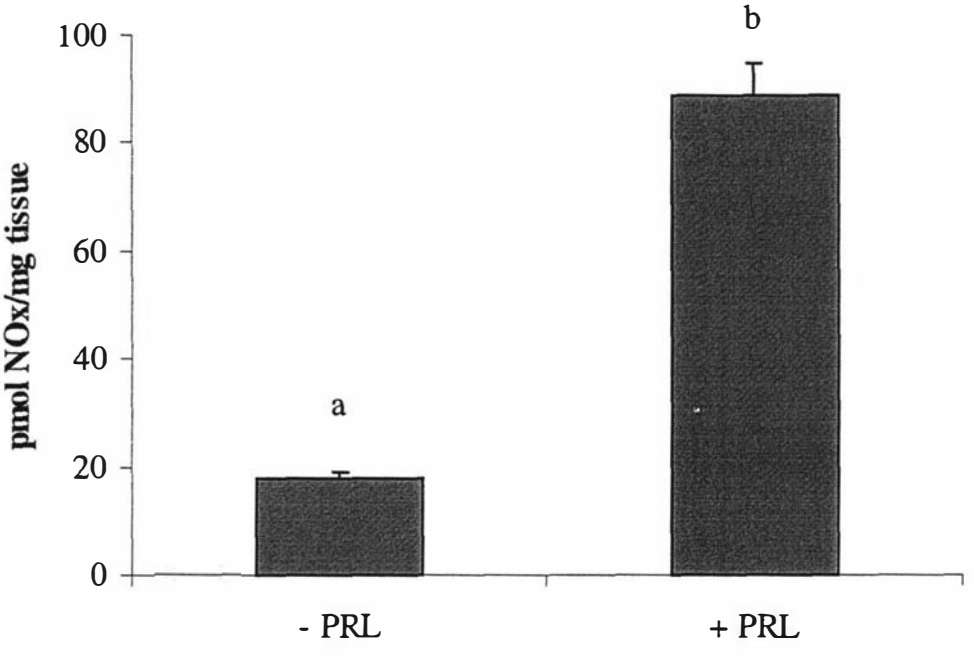

Figure 6.6: Effect of prolactin on the production of NOx in mammary explants from pregnant rats. Explants of mammary tissue excised from 3 pregnant rats were cultured for 24 hours in the absence (- PRL), or presence (+ PRL), of PRL. The media were collected and analysed for NOx. Data are presented as the mean ± SEM. Differences between the means are indicated by superscript letters, where means not sharing common letters are significantly different ($P < 0.0001$).

When treated with LPS explants of mammary tissue from pregnant rats respond by releasing significantly more ($P < 0.05$) NOx into the media than non-treated explants (Figure 6.7). The inclusion of PRL, either during the treatment phase only (+ PRL trt only), or during the whole of the experiment (+ PRL all), did not alter the production when compared to that produced by explants cultured without PRL.

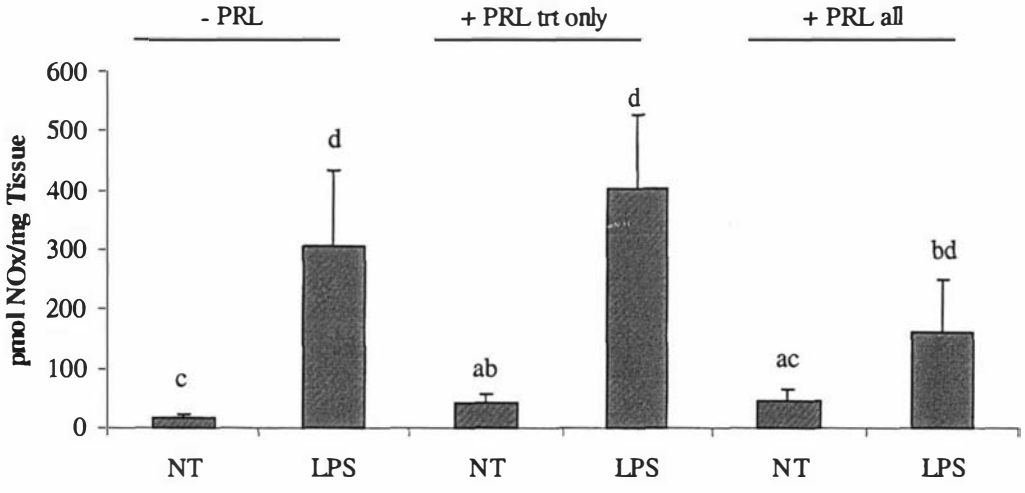

Figure 6.7: Effect of prolactin and LPS on the production of NOx by explants of mammary tissue from pregnant rats. Explants of mammary tissue excised from 3 pregnant rats were cultured for 24 hours in the absence (- PRL or + PRL trt only), or presence (+ PRL all), of PRL. The explants were either left untreated (NT) or were treated with LPS (10 μg/ml) in the absence (- PRL), or presence (+ PRL trt only; + PRL all), of PRL for 24 hours. The media were collected and analysed for NOx. Data are presented as the mean ± SEM. Differences between the means are indicated by superscript letters, where means not sharing common letters are significantly different ($P < 0.05$).

The treatment of explants of mammary tissue from pregnant rats with rIFN-γ results in significant increases ($P < 0.05$) in the concentration of NOx released into the media (Figure 6.8). The inclusion of PRL, either for the whole culture period (+ PRL all), or just during the treatment phase (+ PRL trt only) did not alter this production.

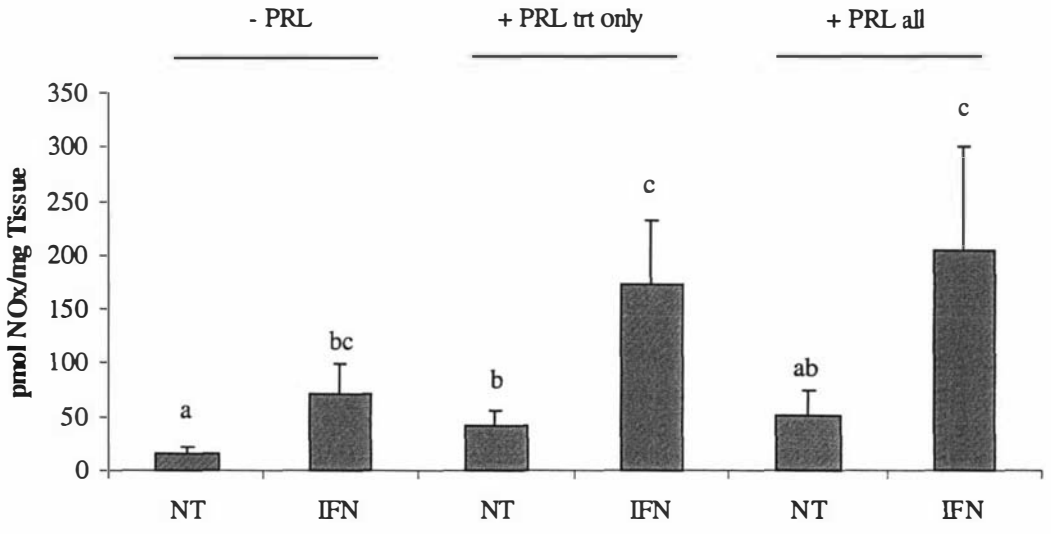

Figure 6.8: Effect of prolactin and rIFN-γ on the production of NOx by explants of mammary tissue from pregnant rats. Explants of mammary tissue excised from 3 pregnant rats were cultured for 24 hours in the absence (- PRL; + PRL trt only), or presence (+ PRL all), of PRL. The explants were then left untreated (NT) or were treated with rIFN-γ (250 U/ml) in the absence (- PRL), or presence (+ PRL trt only; + PRL all), of PRL for 24 hours. The media were collected and analysed for NOx. Data are presented as the mean ± SEM. Differences between the means are indicated by superscript letters, where means not sharing common letters are significantly different ($P < 0.05$).

When explants were cultured with the NOS inhibitors, AG or L-NNA, there was no difference in the NOx production when compared to non-treated explants (Figure 6.9). The inclusion of PRL, either during the whole of the experiment (+ PRL all), or when just included during the treatment period (+ PRL trt only), did not effect this.

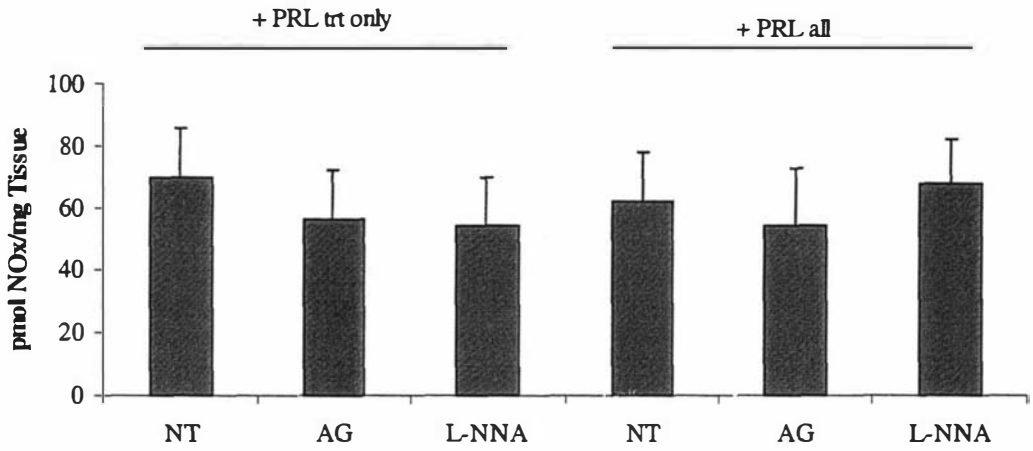

Figure 6.9: Effect of prolactin on the inhibition of unstimulated NOx production in mammary explants from pregnant rats. Explants of mammary tissue excised from 3 pregnant rats were cultured for 24 hours in the absence (+ PRL trt only), or presence (+ PRL all), of PRL. The explants were then treated with AG (100 μM) or L-NNA (1 mM) in the presence (+ PRL trt only; + PRL all) of PRL for a further 24 hours. The media were collected and analysed for NOx. Data are presented as the mean ± SEM.

As the production of NOx by mammary explants was greater in LPS stimulated explants (Figure 6.7) when compared to rIFN-γ stimulated explants (Figure 6.8), the effect of PRL on the inhibition of NOx production by AG and L-NNA was examined in explants stimulated with LPS. When explants cultured with PRL during the whole of the experiment (+ PRL all) were treated with LPS and AG (LPS + AG) there was no difference in the concentration of NOx in the media when compared to explants treated with LPS (Figure 6.10 A). However, if PRL was only present during the treatment period (+ PRL trt only), the production of NOx by LPS + AG cultured explants was significantly lower ($P < 0.01$) than that of explants cultured with LPS alone. The amount of NOx produced by these LPS + AG treated explants was not significantly different from the non-treated (NT) explants. Similar results were also obtained when the inhibitor L-NNA was used (Figure 6.10 B).

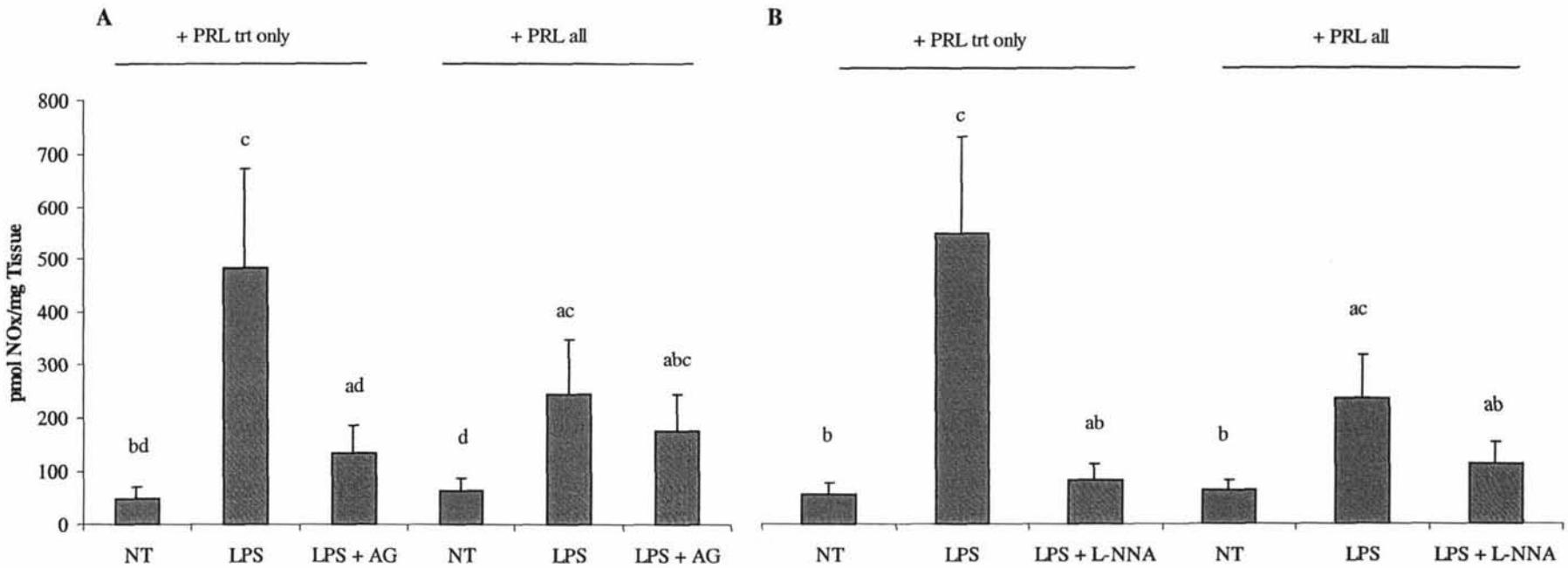

Figure 6.10: Effect of prolactin on the inhibition of stimulated NOx production in mammary explants from pregnant rats. Explants of mammary tissue excised from 3 pregnant rats were cultured for 24 hours in the absence (+ PRL trt only), or presence (+ PRL all), of PRL. The explants were then left untreated (NT), or were treated with **A**: LPS (10 μg/ml) or LPS + AG (100 μM) or **B**: LPS or LPS + L-NNA (1 mM), in the presence of PRL (+ PRL trt only; + PRL all), for 24 hours. The media were collected and analysed for NOx. Data are presented as the mean ± SEM. Differences between the means are indicated by superscript letters, where means not sharing common letters are significantly different ($P < 0.05$).

6.4.3 *Effect of Prolactin on NOx Production by Mammary Explants from Lactating Rats*

Explant weights were not different between treatments (data not shown).

The inclusion of PRL in the medium during the first 24 hours in culture significantly increased (P = 0.0001) the production of NOx in cultured mammary explants from lactating rats when compared to those explants cultured without PRL (Figure 6.11).

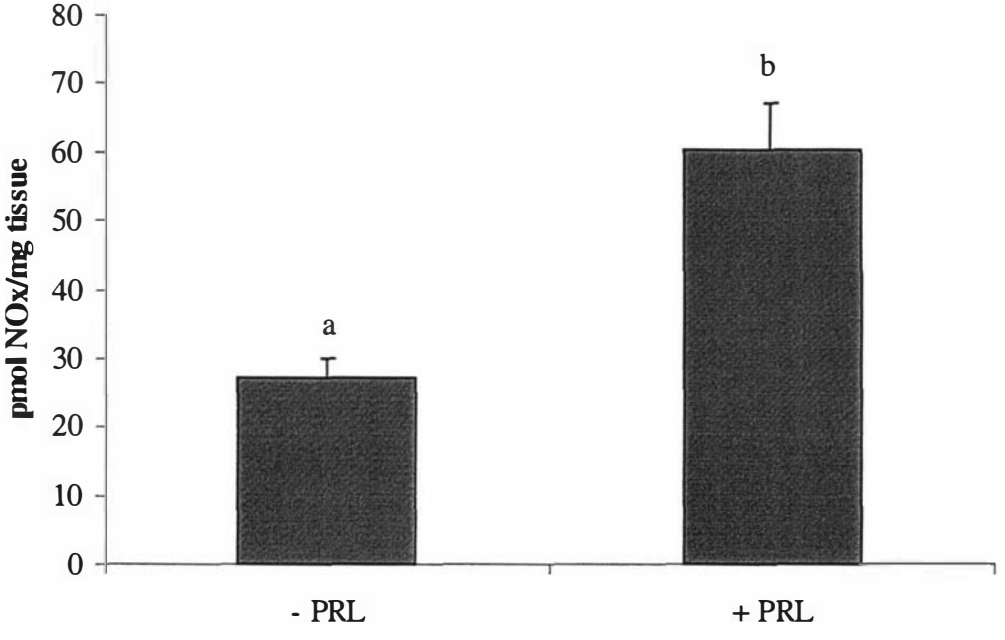

Figure 6.11: Effect of prolactin on the production of NOx from mammary explants from lactating rats. Explants of mammary tissue excised from 3 lactating rats were cultured for 24 hours in the absence (- PRL), or presence (+ PRL), of PRL. The media were collected and subesquently analysed for NOx. Data are presented as the mean ± SEM. Differences between the means are indicated by superscript letters, where means not sharing common letters are significantly different ($P < 0.0001$).

The significant increase ($P < 0.05$) in production of NOx by explants treated with LPS was not affected by the inclusion of PRL in the culture media (Figure 6.12). There was no significant difference between the production by explants without PRL (- PRL), with PRL (+ PRL all) and with PRL during the treatment period only (+ PRL trt only).

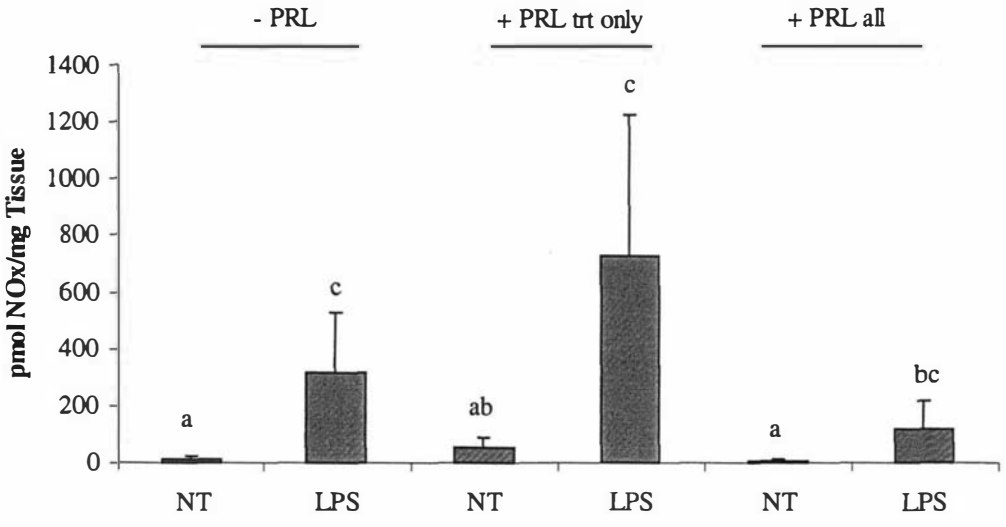

Figure 6.12: Effect of prolactin and LPS on the production of NOx by explants of mammary tissue from lactating rats. Explants of mammary tissue excised from 3 lactating rats were cultured for 24 hours in the absence (- PRL; + PRL trt only), or presence (+ PRL all), of PRL. The explants were either left untreated (NT) or were treated with LPS (10 μg/ml) in the absence (- PRL), or presence (+ PRL trt only; + PRL all), of PRL for 24 hours. Media were collected and analysed for NOx. Data are presented as the mean ± SEM. Differences between the means are indicated by superscript letters, where means not sharing common letters are significantly different ($P < 0.05$).

PRL had no effect on the significant increase ($P < 0.05$) in NOx production when explants were treated with rIFN-γ (Figure 6.13).

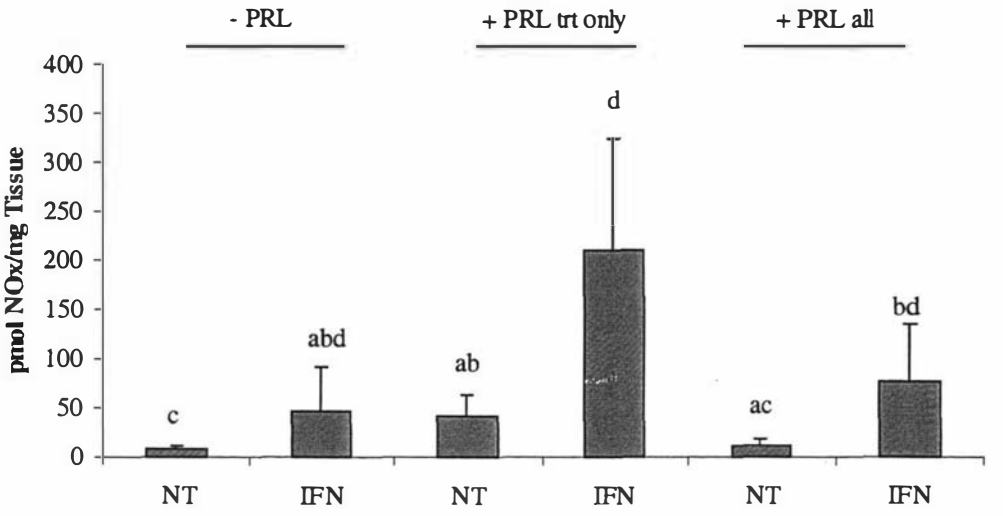

Figure 6.13: Effect of prolactin and rIFN-γ on the production of NOx by explants of mammary tissue from lactating rats. Explants of mammary tissue excised from 3 lactating rats were cultured for 24 hours in the absence (- PRL; + PRL trt only), or presence (+ PRL all), of PRL. The explants were then left untreated (NT) or were treated with rIFN-γ (250 U/ml) in the absence (- PRL), or presence (+ PRL trt only; + PRL all), of PRL for 24 hours. Media were collected and analysed for NOx. Data are presented as the mean ± SEM. Differences between the means are indicated by superscript letters, where means not sharing common letters are significantly different ($P < 0.05$).

When explants were treated with either AG or L-NNA, there was no difference in the production of NOx when compared to the non-treated explants. PRL did not appear to affect this either (Figure 6.14).

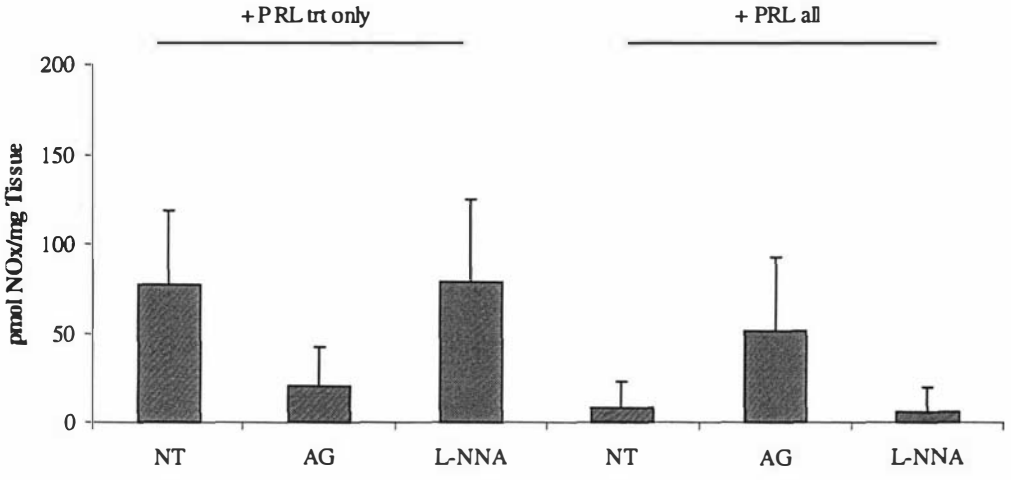

Figure 6.14: Effect of prolactin on the inhibition of unstimulated NOx production in mammary explants from lactating rats. Explants of mammary tissue excised from 3 lactating rats were cultured for 24 hours in the absence (+ PRL trt only), or presence (+ PRL all), of PRL. The explants were treated with AG (100 μM) or L-NNA (1 mM) in the presence (+ PRL trt only; + PRL all) of PRL for 24 hours. The media were collected and analysed for NOx. Data are presented as the mean ± SEM.

When mammary explants, taken from lactating rats were treated in the presence of PRL (+ PRL trt only) with LPS and also co-incubated with either AG or L-NNA there was a significant decrease ($P < 0.05$) in the production of NOx when compared to the explants treated with LPS alone (Figure 6.15 A & B). The production by the LPS+AG or LPS+L-NNA treated explants was decreased to a level that was not different from the production of NOx by non-treated explants. However, when the explants, incubated for the whole of the experiment in PRL (+ PRL all) were treated in the same manner, the inhibitors had no effect on the NOx production.

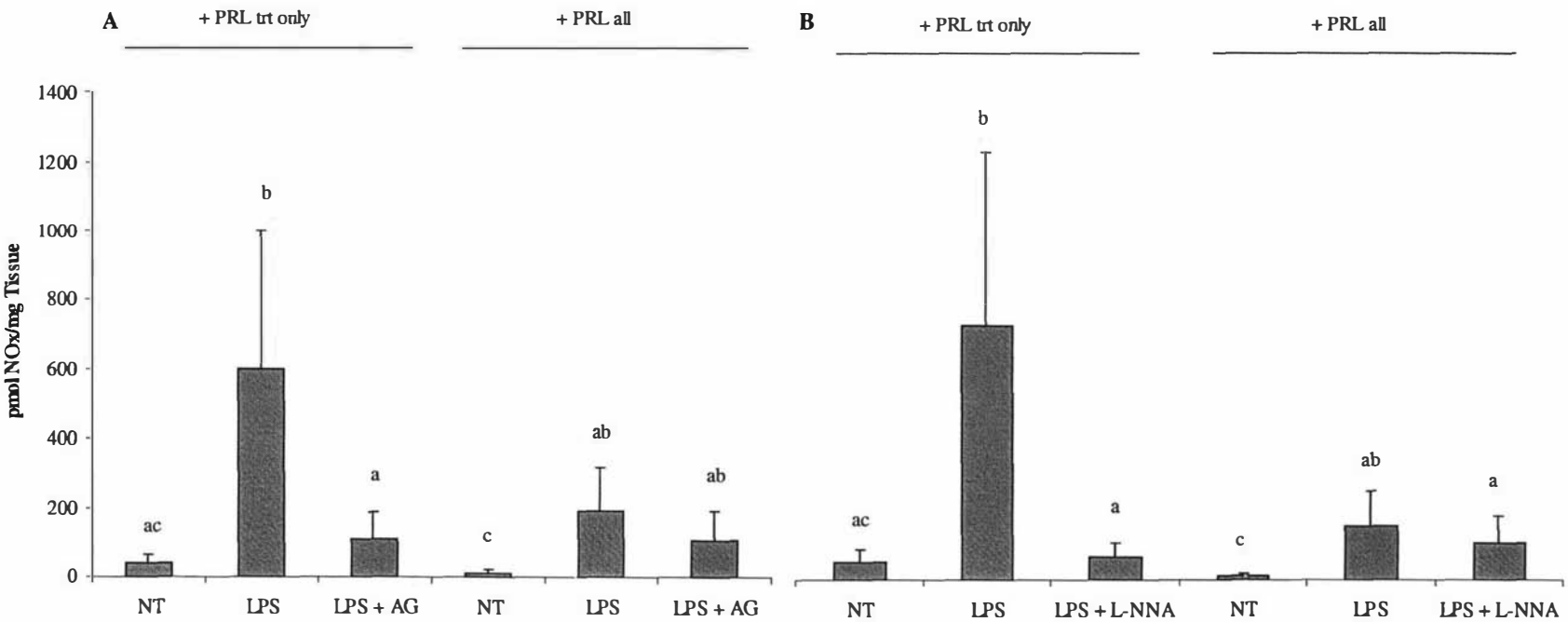

Figure 6.15: Effect of prolactin on the inhibition of stimulated NOx production in mammary explants from lactating rats. Explants of mammary tissue excised from 3 lactating rats were cultured for 24 hours in the absence (+ PRL trt only), or presence (+ PRL all), of PRL. The explants were then left untreated (NT), or were treated with **A**: LPS (10 μg/ml) or LPS + AG (100 μM), **B**: LPS or LPS + L-NNA (1 mM), in the presence of PRL (+ PRL trt only; + PRL all), for 24 hours. Media were collected and analysed for NOx. Data are presented as the mean ± SEM. Differences between the means are indicated by superscript letters, where means not sharing common letters are significantly different ($P < 0.05$).

6.4.4 *Western Analysis*

Western analysis was performed on conditioned media samples in order to determine if proteolytic cleavage of PRL was occurring. In the conditioned medium samples collected from Comma-D cells during the treatment phase (Figure 6.16), there was no evidence to suggest the presence of a 16 kDa fragment of PRL. Conditioned media collected from explants, obtained from either pregnant or lactating rats, show no evidence of a 16 kDa PRL fragment (Figure 6.17) either.

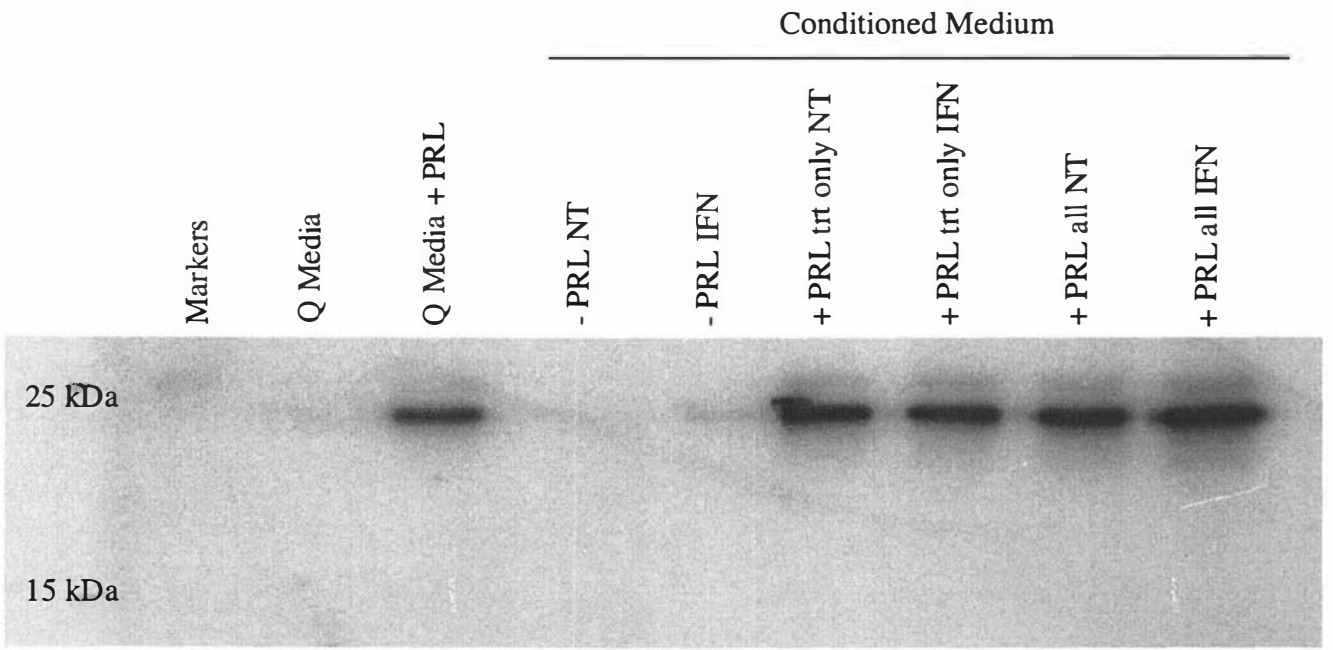

Figure 6.16: Western blot analysis of conditioned medium samples from Comma-D culture experiments. Comma-D cells, cultured without PRL (- PRL), with PRL during the treatment phase (+ PRL trt only), or with PRL for the whole of the treatment period (+ PRL all), were either left untreated (NT), or were treated with 250 U/ml mIFN-γ (IFN) for 24 hours. Conditioned media samples were collected, concentrated 10-fold, reduced by the addition of 2-mercaptoethanol then electrophoretically separated on a 15 % SDS-PAGE gel. Q media ± PRL (Q Media; Q Media + PRL) was also concentrated 10-fold and run as a control. Proteins were transferred to a nitrocellulose membrane, which was probed with a rabbit anti-ovine-PRL antibody.

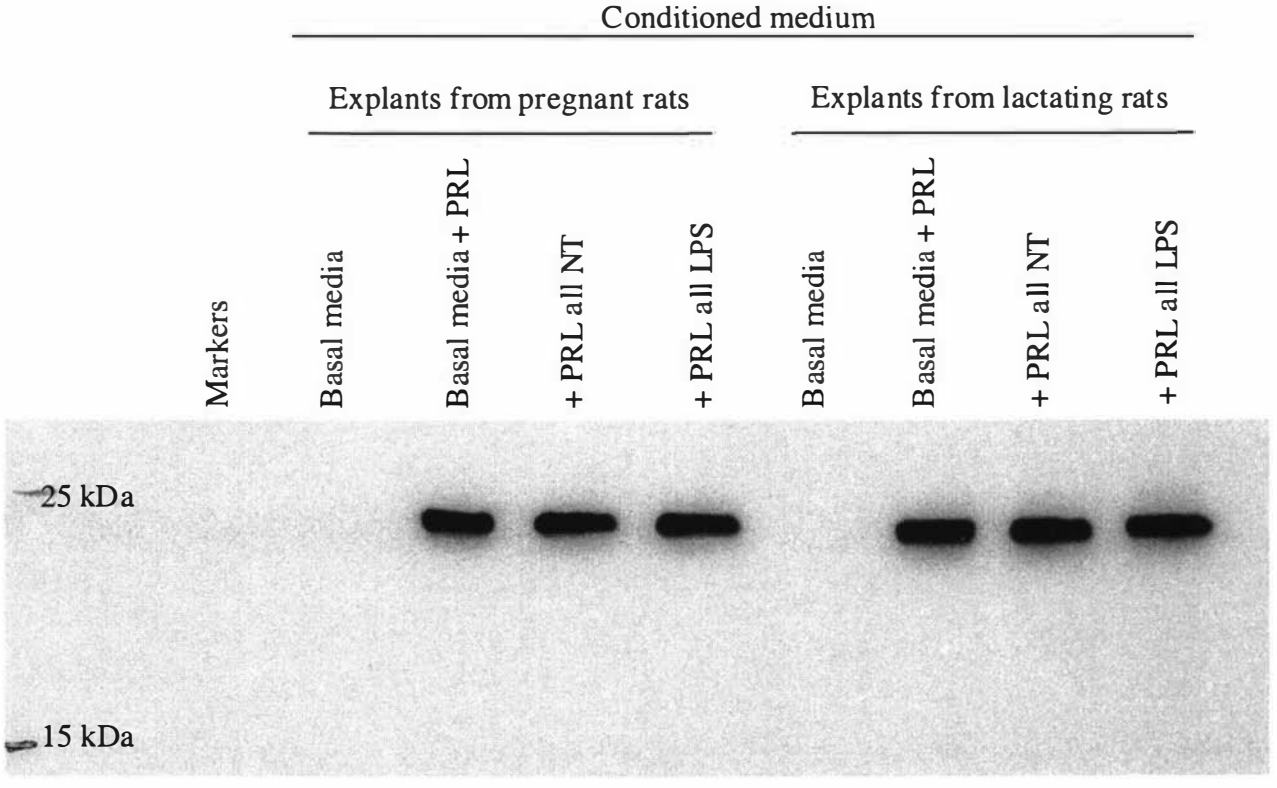

Figure 6.17: Western blot analysis of conditioned medium samples from explant culture experiments. Explants collected from pregnant or lactating rats were cultured for 24 hours without PRL (Basal), or with PRL (Basal + PRL). Those explants cultured with PRL during the 1st 24 hours were then left untreated (+ PRL all NT), or were treated with 10 μg/ml LPS (+ PRL all LPS) for 24 hours. Samples of conditioned medium were collected, concentrated 10-fold, reduced by the addition of 2-mercaptoethanol then electrophoretically separated on a 15 % SDS-PAGE gel. Proteins were transferred to a nitrocellulose membrane, which was probed with a rabbit anti-ovine-PRL antibody.

6.5 Discussion

6.5.1 *Effect of Method of Euthanasia on NOx Production*

Direct comparisons between the effects of PRL on explants from pregnant and lactating rats were not made. This was because the three pregnant rats were euthanased using CO_2, whereas two of the lactating rats were euthanased using anaesthetic, and one using CO_2 (Section 6.3.2). Therefore it was not possible to separate the effects of NOx production by the mammary gland explants of different physiological states (pregnant versus lactating) from the method of euthanasia. Furthermore, since only one of the three lactating rats from which tissue was collected was euthanased with CO_2, it was not possible to test for the effect of method of euthanasia within that group.

6.5.2 *Effect of Prolactin on Basal NOx Production*

PRL (5 μg/ml) increases the production of NOx from Comma-D cells during both the growth and quiescent phases (Figure 6.1), and increases the production of NOx during the first 24 hours in culture by explants of mammary tissue from both pregnant and lactating rats (Figure 6.6 and Figure 6.11). Similarly, stimulation of astrocytes (C6 glioma cell line), murine mammary epithelial cells and murine peritoneal macrophages with PRL (0.4-2.3 μg/ml; 1 μg/ml and 0.1 μg/ml respectively) results in an increase in the production of NO (Kumar *et al.*, 1997; Raso *et al.*, 1999; Bolander, 2001). These results are in direct contrast to those reported by Corbacho *et al.* (2000) who found no effect of PRL (100 nM; ~ 2.3 μg/ml) on rat fibroblasts or Type II cells and also reported decreases in the production of NOx when cells were treated with PRL (Corbacho, personal communication, 2000).

Due to the reported differences in responses between the different cell types it appears that the effect of PRL on the production of NOx is tissue/cell type specific. However, it must be noted that the concentrations of PRL used in all these studies are well above physiological levels, as in rats, the concentrations of PRL in serum vary from approximately 0.07 μg/ml during days 1-8 of lactation to between 0.03 μg/ml

and 0.04 μg/ml at mid-lactation (Amenomori *et al.*, 1970; Grigor *et al.*, 1984). Thus, the biological significance of these effects are still under investigation.

In the experiments presented in this chapter, there is no evidence to suggest PRL cleavage by Comma-D cells (Figure 6.16), or by mammary explants (Figure 6.17), has occurred. In contrast, following incubation with slices of mammary tissue (Baldocchi *et al.*, 1992; Baldocchi *et al.*, 1994) a 16 kDa band was apparent in the medium following SDS-PAGE electrophoresis and immunostaining. Only a 23 kDa band was apparent in the media containing PRL following incubation without tissue (Baldocchi *et al.*, 1992). To further support the findings reported in this chapter, Western analysis needs to be repeated using purified 16K PRL as a positive control.

Both explants (Figure 6.6 and Figure 6.11) and Comma-D cells (Figure 6.1) produce NOx in the absence of PRL and those cells or explants cultured with PRL release more NOx into the media than those cultured without it. Treatment of cells with AG had no effect on NOx production when they were cultured with PRL during the treatment phase, in contrast, when the cells were cultured with PRL for the whole of the experiment, AG significantly decreased the NOx production. As AG is an iNOS specific inhibitor (Corbett & McDaniel, 1996), these results suggest that PRL induction of iNOS has occurred after the extended exposure of the cells to PRL. However, as Raso *et al.* (1999) reported that induction of iNOS protein expression by PRL occurs after 24 hours and is only measurable after immunoprecipitation, it is possible that PRL induction of iNOS was also occurring in those Comma-D cells cultured with PRL for the treatment period only. It may just be that the differences in the production of NOx were not sufficient to measure and thus explain why there was no apparent inhibition by AG. Treatment of Comma-D cells with L-NNA resulted in a significant increase in NOx production regardless of length of exposure to PRL (Figure 6.4). Increases in NOx production from L-NNA treated Comma-D cells has been previously reported (Figure 4.7). The reasons for this effect of L-NNA on basal Comma-D cell production of NOx will be further explored in Chapter 9.

Unlike the effects of AG or L-NNA on the basal production of NOx by Comma-D cells (Figure 6.4), there was no effect of inhibitors on the production of NOx by explants from either the pregnant or lactating rats (Figure 6.9; Figure 6.14). As PRL

was included in the medium of all explants for at least part of the culture period, further experiments are needed to examine whether L-NNA also increases the production of NOx as reported for Comma-D cells (Figure 4.7) when explants are cultured without PRL. These experiments could provide further information on why L-NNA has the effect of increasing NOx production in mammary tissue. However, it should be noted that PRL had no apparent effect on the increase in NOx production in L-NNA treated Comma-D cells (Figure 6.4), with cells treated with PRL for the entire experiment responding in a not significantly different manner to those treated with PRL for the treatment period only. This therefore suggests that PRL is unlikely to be having an effect.

The relationship between the increased production of NOx when cultured in the presence of PRL (explants from pregnant rats, 5-fold increase; Figure 6.6; explants from lactating rats, 2.2-fold increase; Figure 6.11) and the increased basal production of NOx by explants cultured from pregnant rats (Figure 5.5) requires further investigation. Further research is also required to determine how PRL is stimulating the production of NO, what pathways are involved and the biological significance of cytokine role of PRL within the mammary gland.

6.5.3 *Effect of Prolactin on LPS or IFN-γ Stimulated NOx Production*

There was no effect of PRL on the rIFN-γ stimulated production of NOx by mammary explants (Figure 6.8 and Figure 6.13). However, mIFN-γ stimulated production of NOx from Comma-D cells was enhanced in the presence of PRL when the cells were treated with PRL during the treatment phase only (Figure 6.2). In contrast, the inclusion of PRL throughout the entire culture period did not alter the cells' response to stimulation with mIFN-γ when compared to cells cultured in the absence of PRL (Figure 6.2). Enhancement of IFN-γ induction by PRL has been reported in astrocytes (Raso *et al.*, 1999). Both AG and L-NNA significantly decreased the production of NOx by mIFN-γ stimulated Comma-D cells thus showing that the NOx in the medium was due to the activity of NOS (Figure 6.5).

The results presented show no effect of PRL on the LPS stimulation of explants (Figure 6.7 and Figure 6.12). Comma-D cells treated with LPS and cultured without PRL, or with PRL just during the treatment period both showed significant increases ($P < 0.05$) in NOx production whereas those cells cultured in the presence of PRL for the whole of the experimental period did not show a significant response to the LPS (Figure 6.3). Treatment of murine macrophages with LPS and PRL results in a synergistic enhancement of NO production (Kumar *et al.*, 1997). The effect of LPS on different tissue/cell types has been shown to be varied (Geller *et al.*, 1993b; Lorsbach *et al.*, 1993; Lowenstein *et al.*, 1993; Asano *et al.*, 1994) and thus it is suggested that this variability of response is a possible reason why the Comma-D cells and explants did not produce the same response to the LPS and PRL that the macrophages did. Both AG and L-NNA significantly decreased the production of NOx by LPS stimulated explants of mammary tissue (Figure 6.10 and Figure 6.15) thus providing evidence that the increased NOx in the medium was due to the activity of NOS. However, this was only the case in those explants treated with PRL during the treatment phase only. When LPS stimulated explants were exposed to PRL for the entire experiment, neither inhibitor had a significant effect on the NOx production. It is not known why length of exposure to PRL affected the inhibition of NOS.

These results strongly suggest that Comma-D cell response to both LPS and mIFN-γ is altered in the presence of PRL. Unfortunately, the results obtained from the explant experiments were more variable. Thus, although they tended to show similar findings to the Comma-D cell experiments, they were not statistically significant. Further explant culture experiments may help to decrease this variability and reveal the role that PRL plays in mammary gland production of NO.

6.5.4 *General Comments*

In conclusion, the results from the experiments described in this chapter show that mammary explants and Comma-D cells respond to treatment with PRL with an increase in the production of NOx into the media, but there is no evidence to suggest that the response is due to the production of the 16 kDa proteolytic fragment of PRL.

CHAPTER SEVEN

XANTHINE OXIDASE ACTIVITY AND NITRIC OXIDE PRODUCTION

7.1 Abstract

Published reports have indicated that nitric oxide (NO), a product of NOS following the conversion of arginine to citrulline, is also generated by xanthine oxidase (XO) from nitrite and nitrate (NOx). However, the work presented in this Thesis measures the production of NO by the measurement of NOx. This chapter examines whether XO plays a role in the production of NO in the mammary gland to determine if the previously measured NO production has come from this alternative source. Co-culture of Comma-D cells with the XO inhibitor, allopurinol and the NOS inhibitor L-NNA had no effect on the concentration of NOx in the media collected from the cells. When cells were stimulated with mIFN-γ there was no difference in the medium concentration of NOx when comparing cells treated with mIFN-γ alone, or with mIFN-γ in conjunction with 1000 μM allopurinol. Inhibition of this mIFN-γ stimulated NO production was achieved by the addition of either of the NOS inhibitors, AG or L-NNA and allopurinol had no effect on this inhibition. When whole milk samples, collected from 13 cows during the first 4 days postpartum were treated with 50 μM allopurinol, there was a significant decrease ($P < 0.05$) in the activity of XO. Treatment of skim milk samples with allopurinol had no effect on either the XO activity or the NOx concentrations. Although it can not be determined whether the XO activity in skim milk affected the concentration of NOx, the results from the Comma-D culture experiment clearly show that inhibition of XO activity had no effect on the amount of NOx in the medium, suggesting that XO in this system at least, is not converting the NOx to NO and thus affecting the measurement of NOx.

7.2 Introduction

Throughout this Thesis and in many other studies (Bouchard *et al.*, 1999; Corbacho *et al.*, 2000; Boulanger *et al.*, 2001) the activity of NOS and production of NO is determined by measuring the stable metabolites of NO, nitrite and nitrate (NOx). However, recent studies indicate that nitrite and nitrate are substrates for the enzyme xanthine oxidoreductase (XOR), where, under hypoxic conditions and in the presence of NADH, nitrite and nitrate are converted to NO (Millar *et al.*, 1998; Zhang *et al.*, 1998; Godber *et al.*, 2000).

Xanthine oxidoreductase is a complex flavoprotein composed of two identical subunits. In mammals it exists as two interconvertible forms, xanthine oxidase (XO) and xanthine dehydrogenase (XD; Kurosaki *et al.*, 1996; Page *et al.*, 1998; Ichimori *et al.*, 1999; McManaman *et al.*, 1999; Godber *et al.*, 2000). XO catalyses the oxidation of a variety of organic compounds and is also responsible for the conversion of hypoxanthine to xanthine and then urate, both important steps in the degradation of purines (Stryer, 1998). Although found in the cytoplasm of many cells, XO is also associated with the apical plasma membrane of milk-secreting epithelial cells (Jarasch *et al.*, 1981), and is a component of the bovine milk fat globule membrane where it comprises more than 8 % of the membrane protein (Briley & Eisenthal, 1975). The role of XOR in the mammary gland is unclear but it has been postulated to play roles in lipid secretion (Jarasch *et al.*, 1981) and mammary differentiation (Ringo & Rocha, 1983; Hayden *et al.*, 1991; Kurosaki *et al.*, 1996).

The XO activity of mammary tissue collected from mice during the reproductive cycle has been measured and several different patterns have been reported. The activity peaks at parturition at a level approximately 1000-fold the activity of virgin glands, throughout lactation the XO activity is about half that measured at parturition, and a steady decline in activity follows weaning (Lewin, 1957). In contrast, both Ringo & Rocha, (1983) and McManaman *et al.*, (1999) reported that the highest activity of XOR in mouse mammary glands is during mid-lactation. Hayden *et al.* (1991) reported that XOR activity increased at mid-gestation and remained elevated

until weaning at 20 days postpartum. A similar pattern of XOR activity in the mouse mammary gland was reported by Kurosaki *et al.* (1996) who also identified that the increases in activity were accompanied by increases in XOR protein and mRNA concentrations

The control of XOR activity has been investigated in HC11 cells (a murine mammary epithelial cell line derived from Comma-D cells) and it appears that hormones involved in lactogenesis modulate the activity of XOR. For example, during the logarithmic phase of growth, the cells exhibit no significant XOR activity. However, confluent cells express detectable amounts of enzyme activity and treatment with dexamethasone, a synthetic corticosteroid, or cortisol, a natural glucocorticoid increases the XOR activity. Prolactin treatment alone (5 μg/ml) is ineffective, but synergistic increases in XOR activity occur when it is added with dexamethasone or cortisol (Kurosaki *et al.*, 1996). McManaman *et al.*, (2000) also reported that prolactin and cortisol increase the XOR activity of HC11 cells. In contrast, dexamethasone decreases the production of NO by explants of mammary tissue (Onoda & Inano, 1998) and prolactin increases the production of NO by explant of mammary tissue (Figure 6.6 and 6.11) and from Comma-D cells (Figure 6.1). Cytokines, previously shown to activate NOS (Chapters 4 and 5; Lamas *et al.*, 1991; Yoshizumi *et al.*, 1993; Rosenkranz-Weiss *et al.*, 1994; Adler *et al.*, 1995), also increase XOR activity. IFN-γ increases the XOR activity in HB4a cells (human mammary epithelial cell line) in a dose dependent manner. TNF-α and interleukin-1β (IL-1β) also increased the XOR activity to a level significantly different from the activity of those cells cultured without cytokines (Page *et al.*, 1998). Thus, it appears that the same hormones and cytokines that influence the activity of NOS also play a role in the control of the activity of XOR.

XO converts nitrite and nitrate to NO, under hypoxic conditions and in the presence of NADH (Millar *et al.*, 1998; Zhang *et al.*, 1998; Godber *et al.*, 2000). NO is also produced by NOS when incubated under hypoxic conditions, however, the production is independent of the substrate (L-arginine) concentration. Under ambient air conditions, both XO and NOS generate NO (from nitrite and L-arginine respectively), with the amount of NO produced by NOS about double that measured for XO. This

is in contrast to the hypoxic conditions in which XO produced about five times more NO than NOS (Zhang *et al.*, 1998). Cultures of mammary explants and Comma-D cells throughout this Thesis were maintained at 37 °C in humidified 5 % CO_2:95 % air atmosphere.

The suggestion that XO generates NO has implications for the experiments presented in this Thesis. NOS also generates NO, which is then spontaneously, and very rapidly oxidised to nitrite and nitrate. If XO is involved in converting nitrite and nitrate back to NO under hypoxic conditions as described by Millar *et al.* (1998) and Godber *et al.* (2000), or under ambient air conditions as described by Zhang *et al.* (1998), this may impact on the amount of NOx measured in the media. For example, if the NOx in the media is generated by NOS activity of the cells and XO is active, then at the time of measurement, some if not all, of the NOx that was initially generated by NOS may have been converted to NO by XO and thus would not be available to the assay for detection. This is a problem only if the conversion of NOx to NO by XOR is more rapid than the spontaneous oxidation of NO to NOx. It should also be noted that the activity of XOR does not generate any extra NO plus NOx in the system, rather it just converts NOx that is present to NO (which is subsequently oxidised to NOx). Thus, by inhibiting any potential activity of XO we can ensure that once nitrite and nitrate are formed they remain in the media in that state and are not converted back to NO by XO.

7.3 Materials and Methods

7.3.1 *Comma-D Culture*

Experimental Design

Comma-D cells were cultured following the protocols described in Section 2.3. The cells were seeded into 24 well tissue culture dishes, grown for 4 days and made quiescent by the removal of serum and growth factors for 48 hours. The cells were either left unstimulated (n=5 independent experiments; Table 7.1) or stimulated with

mIFN-γ (n=3 independent experiments; Table 7.2). The cells were also concurrently treated with the XO inhibitor, allopurinol and a NOS inhibitor, either AG or L-NNA. Following 24 hours in culture, media and cells were collected and subsequently analysed for NOx and DNA content respectively.

Table 7.1: Treatments used to inhibit the activity of xanthine oxidase and nitric oxide synthase in unstimulated Comma-D cells. Quiescent cells were treated with a NOS inhibitor, either AG, or L-NNA and the XO inhibitor allopurinol, at the concentrations given below. The abbreviations for the treatments are also given.

Treatment Abbreviation	mM L-NNA	μM AG	μM Allopurinol
L-NNA	1	-	
1+1	1	-	1
1+10	1	-	10
1+100	1	-	100
1+1000	1	-	1000
AG	-	100	-
100+1	-	100	1
100+10	-	100	10
100+100	-	100	100
100+1000	-	100	1000
Allo	-	-	1000

Table 7.2: Treatments used to inhibit the activity of xanthine oxidase and nitric oxide synthase in mIFN-γ stimulated Comma-D cells. Quiescent cells were stimulated with mIFN-γ and concurrently treated with a NOS inhibitor, either AG, or L-NNA and the XO inhibitor allopurinol, at the concentrations given below. The abbreviations for the treatments are also given.

Treatment Abbreviation	U/ml mIFN-γ	mM L-NNA	μM AG	μM Allopurinol
IFN	250	-	-	-
Allo	-	-	-	1000
IFN+Allo	250	-	-	1000
IFN+L-NNA	250	1	-	-
IFN+AG	250	-	100	-
IFN+L-NNA+Allo	250	1	-	1000
IFN+AG+Allo	250	-	100	1000

NOx Assay Lower Detection Limit

NOx is measured in media using a two-step method where any nitrate in the sample is converted to nitrite using nitrate reductase (Verdon *et al.*, 1995), and total nitrite is measured using 2-3, diaminonapthalene (Misko *et al.*, 1993). To determine the concentration of NOx in samples, the fluorescent units generated are compared to a standard $NaNO_2$ curve (Section 2.4.3). The lower detection limit (LDL) of the assay was calculated as the value two standard deviations above the blank (average blank from 10 independent assays) and was calculated as 25 pmol. Those samples that fell around or below the LDL were treated as follows: the average sample value from triplicate treatment wells (Figure 2.1) was determined and if it was below the LDL, all samples within the triplicate were deemed to have less than detectable concentrations of NOx.

Statistical Analyses

Differences in DNA content of cultures between experiments, and between treatments, and the effect of treatments, on NOx production were analysed by ANOVA using SAS.

7.3.2 *Xanthine Oxidase and Nitric Oxide in Bovine Milk*

Milk/Colostrum Sample Origin and Preparation

Colostrum/milk samples were collected from 13 cows on days 1 (n=3), 2 (n=2) 3 (n=4) and 4 (n=4) postpartum. Samples were prepared for the assay of XO and NOx as follows. To each of the 13 samples, 60 μl of 0.1 M sodium phosphate buffer (uninhibited) or 60 μl of 0.1 M sodium phosphate buffer containing 2.5 mM allopurinol (50 μM; inhibited) was added to 2940 μl of milk. Each of the inhibited and non-inhibited samples was split into two tubes (1.5 ml/tube), one of which was centrifuged at 2000 rpm for 10 minutes. The skim milk was collected and stored at 4 °C for 48 hours. A subsample (500 μl) of each of the whole milk and skim milk samples was dialysed (Spectra/Por molecularporous membrane; MWCO: 12-14 kDa) for 42 hours against two changes of 100 mM phosphate buffer, pH 7.8. This resulted in eight different treatments for each of the original 13 samples (Figure 7.1). All samples were stored at -20 °C until assayed. All samples were assayed for XO activity; only skim milk samples were assayed for NOx concentration. The dialysis step was carried out following the method of Fried & Fried (1974) as low molecular weight compounds may inhibit XO.

Xanthine Oxidase Activity

Xanthine oxidase (XO) activity was measured in all the milk samples by a method based upon that of Fried & Fried (1974). Briefly, samples (20 μl) were mixed with 180 μl of reagent mix (1.4 mg/ml gelatine, 342 μg/ml nitro blue tetrazolium, 5.7 μg/ml phenazine methosulphate, 0.86 mM EDTA, 100 mM sodium phosphate, pH 7.8) in 96 well assay plates and incubated for 5 minutes in the dark at room temperature. Xanthine (100 μl; 0.14 mM xanthine sodium salt) was added to each well and the plate was read (595 nm) at 2 minute intervals for 10 minutes. Those samples showing either initial or final OD readings over 1.0 were diluted in 100 mM phosphate buffer and re-assayed. Results are presented as OD_{595} units/min/ml, which were calculated as the average change in OD over the linear part of the curve (2-10 minutes)/min/ml milk under the conditions described above.

NOx Assay

NOx was measured in the skim milk samples as described in Section 2.4 and were compared to a standard $NaNO_2$ curve generated in a 1/10 dilution of milk in Milli-Q water.

Statistical Analyses

The effects of treatments on XO activity in milk were analysed by ANOVA.

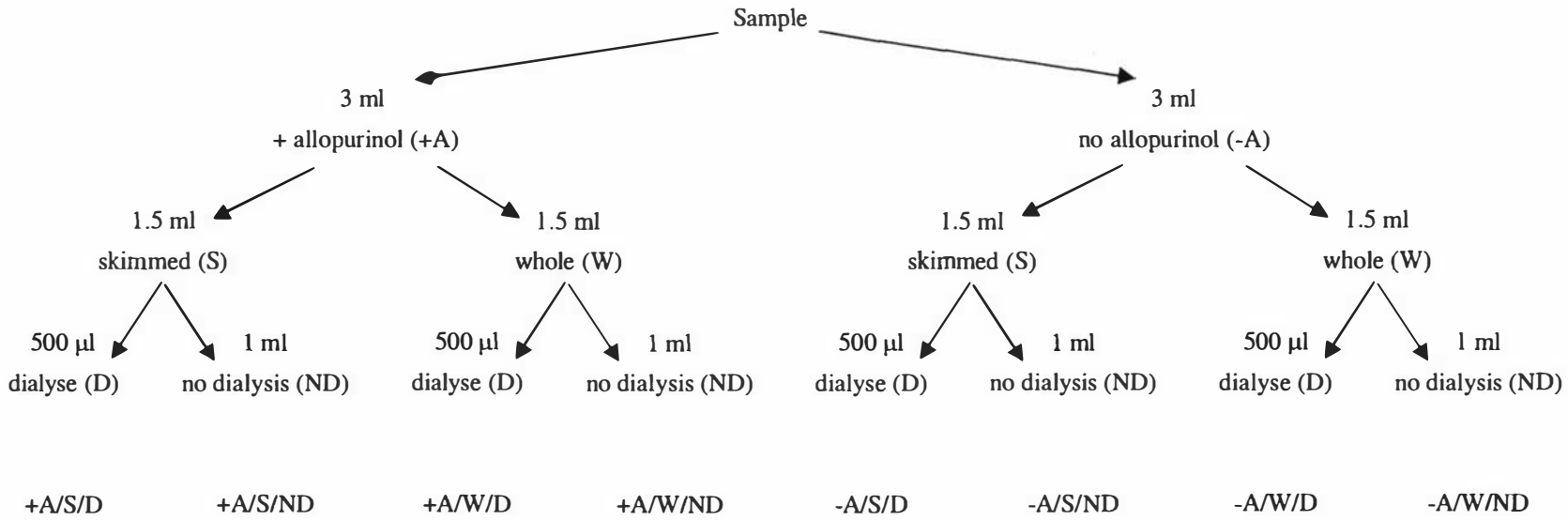

Figure 7.1: Milk sample treatment plan. Each of the 13 milk samples collected from cows during the first 4 days postpartum were split into two and were either treated with 50 µM allopurinol (+A), or left untreated (-A). Each of those samples were split into 2 and were either skimmed of fat by centrifugation (skimmed; S), or were left as whole milk (whole; W). Each of the skimmed and whole samples had 500 µl removed, which was dialysed against 100 mM phosphate buffer for 42 hours (dialyse; D). The remaining 1 ml was left undialysed (no dialysis; ND). All samples were stored at -20 °C until assayed.

7.4 Results

7.4.1 *Effect of Inhibition of Xanthine Oxidase in Comma-D Cells*

There were no significant differences in the DNA content of cultures between treatments. NOx was not detected in the media of non-treated cells, cells treated with allopurinol (1, 10, 100 or 1000 μM) or cells treated with allopurinol and 100 μM AG concurrently (data not shown).

When cells were treated with 1 mM L-NNA and various concentrations of allopurinol concurrently, there was no significant difference in the concentrations of NOx in the media. NOx was not detected in the media of non-treated cells (Figure 7.2).

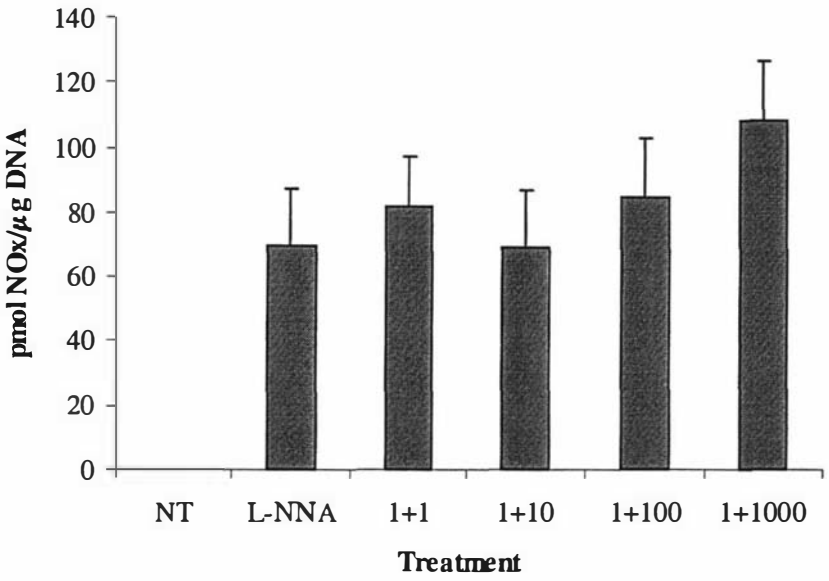

Figure 7.2: Effect of allopurinol and L-NNA on NOx production in Comma-D cells. Comma-D cells, grown for 4 days were made quiescent by the removal of serum and growth factors for 48 hours. The cells were either left untreated (NT) or were treated with 1 mM L-NNA or 1 mM L-NNA with allopurinol (at 1, 10, 100 and 1000 μM) for 24 hours. The media were collected and analysed for NOx. Data are expressed as the mean ± SEM for 5 independent experiments, where no bar indicates the sample contained less NOx than could be detected by the assay.

When Comma-D cells were co-treated with IFN-γ and 100 μM allopurinol the amount of NOx produced was no different from that produced by the cells cultured with IFN-γ alone (Figure 7.3). Untreated cells (NT), or those treated with allopurinol alone, did not produce detectable levels of NOx in the media.

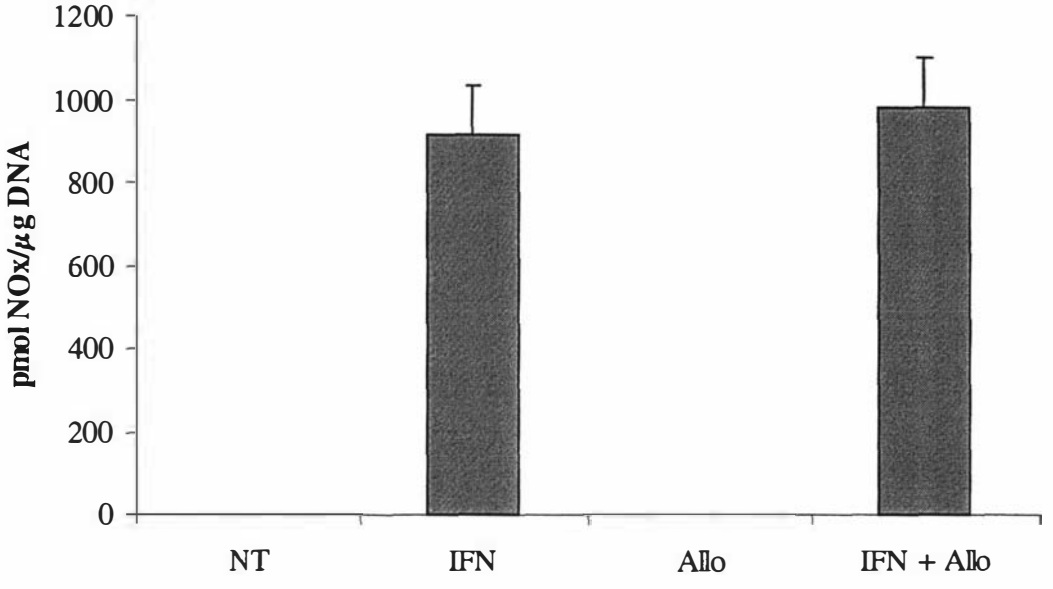

Figure 7.3: Effect of allopurinol on mIFN-γ stimulated NOx production by Comma-D cells. Comma-D cells, grown for 4 days, were made quiescent by the removal of serum and growth factors for 48 hours. The cells were either left untreated (NT), or were treated with either 250 U/ml mIFN-γ (IFN), 1000 μM allopurinol (Allo) or mIFN-γ + allopurinol (IFN + Allo) for 24 hours. The media were collected and analysed for NOx. Data are expressed as the mean ± SEM for 3 independent experiments, where no bar indicates the sample contained less NOx than could be detected by the assay.

Inclusion of the NOS inhibitors L-NNA or AG reduced the NOx production in mIFN-γ stimulated Comma-D cells. This inhibition was not effected by the inclusion of 1000 μM allopurinol (Figure 7.4).

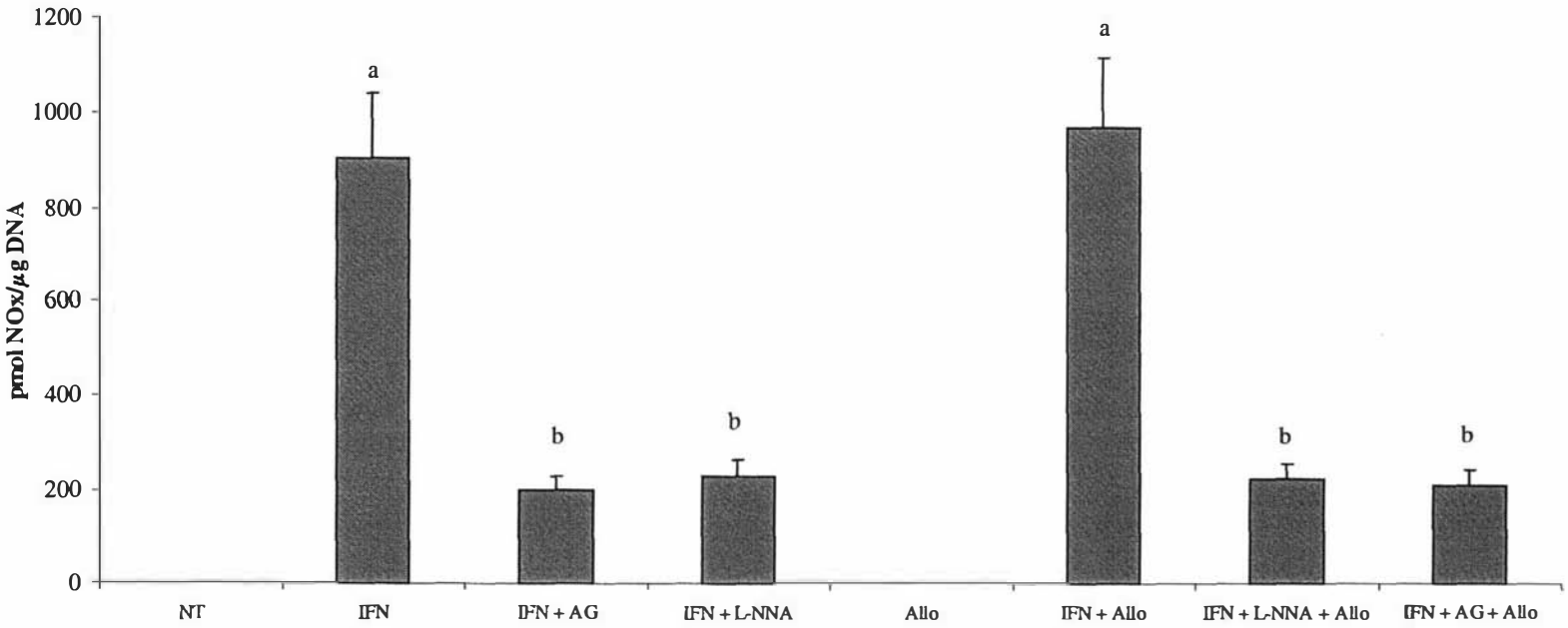

Figure 7.4: Effect of allopurinol on the inhibition of mIFN-γ stimulated NOx production using AG and L-NNA. Comma-D cells, grown for 4 days were made quiescent by the removal of serum and growth factors for 48 hours. The cells were either left untreated (NT), or were treated with either 250 U/ml mIFN-γ (IFN), mIFN-γ + 100 μM AG (IFN-γ + AG), mIFN-γ + 1 mM L-NNA (IFN-γ + L-NNA), 1000 μM allopurinol (Allo), mIFN-γ + allopurinol (IFN + Allo), mIFN-γ + AG + allopurinol (IFN + AG + Allo) or mIFN-γ + L-NNA + allopurinol (IFN-γ + L-NNA + Allo) for 24 hours. The media were collected and analysed for NOx. Data are expressed as the mean ± SEM for 3 independent experiments, where no bar indicates the sample contained less NOx than could be detected by the assay. Differences between the means are indicated by superscript letters, where means not sharing common letters are significantly different ($P < 0.05$).

7.4.2 *Xanthine Oxidase Activity and Nitric Oxide Concentration of Bovine Milk*

There were no significant differences in the XO activity between whole milk samples collected from different days postpartum (Figure 7.5).

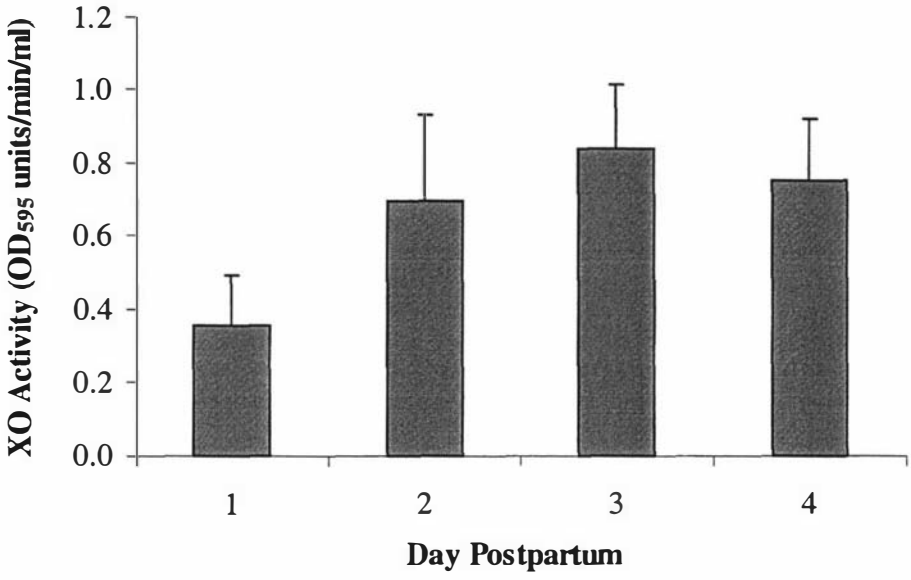

Figure 7.5: Xanthine oxidase activity in bovine milk. Whole milk samples were collected from 13 cows on the indicated days postpartum (Day 1, n=3; Day 2, n=2; Day 3, n=4; Day 4, n=4) and were analysed for XO activity. Data are presented as the mean ± SEM.

The pre-treatment of milk samples with 50 μM allopurinol (Figure 7.6) significantly decreased ($P < 0.05$) the XO activity in whole milk (+ Inhib whole) collected from cows during the first 4 days postpartum when compared to whole milk samples not treated with allopurinol (No Inhib whole). The addition of allopurinol had no significant effect on the XO activity of skim milk samples. There was significantly less ($P < 0.05$) XO activity in undialysed skim milk samples when compared to undialysed whole milk samples. In those samples that were dialysed, the XO activity

in uninhibited whole milk samples was significantly greater (P = 0.0002) than that of the uninhibited skim milk samples. There was no significant difference between the inhibited samples of whole milk and skim milk in XO activity. Dialysis of milk, through a membrane with a 12-14 kDa molecular weight cut off, did not significantly alter the activity of the XO in any samples when comparing the dialysed with the corresponding undialysed samples.

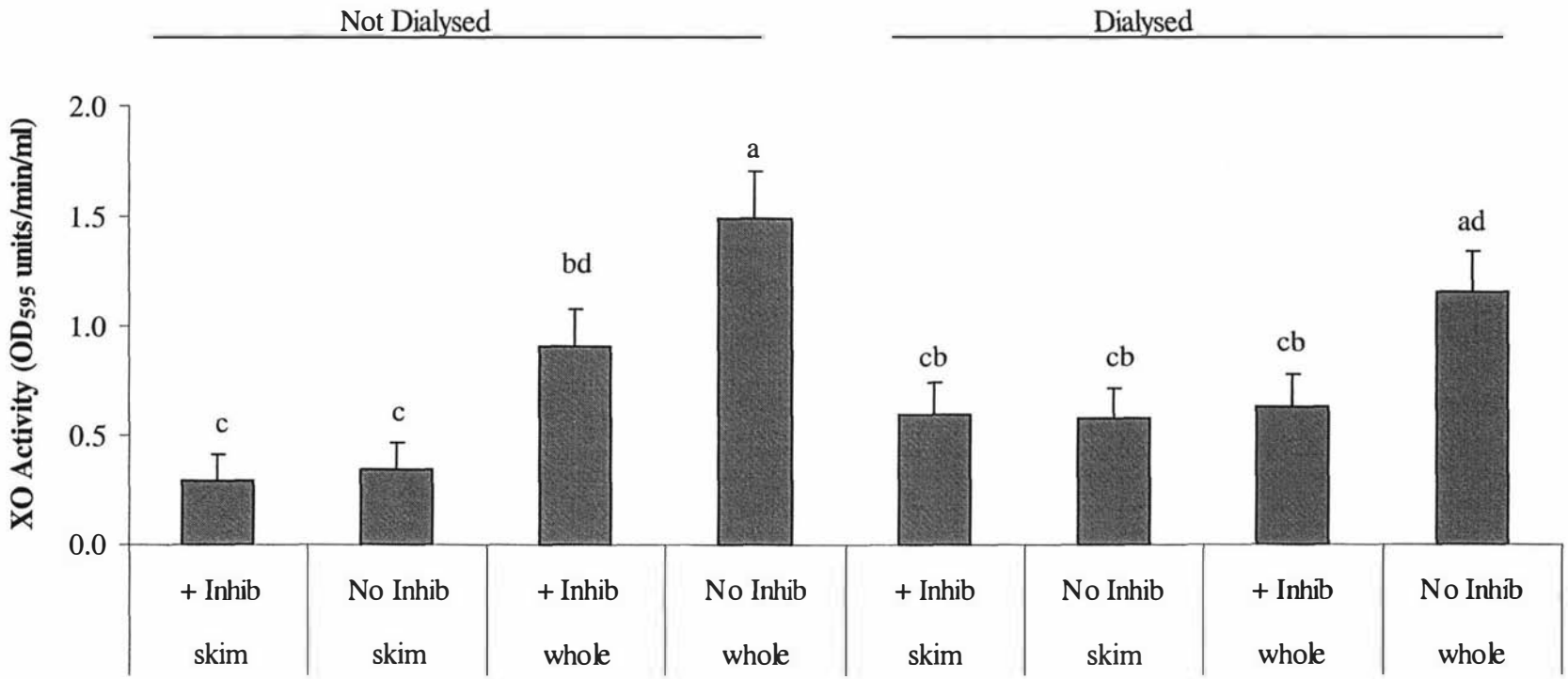

Figure 7.6: Effect of sample treatment on xanthine oxidase activity in bovine milk. Milk samples were collected from 13 cows from days 1-4 postpartum. The samples were treated with 50 μM allopurinol (+ Inhib), or left untreated (No Inhib). Each of the samples was split into two, one was left whole (whole) and the other was centrifuged and the skim milk collected (skim). A subsample of each of the ± allopurinol, whole, or skim samples were dialysed against 100 mM phosphate buffer. All samples were analysed for XO activity. Data are presented as the mean ± SEM. Differences between the means are indicated by superscript letters, where means not sharing common letters are significantly different ($P < 0.05$).

The NOx concentration of skim milk did not appear to be affected by the day postpartum that the sample was collected on (Figure 7.7). Of the 13 samples, NOx was only detected in seven. The six samples that did not contain detectable levels of NOx were all collected on different days postpartum (Day 1, n=1; Day 2, n=1; Day 3, n=2; Day 4, n=2)

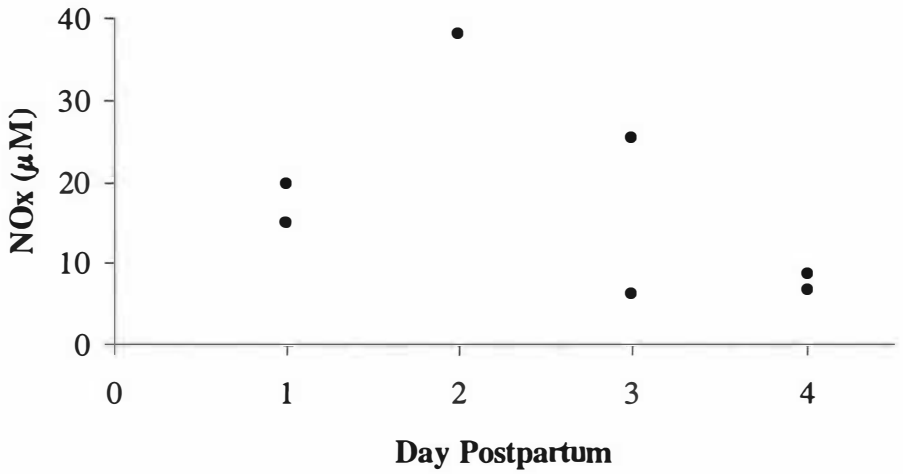

Figure 7.7: Nitrate and nitrite (NOx) concentration of bovine milk. Milk samples, collected from 13 cows on the postpartum days indicated, were skimmed of fat by centrifugation and analysed for NOx concentration.

As previous experiments indicated that whole milk interfered with the NOx assay (Section 2.4.1), only skim milk samples were analysed for NOx concentration. Only five of the 13 undialysed skim milk samples that were treated with allopurinol, and seven of the 13 undialysed skim milk samples that were left untreated contained high enough concentrations of NOx to be detected by the assay. Allopurinol did not appear to have any effect on the NOx concentration in non-dialysed skim milk samples (Figure 7.8).

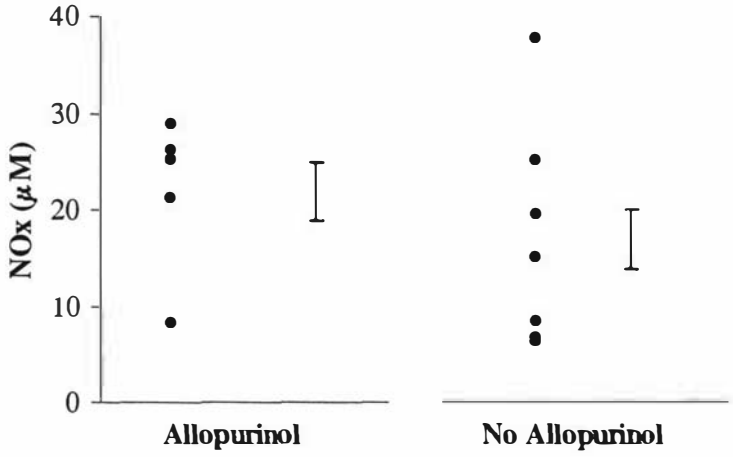

Figure 7.8: Effect of allopurinol on milk NOx concentration. Milk samples were collected from 13 cows during the first 4 days postpartum. Each of the 13 milk samples was split into two and were either treated with 50 μM allopurinol (allopurinol) or left untreated (No allopurinol). The samples were skimmed of fat by centrifugation and analysed for NOx concentration. Scatterplots show raw values for NOx concentration in the allopurinol (n=5) and untreated (n=7; no allopurinol) samples. Standard error bars indicate the mean value ± SEM.

Of the dialysed skim milk samples, only two of the 13 samples that were not treated with allopurinol, and two of the 13 samples that were, contained enough NOx following dialysis to be detected by the assay (data not shown). Two dialysed samples that had detectable levels of NOx, had undetectable levels in the matched undialysed samples (data not shown). The NOx concentrations measured in the dialysed samples were all just above the detection limit of the assay.

There was no correlation (R^2 = 0.0264) between the XO activity in whole milk and NOx concentration of skim milk samples collected during the first 4 days postpartum (Figure 7.9) when a linear regression line was fitted to the data. For the purpose of correlation, a sample with an undetected value of NOx was plotted as 0 μM.

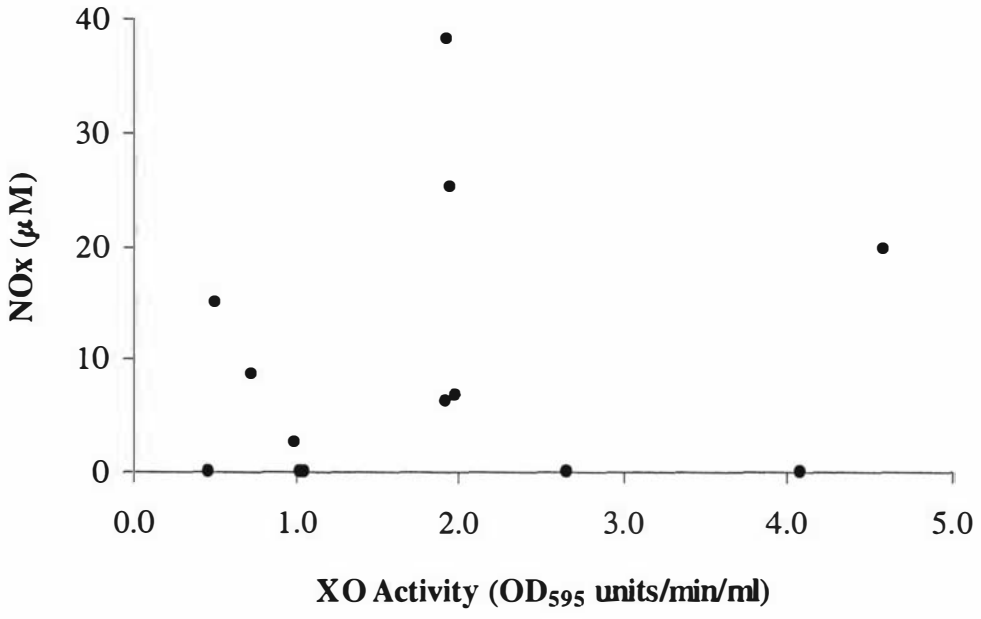

Figure 7.9: Correlation between milk XO activity and NOx concentration. Milk samples, collected from 13 cows between days 1-4 postpartum were analysed for XO activity and NOx concentrations.

7.5 Discussion

7.5.1 *Effect of Inhibition of Xanthine Oxidase in Comma-D Cells*

NO production in Comma-D cells is estimated by measuring NOx concentration in the medium, however, previous studies indicate that the enzyme XO is responsible for the production of NO from NOx (Millar *et al.*, 1998; Zhang *et al.*, 1998; Godber *et al.*, 2000). The results presented in this section suggest that NO is not being produced by XO in unstimulated Comma-D cells, as the inclusion of allopurinol, an

inhibitor of XO did not alter the concentration of NOx in the medium of the cells in any measurable way (data not shown). In addition, the treatment of Comma-D cells with 1 mM L-NNA increased the production of NOx from the cells above the detection level of the assay and when the cells were treated with L-NNA in conjunction with allopurinol, the amount of NOx in the medium was no different between the treatments (Figure 7.2). This increase in NOx production following treatment with L-NNA has been previously reported in this Thesis (Figure 4.7) and the results presented here clearly indicate that the increases in NOx apparent in the media are not affected by the activity of XO. If XO was actively converting NOx to NO in the medium then inhibition of the enzyme with allopurinol would lead to increases in NOx in the medium. Although the cells cultured with 1 mM L-NNA did show increases in the medium, there was no difference between those cultured with or without allopurinol.

Allopurinol had no effect either on the mIFN-γ stimulated production of NOx from Comma-D cells (Figure 7.3), or the inhibition of the production using the NOS inhibitors AG and L-NNA (Figure 7.4). In contrast, IFN-γ, TNF-α and IL-1β increase the XOR (xanthine dehydrogenase plus xanthine oxidase) activity in HB4a (human mammary) cells (Page *et al.*, 1998). Activation of XO in the Comma-D cells by mIFN-γ as reported for HB4a cells would have resulted in increased XO activity and thus decreases in the NOx in the medium as XO converted it to NO. This clearly did not occur. The lack of secretory activity of Comma-D cells may explain why other mammary epithelial cell types such as HC11 and HB4a cells exhibit XOR activity and Comma-D cells do not appear to. Within the bovine mammary gland, milk-secreting alveolar cells exhibit significant levels of XO whereas in non-secreting ductal epithelial cells the enzyme is not detectable (Jarasch *et al.*, 1981). Although Comma-D cells exhibit several characteristics distinctive of normal mammary epithelial cells (Danielson *et al.*, 1984), the expression of milk protein requires both prolactin and Matrigel (an extracellular matrix preparation; Bennett, 1995). In contrast HC11 cells can be induced with prolactin to produce β-casein without the requirement for an extracellular matrix (Ball *et al.*, 1988). Whether Comma-D cells would exhibit XOR activity if grown on Matrigel and treated with PRL requires further investigation.

Given that the inclusion of the specific XO inhibitor, allopurinol, had no effect on the production of NOx from either unstimulated or IFN-γ stimulated Comma-D cells, this suggests that XO was not actively affecting the NOx concentration of the media. This leads to the conclusion that either the XO enzyme was not active, or not present in sufficient quantities to significantly alter the amounts of NOx in the medium. In conclusion, these results clearly indicate that the activity of XOR does not affect the measurement of NOx in conditioned Comma-D cell culture medium.

7.5.2 *Xanthine Oxidase Activity and Nitric Oxide Concentration of Bovine Milk*

NO production in milk is determined by measuring the concentrations of NOx. However, given the recent suggestion that XO converts NOx to NO (Millar *et al.*, 1998; Zhang *et al.*, 1998; Godber *et al.*, 2000), the activity of XO could affect the accurate measurement of NOx and thus estimation of NO production. This effect was examined by measuring the NOx concentrations and XO activity of both whole and skim milk samples, and in samples that were or were not treated with the XO inhibitor allopurinol.

The treatment of the milk with allopurinol had no significant effect on the XO activity measured in skim milk but significantly decreased ($P < 0.05$) the XO activity of both the dialysed and undialysed whole milk samples (Figure 7.6). Why inhibition of XO activity did not occur in the skim milk samples is not known. Allopurinol was used at a concentration of 50 μM in these experiments, which should have been sufficient to abolish the activity of the XO in the milk samples. As an inhibitor of XO purified from bovine spleen, allopurinol has an inhibition constant of 4.88 μM and using a substrate (xanthine) concentration of 100 μM, 50 μM allopurinol almost totally abolished the activity of the enzyme (Lewis *et al.*, 1984). XO activity in bovine milk is also inhibited using allopurinol, which has an inhibition constant of 0.91 μM (Ho & Clifford, 1976). Although the concentration of xanthine (substrate) used in the XO assay (0.14 mM) described in this chapter was nearly 1.5 times greater than that used by Lewis *et al.* (1984), the concentration of allopurinol used was the same and therefore should still have been sufficient to inhibit the activity in the skim milk

samples. The assay used in this chapter to measure XO activity is actually more specific for XD than XO (Fried & Fried, 1974) and therefore it could be suggested that the uninhibitable activity in the skim milk is due to the XD form of XOR. However, in bovine milk, XOR is obtained exclusively as XO (Battelli *et al.*, 1973), suggesting that this activity can not be attributed to xanthine dehydrogenase.

The treatment of skim milk samples with allopurinol had no apparent effect on the concentration of NOx in the milk (Figure 7.8). XO, under hypoxic conditions converts nitrite and nitrate to NO (Millar *et al.*, 1998; Godber *et al.*, 2000). Thus, if XO was actively converting the NOx into NO, the sample containing the allopurinol should contain increased levels of NOx. This was not the case and is in direct contrast to the results of Stevens *et al.* (2000) who report that human milk generates NO, and the addition of oxypurinol (also a specific XO inhibitor) inhibits this generation, indicating that XO is critical in the reaction. There was no correlation between the activity of XO and the concentration of NOx in milk (Figure 7.9). If XO was actively converting NOx to NO, a negative correlation should exist between the two (XO activity and NOx concentration), that is with high XO activity, lower levels of NOx should be apparent in the milk. Unfortunately, the NOx assay used in this Thesis is unable to measure NOx in whole milk samples and therefore it can not be conclusively determined if the successful inhibition of XO (as apparent in the whole milk samples) would also result in changes in the NOx concentration of the milk.

Given that XO represents more than 8 % of the protein of the bovine milk fat globule membrane (Briley & Eisenthal, 1975) it is unsurprising that skim milk was shown to contain significantly less ($P < 0.05$) XO activity than whole milk (Figure 7.6). XO in bovine milk exists both membrane bound and free (Briley & Eisenthal, 1975; Bhavadasan & Ganguli, 1980). Therefore the activity measured in the skim milk was more than likely due to either fat remnants left in the milk, or to free (not membrane bound) XO. All samples analysed for XO activity were stored at 4 °C for 48 hours prior to dialysis then stored at -20 °C prior to assay. Storage of samples at 5 °C for 24 hours prior to assay increases the enzyme activity of XO in skim milk. The increase in activity is due to an increase in the activity of the free enzyme (Bhavadasan & Ganguli, 1980). It is unknown whether the storage of the samples analysed in this chapter resulted in increased activity of the samples, as no analysis was done prior to,

or throughout the sample preparation. Further experiments would have to address this, if a comparison of fresh versus stored milk was required.

Dialysis had no significant effect on the activity of XO in the various milk samples (Figure 7.6) indicating that low molecular weight compounds such as xanthine, hypoxanthine and uric acid which are known inhibitors of XOR activity (Fried & Fried, 1974) were present in insignificant amounts in the milk. However, as dialysis significantly decreased the amount of NOx in the milk of the dialysed samples this indicates that the dialysis successfully removed small compounds (data not shown), such as nitrite and nitrate, which would have readily, passed through the dialysis membrane (12-14 kDa molecular weight cut off).

As the generation of NO in human milk occurred in a low O_2 environment (< 5 %; (Stevens *et al.*, 2000) and the NOx in this Thesis is measured in milk collected from a lactating mammary gland, the NOx measured in the milk is not likely to be affected by the activity of XO. Further investigation of the production of NOx in milk is therefore warranted (Chapter 8).

7.5.3 *General Conclusions*

As allopurinol had no effect on either the activity of XO in skim milk, or on the concentration of NOx in skim milk, the results are inconclusive as to whether XO affects the measurement of NO production (i.e. NOx concentrations) in bovine milk. However, the experiments with Comma-D cells in culture clearly show that inhibiting XO has no effect on the amount of NOx in the culture medium, both in unstimulated and in mIFN-γ stimulated cells. In conclusion, it is unlikely that in the lactating mammary gland or in the culture systems utilised in this Thesis (cells are grown in 95 % air:5 % CO_2) that XO activity is affecting the estimation of NO production by the measurement of NOx.

CHAPTER EIGHT

NITRIC OXIDE AND BOVINE MILK

8.1 Abstract

Streptococcus uberis was infused into one quarter of the mammary glands of 10 lactating dairy cows resulting in signs of clinical mastitis in six of the animals. Milk from nine of the 10 animals had elevated somatic cell counts (SCC) within 24 hours post-infusion. Electrical conductivity (EC) and nitrite and nitrate (NOx) concentrations were also elevated in those animals that showed signs of clinical mastitis. Treatment with sodium cloxacillin (intramammary antibiotic) returned EC and NOx concentrations in the milk to the same level as the non-infused quarters although SCC remained elevated in those cows that showed clinical signs of mastitis. In a second trial, the infusion of interleukin-1β (IL-1β) into the mammary gland of six lactating dairy cows also resulted in elevated SCC. Both EC and NOx were elevated in animals infused with IL-1β when compared to phosphate buffered saline (PBS) infused quarters. To provide insight as to whether the somatic cells or the mammary epithelium were responsible for the production of NOx, a comparison between NOx and EC, and NOx and SCC were made. A positive relationship existed between NOx concentration and EC in the milk of cows that showed clinical mastitis following the infusion of *S. uberis.* In contrast, no relationship was apparent between NOx and EC of the milk from those cows that did not show clinical signs of mastitis or between NOx concentration and EC of the milk from cows infused with either IL-1β or PBS. No relationship was apparent between the NOx concentration and SCC of milk samples collected from either the *S. uberis* or IL-1β treated cows or any of the associated controls. It is suggested that increased production of NOx from damaged epithelial cells and increased number and activity of somatic cells are all in part responsible for increased levels of NOx in the milk of mastitic cows. Further work is required to elucidate the contribution of each population of cells.

8.2 Introduction

Mastitis (inflammation of the mammary gland) has important economic consequences for the dairy industry. Bacterial infection of the udder, the most common cause of mastitis, results in a loss of productivity and a change in milk composition (Wheelock *et al.*, 1966).

A healthy mammary gland produces milk that contains some somatic cells; these are primarily white blood cells and include macrophages, lymphocytes, polymorphonuclear neutrophils and a small percentage of epithelial cells (Harmon, 1994; Sordillo *et al.*, 1997). Following bacterial invasion, the macrophages in the milk release chemo-attractants, including cytokines, which recruit neutrophils to the infected gland. Indeed more than 95% of the somatic cells in the milk of the gland with mastitis are neutrophils (Kehrli & Shuster, 1994). The recruited neutrophils are subsequently activated and thus form an essential part of the udder's defence against pathogens (Wedlock *et al.*, 2000). For example, neutrophils isolated from milk from quarters of lactating cows infused with recombinant bovine IL-2 show enhanced bactericidal activity against *Staphylococcus aureus* (Wedlock *et al.*, 2000). SCC is most commonly used to assess udder health (Harmon, 1994) although there are other changes in milk composition as well. Intramammary infection can also lead to damage to the mammary epithelium and this results in an increase in the levels of sodium and chloride ions in the milk (Wheelock *et al.*, 1966). This change in ionic composition leads to an increase in electrical conductivity (EC) of the milk, which is often used as a means of detecting mastitis. Mastitis and its effects on the mammary gland have been reviewed extensively (Harmon, 1994; Kehrli & Shuster, 1994).

The recruitment of neutrophils and other leukocytes from blood into milk is controlled in part by the release of cytokines such as IL-1, IL-6, IL-8 and tumor necrosis factor (TNF; Kehrli & Shuster, 1994). IL-1β and TNF-α both stimulate the production of NO from various tissue and cell types (Lamas *et al.*, 1991; Rosenkranz-Weiss *et al.*, 1994; Schini *et al.*, 1994), including Comma-D cells where there is a synergistic response when cells are cultured with TNF-α, IL-1β and IFN-γ (Low *et al.*, 1997). Previous experiments reported in this Thesis also show that Comma-D

cells (Section 4.8.3) and explants of mammary tissue from both pregnant and lactating rats (Section 5.4.4) respond to IFN-γ with increased production of NOx into the medium.

Somatic cells may play a role in the control of intramammary infection by producing NO. Both the direct infusion of pathogens (e.g. *Streptococcus uberis* or *S. aureus*) or infusion of bacterial endotoxins have been used to induce mastitis in cows. The infusion of LPS or *S. aureus* results in a significant increase in SCC within 24 hours (Bouchard *et al.*, 1999; Shoshani *et al.*, 2000). There is an increase in nitrite in the milk of cows infused with *S. uberis* (Lacasse *et al.*, 1997) and serum NOx concentrations are elevated following intramammary challenge of cows with 1500 cfu *E. coli* (Hirvonen *et al.*, 1999). Both were accompanied by the appearance of clinical signs of mastitis. When cows were infused with *E. coli* endotoxin (LPS), the NOx concentration in the milk peaked 3 hours post-infusion and returned to pre-infusion levels within 48 hours. However, co-infusion of aminoguanidine (a specific iNOS inhibitor) with LPS prevented the NOx concentrations increasing in cows treated with LPS (Bouchard *et al.*, 1999).

The actual cells involved in the production of the NOx are still under investigation. Bovine macrophages stimulated with *Salmonella dublin* or LPS produced NO (Adler *et al.*, 1995). Somatic cells obtained from both infected and non-infected quarters of cows with spontaneous mastitis, when cultured and treated with LPS, increase the production of NOx into the culture medium (Lacasse *et al.*, 1997). Although approximately 95% of somatic cells present during mastitis are neutrophils (Kehrli & Shuster, 1994), neither Goff *et al.* (1996) or Boulanger *et al.* (2001) have been able to stimulate cultured bovine neutrophils to produce NOx using LPS, recombinant bovine IFN-γ (rboIFN-γ), and a variety of other cytokine and cytotoxins. However, there is an increase in iNOS mRNA in bovine blood neutrophils following LPS and rboIFN-γ stimulation (Boulanger *et al.*, 2001).

The aim of the experiments described in this chapter was to investigate the production of NOx in milk during induced mastitis and its relationship to the changes in SCC and EC.

8.3 Materials and Methods

8.3.1 *Streptococcus uberis Trial*

The objectives of the *S. uberis* trial described below were to investigate the effect of induced mastitis on the concentration of various milk components. The trial also provided the opportunity to determine if and how the concentration of these various milk components changed throughout the course of the infection. The trial was designed and run by Vicki Farr, Steve Davis and Colin Prosser (AgResearch, Hamilton, New Zealand) for their own research purposes. I gratefully acknowledge the supply of milk samples for analysis of NOx and other relevant information. The results presented in this chapter focus on the effect of induced mastitis on NOx concentration, SCC and EC of the milk throughout the course of the infection. The effects of intramammary infusion of *S. uberis* on other milk components not mentioned in this chapter will be reported elsewhere by Farr, Davis and Prosser.

Trial Design

This trial was undertaken with the approval of the Ruakura Animal Ethics Committee (Hamilton, New Zealand) and was conducted at the No 6 Dairy, Ruakura, Hamilton

Ten healthy, Friesian and Jersey cows in mid lactation (121 ± 5 days in milk), yielding an average of 10.9 ± 0.8 litres of milk/day and with no recent (3–4 months) history of mastitis infection (SCC < 50,000 cell/ml) were chosen. The cows which were in their first or second lactation, grazed ryegrass and white clover pasture, were milked twice daily at normal milking times (0800 and 1600 h) throughout the study.

The strain of *S. uberis* used in the trial had previously been isolated from a cow suffering from sub-clinical mastitis and was shown to be highly sensitive to cloxacillin both *in vitro* and *in vivo*. Following overnight culture in Todd Hewitt broth the inoculum was serially diluted in quarter strength Ringers solution (Fort Richard Laboratories, Auckland, New Zealand) and incubated overnight at 37 °C on Columbia blood agar containing 5 % whole bovine blood and 0.1 % aesculin (Fort Richard Laboratories, Auckland, New Zealand). The diluted inoculum was loaded

into 1 ml disposable syringes with attached infusion cannulae (Interlink™ Syringe Cannula, Becton Dickenson & Co., NY) and stored at 4 °C until required.

On the 2 days prior to intramammary infusion of the pathogen, cows were checked for infection. Foremilk samples were collected aseptically from all quarters at the first milking for bacteriology, SCC and EC (Technipharm Digital Mastitis Checker, Rotorua, New Zealand). At the following three milkings, EC of the milk was measured and foremilk samples were collected from the two hind quarters for the measurement of NOx and SCC. SCC was analysed on all pre-infusion samples to assure that quarters were still clear of infection.

Following the afternoon milking on the second day, the teat end was scrubbed with an alcohol swab and approximately 1000 cfu (1ml of innoculum) of *S. uberis* was introduced into one hind quarter. Following infusion the innoculum was gently massaged up into the gland.

At each milking post infusion all cows were observed for visual signs of infection such as redness, tenderness or swelling of the quarter and/or the appearance of clots in the foremilk. Rectal temperatures were monitored at milking time on sampling days throughout the experiment.

At the first milking after the infusion, foremilk samples were taken aseptically from both hind quarters for bacteriological examination. Foremilk samples were also collected from both hind quarters under non-aseptic conditions at each milking either until there were signs of clinical mastitis or until 3 days (72 h) after infusion. Aseptic samples of foremilk were collected for bacteriology from the infused quarters that showed clinical symptoms of mastitis. Infected quarters were treated with a course of intramammary antibiotics (sodium cloxacillin, Orbenin L.A.,Beecham Veterinary Products, Auckland, New Zealand, three tubes of 200 mg, one every 2 days). If no clinical signs were shown earlier, cows were treated with cloxacillin at 72 hours post-infusion. To determine gland recovery from infusion and effectiveness of antibiotic treatment, milk samples were collected from both hind quarters on days 10 and 14 following the infusion of the innoculum for bacteriology, SCC, EC and the other components of interest.

8.3.2 *Recombinant Cytokine Trial*

The objectives of the interleukin-1β (IL-1β) trial described below were to observe the effect of IL-1β on somatic cell concentration and activation. The trial was designed and run by Jane Lacy-Hulbert, John Williamson and Alan Napper (Dexcel, Hamilton, New Zealand) for their own research purposes. I gratefully acknowledge the supply of milk samples for analysis of NOx and other relevant information. The results presented in this chapter examine the effect of infusion of IL-1β on NOx concentration, SCC and EC of milk samples collected during a small part of the trial. Further information will be reported elsewhere by Lacy-Hulbert, Williamson and Napper.

Trial Design

This trial was undertaken with the approval of the Ruakura Animal Ethics Committee (Hamilton, New Zealand) and was conducted at the Dexcel No 1 Dairy, Ruakura, Hamilton. Six healthy, lactating cows, all free from intramammary infection, were selected from the No 1 dairy herd. Two quarters of each cow were infused with either a 10 μg (L) or 50 μg (H) dose of recombinant bovine IL-1β (expressed in *Escherichia coli*, purified and supplied by Wedlock *et al.*, 1999) in 10 ml sterile PBS containing 0.1 % BSA. The other two quarters received 10 ml PBS containing 0.1 % BSA (Table 8.1).

Foremilk samples were collected from all quarters on the day prior to infusion, on the day of infusion (day 0) and on days 1-6 after infusion. Infusions took place immediately after the PM milking on day 0. Samples from all milkings were analysed for SCC and EC (Technipharm Digital Mastitis Checker, Rotorua, New Zealand) was measured on samples collected aseptically at the AM milkings on days 0, 1 and 2. Bacteriology was carried out on samples collected aseptically from the AM milkings on days 0, 2 and 6. At the AM milking on day 2, a 200 ml sample was collected and subsequently analysed for neutrophil content and bactericidal activity. Samples from the 2nd, 3rd, 4th and 5th milkings after infusion were analysed for NOx. All cows remained free of new intramammary infection throughout the experimental period, as determined by bacteriological analysis of the foremilk.

Table 8.1: Treatment of lactating cows with interleukin-1β. Either PBS + 0.1 % BSA (PBS) or a 10 μg (L) or 50 μg (H) dose of recombinant bovine interleukin-1β (IL-1) in PBS were infused into 2 quarters (LF: left front; RF: right front; LH: left hind; RH: right hind) of the udder of lactating dairy cows.

Cow	LF	RF	LH	RH
633	IL-1 H	PBS	PBS	IL-1 H
634	IL-1 L	PBS	PBS	IL-1 L
681	PBS	IL-1 L	IL-1 L	PBS
6833	IL-1 L	PBS	PBS	IL-1 L
6923	PBS	IL-1 H	IL-1 H	PBS
6943	IL-1 H	PBS	PBS	IL-1 H

Bacteriological Analysis

Foremilk samples for bacteriological analysis were collected aseptically. The teat ends were scrubbed with 70 % alcohol-soaked cotton wool swabs, the first 2-3 squirts of milk were discarded and the next 5-10 ml of milk were collected in a sterile container. A 0.01 ml sub-sample of this milk was plated onto a tryptose blood agar plate containing 0.1 % w/v esculin and incubated at 37 °C for 48 hours. Presumptive identification of isolates was by colony morphology, haemolysis, esculin reaction, catalase reaction and Gram stain.

8.3.3 *Sample Analysis*

Electrical Conductivity

EC of milk samples were analysed in the field using a Technipharm Digital Mastitis Checker (Rotorua, New Zealand). Conductivity of milk is commonly measured in seiverts, and usually reported as a ratio between the EC of the three highest quarters and the lowest one (Woolford *et al.*, 1998). However, as the instruments were not calibrated and due to the nature of the trials (quarters receiving different treatments) the raw values from the instruments are reported as EC Units.

Somatic Cell Count

SCC were analysed on milk samples using a cell counter (Fossomatic 450; Foss Electric, Hillerød, Denmark) which had an upper limit of 9.999 x 10^6 cells/ml.

NOx Assay

Skim milk samples were analysed for NOx as described in Section 2.4.

8.4 Results

8.4.1 *Effect of Streptococcus uberis Infection on Conductivity of Milk, SCC and NOx Concentration.*

Of the 10 cows treated, six developed clinical mastitis as indicated by the appearance of clots in the milk. Three cows showed slight increases in the conductivity (EC) of the milk, and in SCC, but did not show any clinical signs of infection. One cow did not appear to respond to the infusion of *S. uberis* and there were no apparent changes in the EC of the milk or SCC. As the response amongst the 10 cows was so variable, the following results presented in this chapter are divided into three groups (clinical cows; sub-clinical cows; non-responder). Results for individual animals are given in the appendix.

There were no significant differences in EC between infused and control quarters of the sub-clinical cows (Figure 8.1 A & C). The difference in EC between the control and infused quarters of the cows with clinical mastitis increased from a pre-infusion average of -0.10 to a maximum of 4.4, 40 hours following the infusion (Figure 8.1 B). At 24, 40 and 64 hours post-infusion the conductivity of the infused quarter was significantly higher ($P < 0.0001$) than the control quarter.

There was an increase in milk SCC in the cows showing clinical signs of mastitis (Figure 8.2 B) with numbers increasing from a pre-infusion average difference of 9 x10^3 cells/ml, to a maximum difference between the infused and control quarters of

9761 x 10^3 cells/ml, 40 hours following infusion of *S. uberis*. All samples collected from the infused quarters from 16 hours post-infusion onward had significantly higher ($P < 0.0001$) SCC than the control quarters. The SCC of milk from the infused quarters of the sub-clinical cows were significant greater ($P < 0.01$) than the control quarters from 24 hours following infusion until 232 hours where no difference was apparent.

There was an increase in the concentration of NOx in the milk in the cows that showed a clinical response to the infusion of *S. uberis* (Figure 8.3 B). The difference in NOx concentration between the infused and control quarters rose from a pre-infusion average of -0.07 μM to a maximum of 12.87 μM, 40 hours after infusion. Concentrations in the infused gland had returned back to the same as the control gland 232 hours following infusion. The NOx concentrations of the infused gland were significantly higher ($P < 0.01$) than that in the control gland at 24, 40 and 64 hours post-infusion. There were no differences in the concentrations of NOx between control and infused glands in those cows classified as sub-clinical or non-responder (Figure 8.3 A & C). The average NOx concentration of both quarters of all cows prior to the infusion was 8.2 ± 0.3 μM.

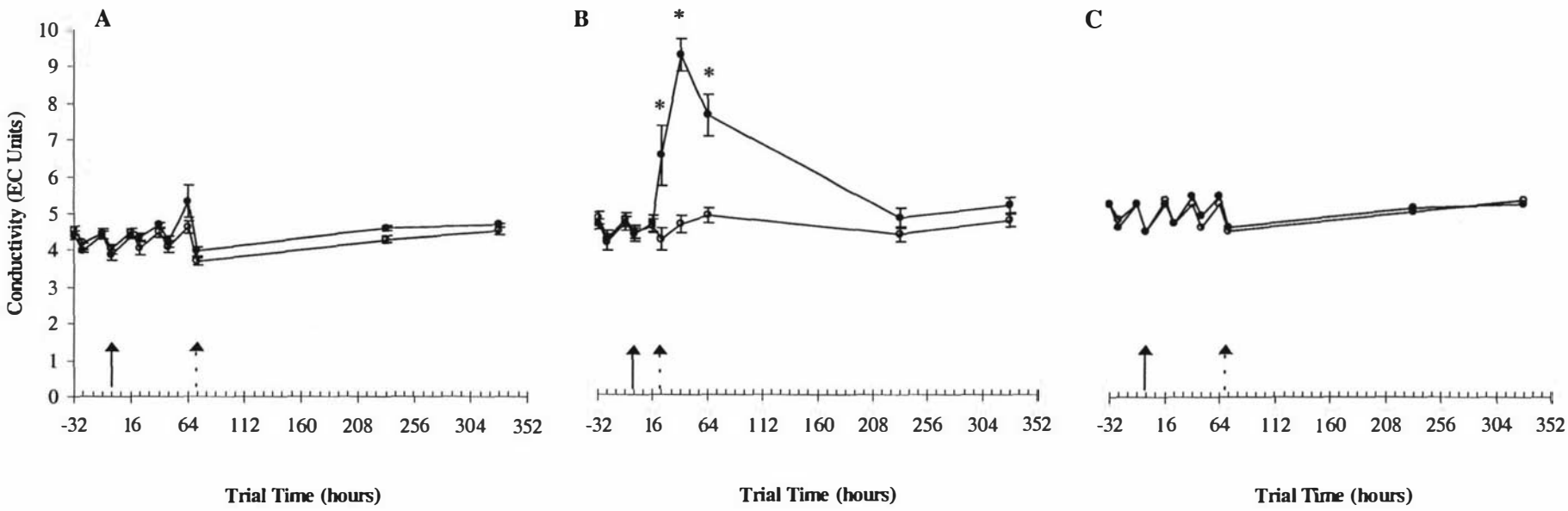

Figure 8.1: Effect of intramammary infusion of *Streptococcus uberis* on milk conductivity. The EC of milk was measured in samples taken from both the right hind quarter (-o-) and infused left hind quarter (-●-). *S. uberis* (1000 cfu) infusion followed the afternoon milking on the 2nd day (solid arrow), treatment with cloxacillin occurred either when the animals showed clinical symptoms of mastitis, or 72 hours after infusion (striped arrow). **A:** Sub-clinical cows (n=3). **B:** Clinical cows (n=6). **C:** Non-responder (n=1). Data are presented as the mean ± SEM. Statistical differences between the means are indicated by an asterisk (*; $P < 0.05$).

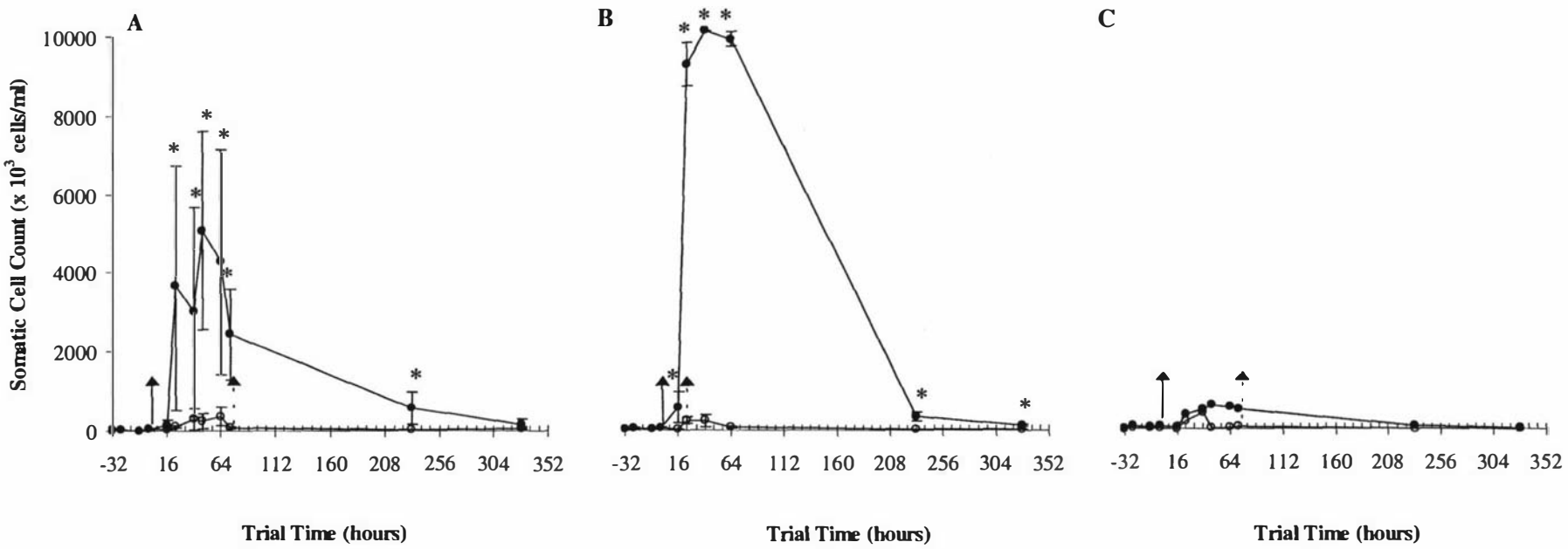

Figure 8.2: Effect of intramammary infusion of *Streptococcus uberis* on milk somatic cell count. The milk SCC was measured in samples taken from both the right hind quarter (-o-) and infused left hind quarter (-●-). *S. uberis* (1000 cfu) infusion followed the afternoon milking on the 2nd day (solid arrow), treatment with cloxacillin occurred either when the animals showed clinical symptoms of mastitis, or 72 hours after infusion (striped arrow). **A:** Sub-clinical cows (n=3). **B:** Clinical cows (n=6). **C:** Non-responder (n=1). Data are presented as the mean ± SEM. Statistical differences between the means are indicated by an asterisk (*; $P < 0.05$).

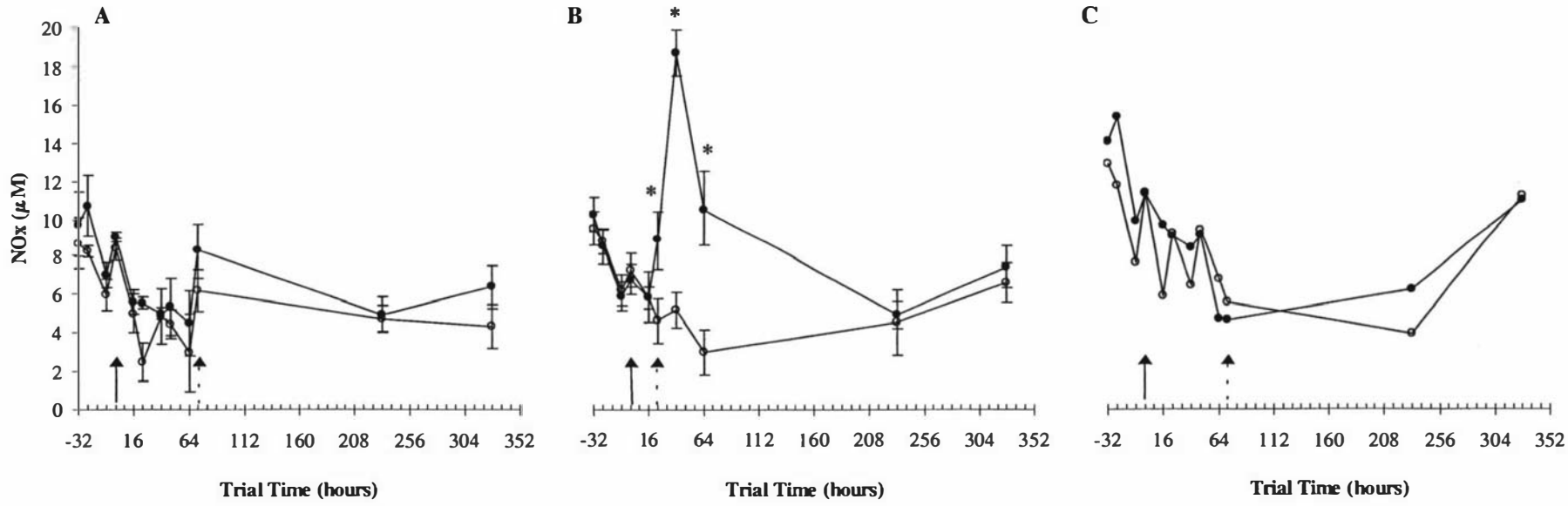

Figure 8.3: Effect of intramammary infusion of *Streptococcus uberis* on milk NOx concentration. The amount of NOx in the milk was measured in samples taken from both the right hind quarter (-o-) and infused left hind quarter (-●-). *S. uberis* (1000 cfu) was infused following the afternoon milking on the 2nd day (solid arrow), treatment with cloxacillin occurred either when the animals showed clinical symptoms of mastitis, or 72 hours after infusion (striped arrow). **A:** Sub-clinical cows (n=3). **B:** Clinical cows (n=6). **C:** Non-responder (n=1). Data are presented as the mean ± SEM. Statistical differences between the means are indicated by an asterisk (*; $P < 0.05$).

Both EC and the concentration of NOx in the milk show a similar response to the infusion of *S. uberis* (Figure 8.4). Those animals that were classified as having clinical mastitis showed an increase in both EC and NOx concentration, whereas those animals that were classified as sub-clinical responders showed a small decrease in NOx and a small increase in EC. Data are presented from the 40 hour time point, as it is the time at which NOx, EC and SCC were all at a maximum level. Eight hours pre-infusion was used as the basal value as it was the last AM milking prior to infusion (40 hours post-infusion was also an AM collection).

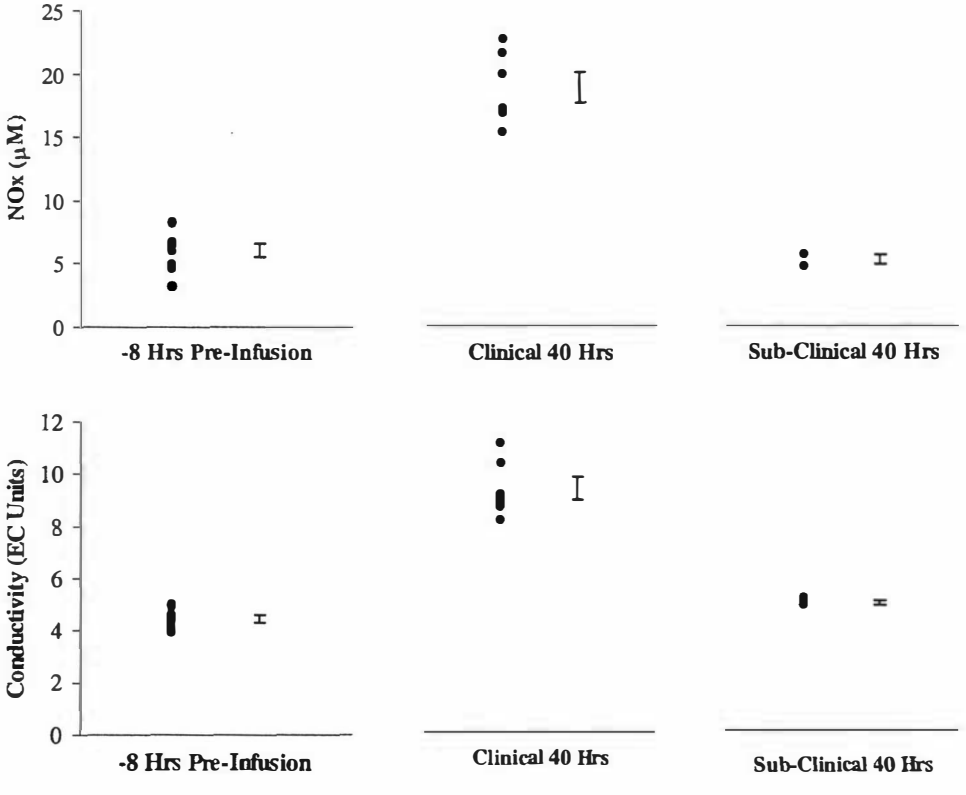

Figure 8.4: Effect of intramammary infusion of *Streptococcus uberis* on milk NOx concentration and conductivity. Infusion of *S. uberis* (1000 cfu) into the left hind quarters of 10 lactating dairy cows occurred following the afternoon milking on Day 2 (0 hours). Conductivity and NOx concentrations were measured in milk samples taken from the infused quarters. Scatterplots show raw values for 9 animals at 8 hours pre-infusion (-8 Hrs), and at 40 hours post-infusion, separately for the cows which showed signs of clinical mastitis (Clinical; n=6) and for those, which were sub-clinical (Sub-Clinical; n=3). The standard error bars indicate the mean value ± SEM.

Further examination of the data from all cows over the entire experimental period shows that there is only a positive relationship between EC and NOx concentration in milk samples collected from the infused left hind quarter of those cows that showed signs of clinical mastitis (Figure 8.5 B). When linear regression equations were fitted to the data from each cow in the Clinical group, there was a range in slopes of 1.25 to 3.0 μM NOx/EC Units with an average slope of 1.93 ± 0.29 μM NOx/EC Units. The range in slopes for the three cows in the sub-clinical group was -1.10 to -6.47 μM NOx/EC Units with an average slope of -3.25 ± 1.64 μM NOx/EC Units. No relationship between NOx and EC was apparent in the right hind quarter of any of the groups (data not shown).

There was no apparent relationship between SCC and NOx concentrations in milk samples collected from either the infused left hind quarter or the untreated right hind quarter of any cows (Figure 8.6). Many of the samples collected from cows in the clinical group had SCC values measured as 9.999×10^6 (Figure 8.6 B). The NOx concentrations of these samples varied with a range from 21.4 to 6.4 μM and an average of 13.8 ± 4.5 μM. This range shows considerable overlap with the NOx concentrations of the rest of the clinical group (SCC values below 9.999×10^6) as well as with the sub-clinical group (Figure 8.6 A) and the non-responder (Figure 8.6 C).

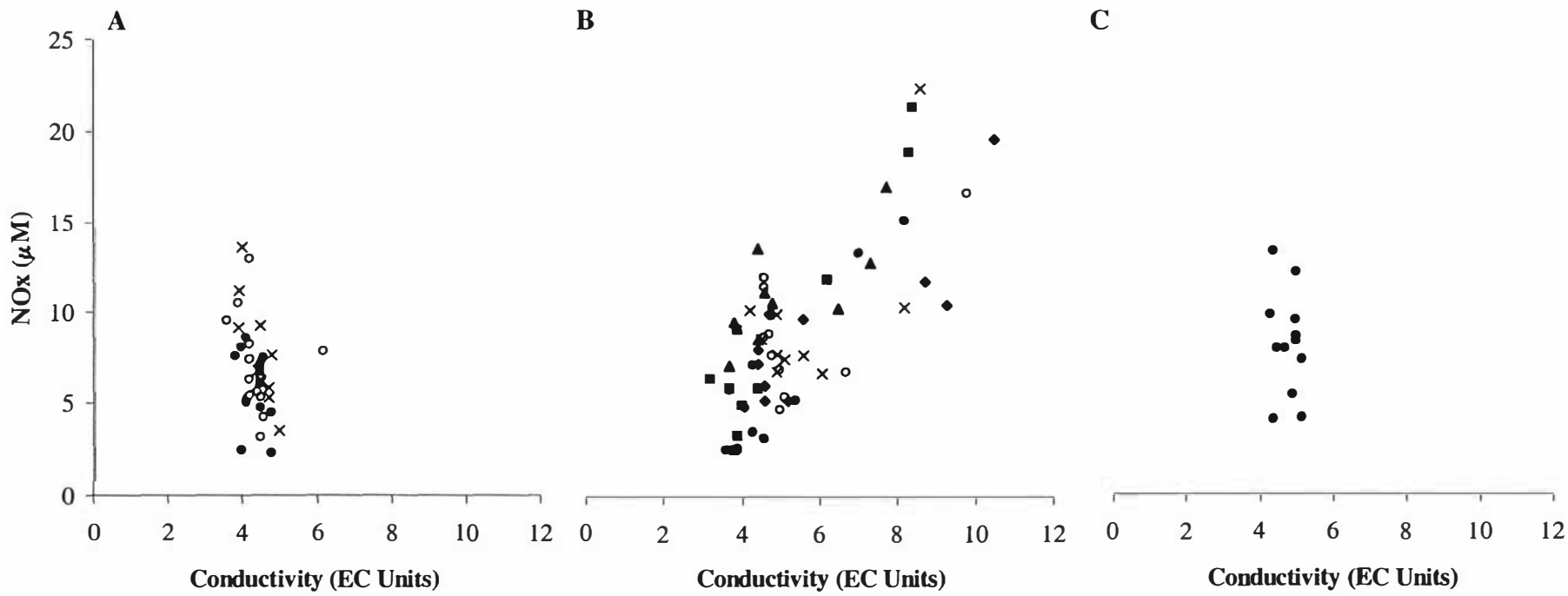

Figure 8.5: Effect of intramammary infusion of *Streptococcus uberis* on the correlation between milk NOx concentration and conductivity. Conductivity and NOx concentrations were measured in milk samples taken from the infused left hind quarters of all cows throughout the experimental period (-32, -24, -8, 0, 16, 24, 40, 48, 64, 232, 328 hours). Infusion of *S. uberis* (1000 cfu) occurred following the afternoon milking on the 2nd day, treatment with cloxacillin occurred either when the animals showed clinical symptoms of mastitis, or 72 hours after infusion. **A:** Sub-Clinical cows (n=36, 12 samples/3 cows; o,•,x); **B:** Clinical cows (n=60, 12 samples/6 cows; o,•,x,■,▲,♦: except 48 hours 1 cow only •; 232 hours 1 cow missing ▲); **C:** Non-responder (n=12 samples; •).

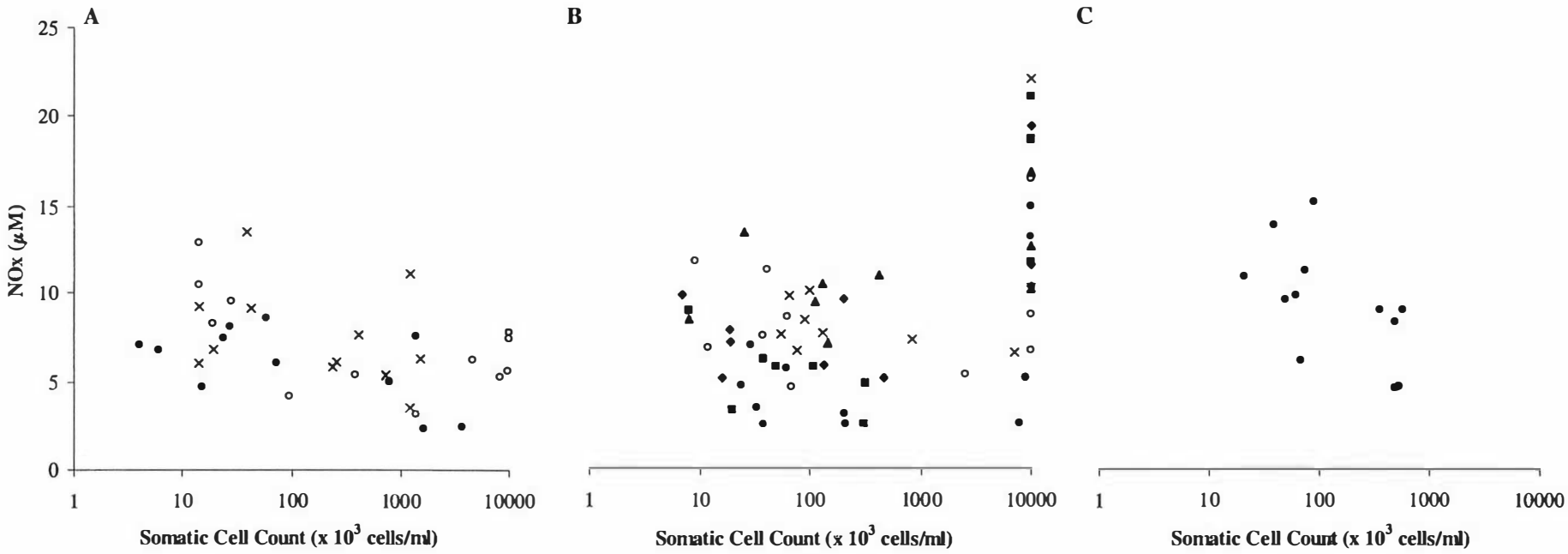

Figure 8.6: Effect of intramammary infusion of *Streptococcus uberis* on the correlation between milk NOx concentration and somatic cell count. SCC and NOx concentrations were measured in milk samples taken from the infused left hind quarters of all cows throughout the experimental period (-32, -24, -8, 0, 16, 24, 40, 48, 64, 232, 328 hours). Infusion of *S. uberis* (1000 cfu) occurred following the afternoon milking on the 2nd day, treatment with cloxacillin occurred either when the animals showed clinical symptoms of mastitis, or 72 hours after infusion. **A:** Sub-Clinical cows (n=36, 12 samples/3 cows; o,•,x: except 40 hours 1 cow missing •); **B:** Clinical cows (n=60, 12 samples/6 cows; o,•,x,■,▲,♦: except 48 hours 1 cow only •; 232 hours 1 cow missing ▲); **C:** Non-responder (n=12 samples; •).

8.4.2 *Effect of Intramammary Infusion of Interleukin-1β on Conductivity of Milk, SCC and NOx Concentration.*

The average somatic cell counts across all four milkings of the PBS infused quarters was 184 ± 89 x 10^3 cells/ml. In contrast, the SCC in nearly all of the IL-1β infused quarters were in excess of 9.999 x 10^6 cells/ml throughout the experimental period. Only six of the samples collected from the IL-1β treated quarters over the four milkings had SCC values below 9.999 x 10^6 cells/ml (average SCC of these six samples was 4902 ± 1183 x 10^6 cells/ml).

Both EC and NOx were elevated in the milk collected at the 3rd and 5th milkings following infusion in those quarters infused with IL-1β (Figure 8.7). The infusion of IL-1β significantly increased ($P < 0.001$) the EC of the milk when compared to that of milk samples collected from PBS infused quarters (4.6 ± 0.1 EC Units). The average EC of the quarters receiving an infusion of the 50 μg IL-1β (H) was 7.5 ± 0.3 EC Units and was significantly higher ($P < 0.0001$) than that of the quarters that received the 10 μg (L) infusion (6.0 ± 0.2 EC Units).

Average NOx concentrations were significantly higher ($P < 0.01$) in the milk collected from IL-1β infused quarters when compared to PBS infused quarters (4.72 ± 0.56 μM), however, there was no significant difference in the NOx concentrations between the two doses of IL-1β (IL-1 L, 8.03 ± 0.74 μM; IL-1, 8.64 ± 1.03 μM; Figure 8.7).

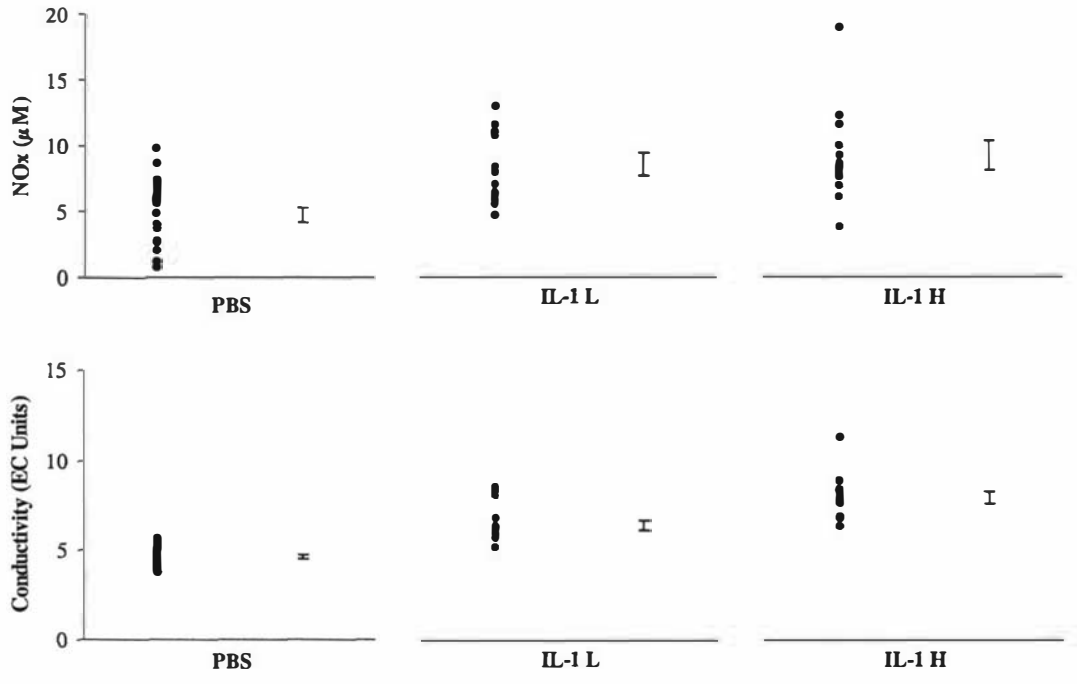

Figure 8.7: Effect of intramammary infusion of interleukin-1β on milk NOx concentrations and conductivity. Intramammary infusion of PBS (PBS; n=24), IL-1β at 10 μg (IL-1 L; n=12) or IL-1β at 50 μg (IL-1 H; n=12) into separate quarters of 6 lactating dairy cows occurred after the PM milking on Day 0. Conductivity and NOx concentrations were measured in milk samples taken from the 3rd and 5th milkings following infusion. Scatterplots show raw values for 6 animals at the 2 milkings, separately for the quarters, which were infused with PBS, IL-1 L and IL-1 H. The standard error bars indicate the mean value ± SEM.

Although NOx concentrations in the milk of most cows increased between the 3rd and 5th milkings, there was no concurrent increase in the EC of the milk suggesting that no apparent relationship exists between the NOx concentration of the milk and the EC in either the PBS (Figure 8.8 A) or IL-1β infused quarters (Figure 8.8 B and C). As samples were analysed for conductivity only twice for each cow (3rd and 5th milkings following infusion), regression analysis was not considered appropriate.

No relationship exists between the SCC and NOx concentrations of milk collected from the 2nd, 3rd, 4th and 5th milkings from the PBS infused quarters of cows (Figure 8.9 A). Nor does there appear to be a relationship between NOx concentration and SCC, as the variability between NOx values for the cows with SCC of 9999 x 10^3 was so large (Figure 8.9 B and C). Due to limitations of the instrument used to measure SCC, values above 9999 x 10^3 are out of the range of the instrument and can therefore not be reported accurately, thus, regression analysis of this data was not considered to be appropriate. The average SCC of the IL-1β L and IL-1β H infused quarters collected from the four milkings were 9060 x 10^3 cells/ml, and 9664 x 10^3 cells/ml respectively. However, the range in NOx concentrations of those samples in the IL-1β L group with SCC of 9999 x 10^3 was 17.8 to 3.4 μM, and range in NOx concentrations of those samples in the IL-1β H group with SCC of 9999 x 10^3 was 37.0 to 3.5 μM. Both of these ranges show considerable overlap with the NOx concentrations of the PBS infused group (17 to 0.8 μM) that had an average SCC of 184 x 10^3 cells/ml.

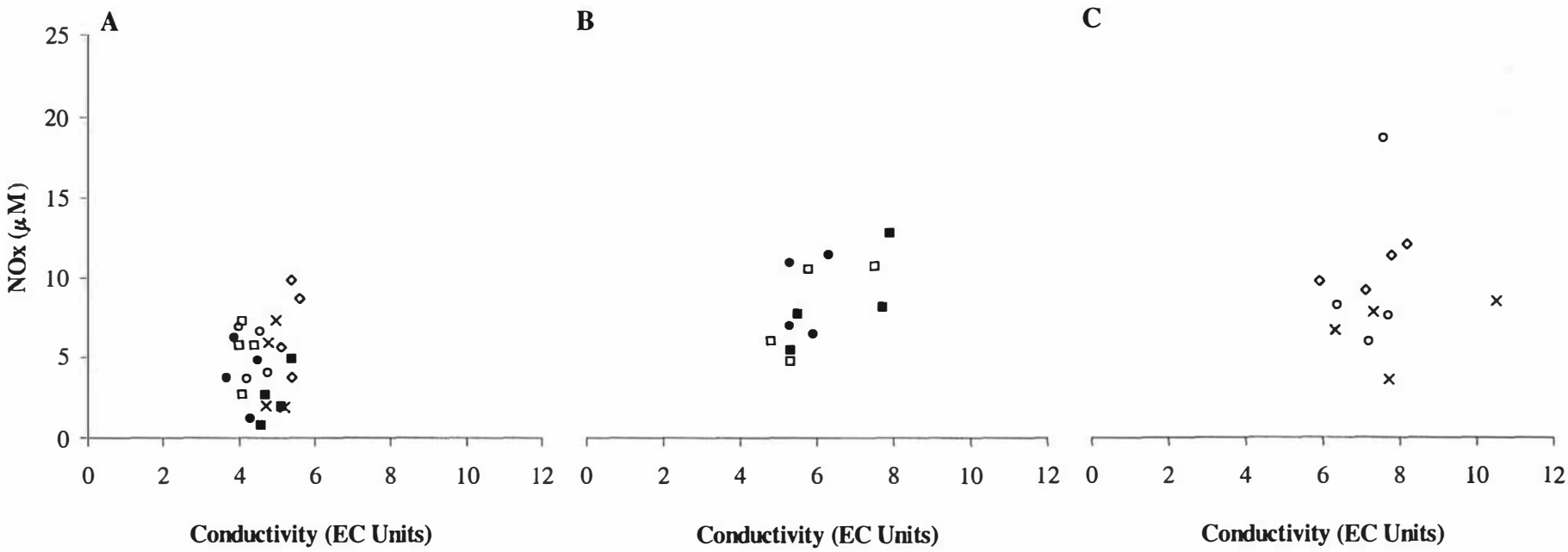

Figure 8.8: Effect of intramammary infusion of interleukin-1β on the correlation between milk NOx concentrations and conductivity. Conductivity and NOx concentrations were measured in milk samples taken from 4 quarters of 6 cows at the 3rd and 5th milkings following infusion. Infusion of **A:** PBS (2 quarters/cow/6 cows; o,•,□,■,◊,x), **B:** IL-1β at 10 μg (2 quarters/cow/3 cows; o,•,■) and **C:** IL-1β at 50 μg (2 quarters/cow/3 cows; o,◊,x) occurred after the PM milking on Day 0.

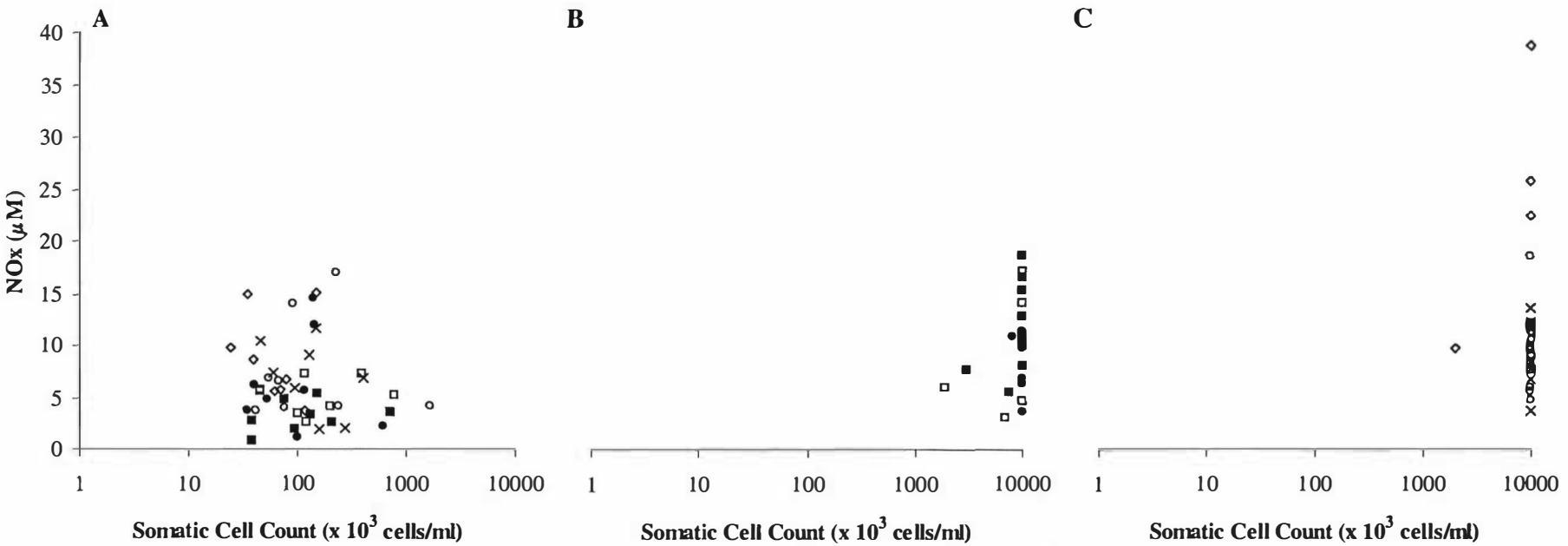

Figure 8.9: Effect of intramammary infusion of interleukin-1β on the correlation between milk NOx concentrations and somatic cell count. SCC and NOx concentrations were measured in milk samples taken from 4 quarters of 6 cows at the 3rd and 5th milkings following infusion. Infusion of **A:** PBS (2 quarters/cow/6 cows; o,•,□,■,◊,x), **B:** IL-1β at 10 μg (2 quarters/cow/3 cows; o,•,■) and **C:** IL-1β at 50 μg (2 quarters/cow/3 cows; o,◊,x) occurred after the PM milking on Day 0.

8.5 Discussion

The results presented in this chapter give some insight into the effect that mastitis had on the concentration of NOx measured in the milk as well as its sources. The mammary epithelium, milk somatic cells and serum NOx were considered as potential sources. Both SCC and EC, which are used as indicators of mastitis, were compared with the NOx concentrations in milk samples collected from lactating cows under two different experimental regimes.

The intramammary infusion of *S. uberis* resulted in an increase in the SCC, EC and NOx concentrations in the milk of those cows that showed clinical signs of mastitis (Figure 8.1 B, Figure 8.2 B and Figure 8.3 B). An increase in NOx concentration of the milk of cows following the intramammary infusion of *S. uberis* has been previously reported (Lacasse *et al.*, 1997). The average NOx concentration in the milk of the *S. uberis* trial cows prior to infusion, was 8.2 ± 0.3 μM. This is similar to that reported by Bouchard *et al.* (1999) who found milk NOx concentrations of approximately 10 μM prior to infusion of LPS. However, the results are vastly different from the 8.5 mM reported in the milk from an untreated quarter by Lacasse *et al.* (1997). The maximal NOx concentration in the milk following *S. uberis* infusion was 17.8 ± 1.1 μM (Figure 8.3), this is in agreement with Bouchard *et al.* (1999) who reported a maximum NOx concentration of 24.4 μM following LPS infusion. However, as the maximum NOx concentration in the milk of cows infused with *S. uberis* was 24.1 mM (Lacasse *et al.*, 1997), it is suggested that the basal and treated values quoted by Lacasse *et al.* (1997) are probably out by a factor of 1000 due to the micro (μ) being transcribed as a milli unit (m) in the published abstract.

The intramammary infusion of IL-1β resulted in an increase in the NOx concentration of the milk when compared to PBS infused quarters (Figure 8.7). Although the effect of the infusion of cytokines such as interleukin and IFN-γ on the mammary gland have been studied (Sordillo *et al.*, 1991; Hogan *et al.*, 1995; Wedlock *et al.*, 2000), the results presented in this chapter appear to be the first time that NOx has been measured in the milk following such an infusion.

To further examine the relationship between NO production and mastitis, the EC of the milk and NOx concentrations were compared. There appears to be a reasonably good relationship between electrical conductivity of the milk and NOx concentrations in the animals that showed clinical signs of mastitis following infusion of *S. uberis* (Figure 8.5 B). No consistent relationship between the two was apparent in the cows that did not show any clinical signs of mastitis, or in milk from the non-infused quarters (Figure 8.5 A and C). Although intramammary infusion of IL-1β resulted in an increase in both the EC and NOx concentration of the milk when compared to PBS infused quarters (Figure 8.7), further examination of the data suggested that there is no relationship between the two (Figure 8.8).

A possible interpretation of the relationship between NOx and EC (a measurement of the integrity of the epithelium) in the milk of those cows showing signs of clinical mastitis following infusion of *S. uberis* (Figure 8.5 B) is an effect on epithelial integrity. Tight junctions, which form a narrow continuous seal that surround each epithelial cell at the apical border, function to regulate the paracellular movement of material (Nguyen & Neville, 1998) are integral to the maintenance of mammary gland integrity. While there are conflicting reports in the literature as to the effect of nitric oxide donors on the maintenance of tight junctions (Salzman *et al.*, 1995; Guo *et al.*, 1998; Menconi *et al.*, 1998; Zech *et al.*, 1998), there is the overwhelming support for the negative effect of cytokines and cytotoxins on the integrity of various epithelia. For example, when rat retinal pigment epithelial cells were treated with IFN-γ, TNF-α and LPS there was a decrease in the transepithelial resistance (Zech *et al.*, 1998). An increase in permeability was also apparent in IFN-γ, IL-1β and TNF-α treated Caco-2 monolayers (Chavez *et al.*, 1999) and in IFN-γ treated Caco-2BBe monolayers (Unno *et al.*, 1999). The intramammary infusion of *S. uberis* resulted in a positive relationship between NOx and EC in those cows showing signs of clinical mastitis (Figure 8.5 B) further demonstrating the negative effect of cytotoxins on the mammary gland integrity.

There does not appear to be a strong relationship between milk NOx and SCC, however, it may be that only a small sub-population of somatic cells produce NO. No clear trend for increasing SCC and increasing NOx is apparent following either the

infusion of *S. uberis* (Figure 8.6) or IL-1β (Figure 8.9). SCC were not measured above 9999 x 10^3 cells/ml, due to equipment limitations, and therefore a correlation between SCC and NOx was not possible especially given that it is also highly likely that some of the samples had SCC above this value. However, if measurement of SCC above 9999 x 10^3 cells/ml was carried out (for example by dilution of the milk sample), this would probably not improve the relationship, as, in those cases where SCC were measured at 9999 x 10^3 cells/ml, there is also a wide range in the NOx concentrations for those samples. This therefore suggests that may be not all somatic cells produce NOx, a concept supported by Goff *et al.*, (1996) and Boulanger *et al.* (2001) who reported that bovine neutrophils in culture do not produce NOx following stimulation with a variety of cytokines and cytotoxins. Nor was homologous IFN-γ able to induce iNOS expression and accumulation of nitrite in bovine macrophages (Adler *et al.*, 1995). In contrast, bovine monocytes produce NO in response to IFN-γ and LPS (Goff *et al.*, 1996; Zhao *et al.*, 1996; Boulanger *et al.*, 2001), although LPS alone was ineffective in stimulating NOx production (Boulanger *et al.*, 2001). However, there is conflict in the literature as Boulanger *et al.* (2001) reported that when bovine blood neutrophils were stimulated with LPS and recombinant bovine IFN-γ there were increases in iNOS mRNA. Further, differences are also apparent in the production of NOx and expression of iNOS by rodent and human neutrophils when stimulated with cytokines and LPS (Carreras *et al.*, 1994; Yan *et al.*, 1994; Yamashita *et al.*, 1997; Greenberg *et al.*, 1998; Fierro *et al.*, 1999; Tsukahara *et al.*, 2001). Further study is required to determine which somatic cells are responsive to activation by cytokines and cytotoxins, with careful consideration of potential differences between *in vitro* and *in vivo* stimulation made. Thus the lack of apparent relationship between NOx and SCC may be due to the activation of only a small population of cells within the milk. Further work would have to be done to elucidate the exact mechanism of action and the proportions of activity by each cell type.

Serum NOx is not considered to be a likely source of NOx measured in the milk. In human breast milk collected from healthy women on days four or five postpartum, no correlation was found between the concentration of NOx in milk and plasma (Iizuka *et al.*, 1997). In ten healthy cows, Hirvonen *et al.*, (1999) reported that the serum NOx concentrations ranged from 1-30 μM. These concentrations are within the same

range for both milk NOx (reported in this chapter) and by Bouchard *et al.* (1999). However, previous reports clearly show increases in milk NOx following infusion of LPS (Bouchard *et al.*, 1999) or *S. uberis* (Figure 8.3). Thus it could be argued that the increase in the milk is due to an influx from the plasma. However, the infusion of *E. coli* only slightly decreased the NOx concentration of the serum and was followed by an increase (to approximately 40 μM; compared with a prechallenge serum NOx range of 12-51 μM) in the most severely affected cows (Hirvonen *et al.*, 1999). Further, in unstimulated cows, NOx concentrations in the milk were four times higher than that in the serum (Blum *et al.*, 2000) and although both the infusion of LPS and *E. coli* significantly increased the NOx concentrations in the milk, there was no effect on plasma NOx. Thus, NOx from the blood is not considered to be a likely source of the NOx measured in the milk.

Thus the results from this chapter would suggest that the increase in NOx concentration in milk during mastitis might not be due to production solely from somatic cells, but involves other cells such as the mammary epithelium as well, which is consistent with Bouchard *et al.*, (1999). Given that epithelial cells in culture respond to LPS and IFN-γ (see Sections 4.5, 5.4 and Low *et al.*, 1997), with increases in NOx, and that somatic cells produce NOx upon stimulation (Lacasse *et al.*, 1997), it is likely that the somatic cells, being either an increase in number due to infection, or an increase in activation by the infection, and the mammary gland epithelium, due to damage, are both responsible for the milk NOx measured. However, without further investigation it still can not be determined if the loss of integrity of the mammary epithelium results from the increase in NO production, or whether the loss of integrity results in an increase in NOx production. Further, as to why there is a relationship between milk EC and NOx concentrations following intramammary infusion of *S. uberis* (Figure 8.5) but not IL-1β (Figure 8.8) is not known and also requires further investigation. The NOx concentrations in the milk from cows from the *S. uberis* trial peaked at an average of 17.8 ± 1.1 μM, 40 hours following infusion, the average concentration of NOx in the milk of the IL-1β infused cows on the 2nd day post infusion was 9.1 ± 1.4 μM, more like the NOx concentrations of the milk from the sub-clinical and non-responder groups of the *S. uberis* trial which also did not show any relationship between milk NOx and EC. Thus it appears that the

infusion of *S. uberis* has a greater affect on the milk NOx concentrations than does the infusion of IL-1β. Further work is required to address why there was only a relationship between milk NOx and EC in those cows that showed clinical signs of mastitis following infusion of *S. uberis*, and not in the milk from cows infused with IL-1β.

CHAPTER NINE

GENERAL DISCUSSION

The many different experiments described in the previous chapters were performed with the aim of investigating the control of nitric oxide (NO) production within the mammary gland. Different techniques have been used as well as different systems including the use of the murine mammary epithelial cell line, Comma-D and the use of explants taken from both pregnant and lactating rats. The source of nitrite and nitrate (NOx) in milk from mastitic cows was also investigated. Each of the experiments focuses on different mechanisms of control within the gland. This discussion shows that although presented as distinct experiments, the results are interrelated and can all be used to draw a clearer picture of what controls the production and use of NO within the mammary gland.

7.1 Detection of Nitric Oxide Synthase in the Mammary Gland

The aims of this Thesis were to examine the production and control of NO within the mammary gland. Thus, the first experiments focused on the detection of the NOS and the production of NO.

7.1.1 *Method Assessment*

Several systems were assessed for their ability to measure nitric oxide synthase (NOS) activity or NO production. Western analysis using specific antibodies did not detect iNOS immunoreactive protein in tissues collected from lactating rats, although a small amount of iNOS was detected in the liver and lung collected from a lactating rat 15 hours following intramammary infusion of LPS (Section 3.5.1). Antibodies against eNOS also failed to detect eNOS in tissues from lactating rats, although a

small amount of immunoreactivity was detected in the particulate fractions of the mammary gland and lung (Figure 3.6). All antibodies detected several small molecular weight proteins whose identity was not determined. Antibodies against the various NOS isoforms have been used to detect NOS from many different cell and tissue types (Schmidt *et al.*, 1992a; Huang *et al.*, 1995; Dong *et al.*, 1996; Bandyopadhyay *et al.*, 1997), including the mammary gland (Onoda & Inano, 1998). Why the antibodies used in the present study were unable to detect NOS in mammary tissue and why many different small molecular weight proteins were detected by the antibodies is unknown. As the method was not reliable, a different detection method was sought.

NOS activity utilising conversion of ^{3}H-arginine to citrulline was also examined but this too failed to detect any significant activity within the mammary gland (Section 3.5.2). Further extraction of the enzyme from the tissue failed to increase the activity to any appreciable level. The assay method itself was successful, as there was considerable NOS activity measured in samples of murine brain. The assay had previously been used in several tissue types (Bredt & Snyder, 1989; Pollock *et al.*, 1991; Ribiere *et al.*, 1996), but not in mammary tissue.

The third method investigated detection of nitrite and nitrate (NOx), the stable metabolites of NO, in the medium of cultured cells and explants (Section 3.5.3). An assay method was developed that proved to be particularly sensitive and robust in detecting the NOx released into the medium (Section 2.4). The drawback to this procedure is that it is possible that NOx may have been derived from sources other than NOS, additionally the method does not determine the NOS isoform responsible for the production of NOx. More importantly, as this study was being conducted, it was suggested that xanthine oxidoreductase (XOR) could be producing NO from NOx (Godber *et al.*, 2000; Stevens *et al.*, 2000). Therefore it was necessary to conduct experiments with inhibitors of NOS to determine how much of the NOx measured in the medium was derived from this source. The use of specific inhibitors of the different isoforms of NOS would give some indication as to which isoform was responsible for the NOx measured in the medium.

7.1.2 *Effect of Inhibitors of Nitric Oxide Synthase on Nitric Oxide Production*

The two inhibitors of NOS used in these experiments were aminoguanidine (AG), which is specific for iNOS (Corbett & McDaniel, 1996), and N^{ω}-nitro-L-arginine (L-NNA) which is a general NOS inhibitor, but has greater affinity for the constitutive NO synthases (eNOS or nNOS; Griffith & Kilbourn, 1996; Moore & Handy, 1997). Both inhibitors decreased the NOx in the medium of interferon-γ (IFN-γ) stimulated Comma-D cells (Figure 4.8), and mammary explants from both pregnant (Figure 5.11) and lactating rats (Figure 5.12). However, the extent of the inhibition varied.

The production of NOx by the Comma-D cells and explants of mammary tissue was not completely inhibited by AG, which suggests that NOx produced by unstimulated cells and explants could be due to eNOS. Thus NOx production by mIFN-γ stimulated Comma-D cells (Figure 4.8 A and C), rIFN-γ stimulated explants from lactating rats (Figure 5.12 C) and LPS stimulated explants from pregnant rats (Figure 5.11 A) was not reduced to basal concentrations even by 100 μM AG. As AG is a specific inhibitor of iNOS, this suggests the presence of another isoform of NOS. Indeed, the presence of eNOS in the mammary gland of rats and cows has been detected using immunohistochemistry (Lacasse *et al.*, 1996; Iizuka *et al.*, 1998; Onoda & Inano, 1998). Further, although mM quantities of AG are required for the inhibition of eNOS from bovine pulmonary arterial endothelial cells, the concentration of AG required for half-maximal inhibition (*Ki*) of IFN-γ stimulated iNOS from murine macrophages was 16 μM (Wolff & Lubeskie, 1995). AG inhibition of interleukin-1 (IL-1) induced NOx production by RINm5F cells (rat insulinoma cell line) had a *Ki* of 10 μM. These are much lower than the 100 μM used in the explant and Comma-D culture experiments and therefore suggest that any iNOS present would have been fully inhibited and thereby providing further support for the theory of an alternative NOS isoform. Future experiments could investigate this further by the use of two inhibitors in conjunction with each other, for example AG and L-NNA. However, whether or not the assay could detect differences in NO production when the cells or explants are inhibited with one versus both inhibitors is not known and would also need to be examined.

The experiments presented in this Thesis have cast doubt on the action of L-NNA as an inhibitor of NOS in cells and explants not stimulated with ctyokines and cytotoxins. A consistent finding throughout the experiments was the significant increase in NOx in the medium of Comma-D cells (Figure 4.7) cultured with L-NNA in the absence of stimulation by cytokines or cytotoxins. This was also apparent when the cells were cultured in the presence of PRL and co-treated with L-NNA (Figure 6.4). Up-regulation of iNOS by L-NNA and the action of arginase were considered as potential mechanisms for the increased NOx in the medium of cells following incubation with L-NNA.

L-NNA is an amino acid substrate analogue of arginine (Knowles & Moncada, 1994; Griffith & Kilbourn, 1996), however, unlike N^G-monomethyl-L-arginine, it is not metabolised to citrulline and subsequently to arginine (Hecker *et al.*, 1990), ruling out the possibility that metabolism of L-NNA is responsible for the increased NOx in the medium of the cells. The methyl ester of L-NNA, N^G-nitro-L-arginine methyl ester (L-NAME) which failed to inhibit NOS in the blood vessels and intestines of rats, also resulted in an increase in nitrite concentrations (Miller *et al.*, 1996). Further, the administration of L-NAME also resulted in an induction of iNOS gene expression (Miller *et al.*, 1996). Thus, further work is needed to determine if the increases in NOx in the medium of the Comma-D cells treated with L-NNA were the result of up-regulation of iNOS. This could be addressed with the use of Western or Northern analysis of the cells following incubation with L-NNA.

Arginase may be a potential factor in the increased NOx in the medium of L-NNA treated cells (Figure 4.7 and Figure 6.4). As discussed in Section 4.10.4 arginase is responsible for the conversion of arginine to ornithine (Figure 9.1) and it is suggested that the inhibition of arginase by L-NNA might result in more arginine being available for NOS to produce NO. However, although L-NNA inhibits rat liver arginase (AI; (Robertson *et al.*, 1993) it is not known whether it inhibits non-hepatic arginase (AII) which is the predominant form in the mammary gland (Jenkinson & Grigor, 1994). Further experiments should be done to address this.

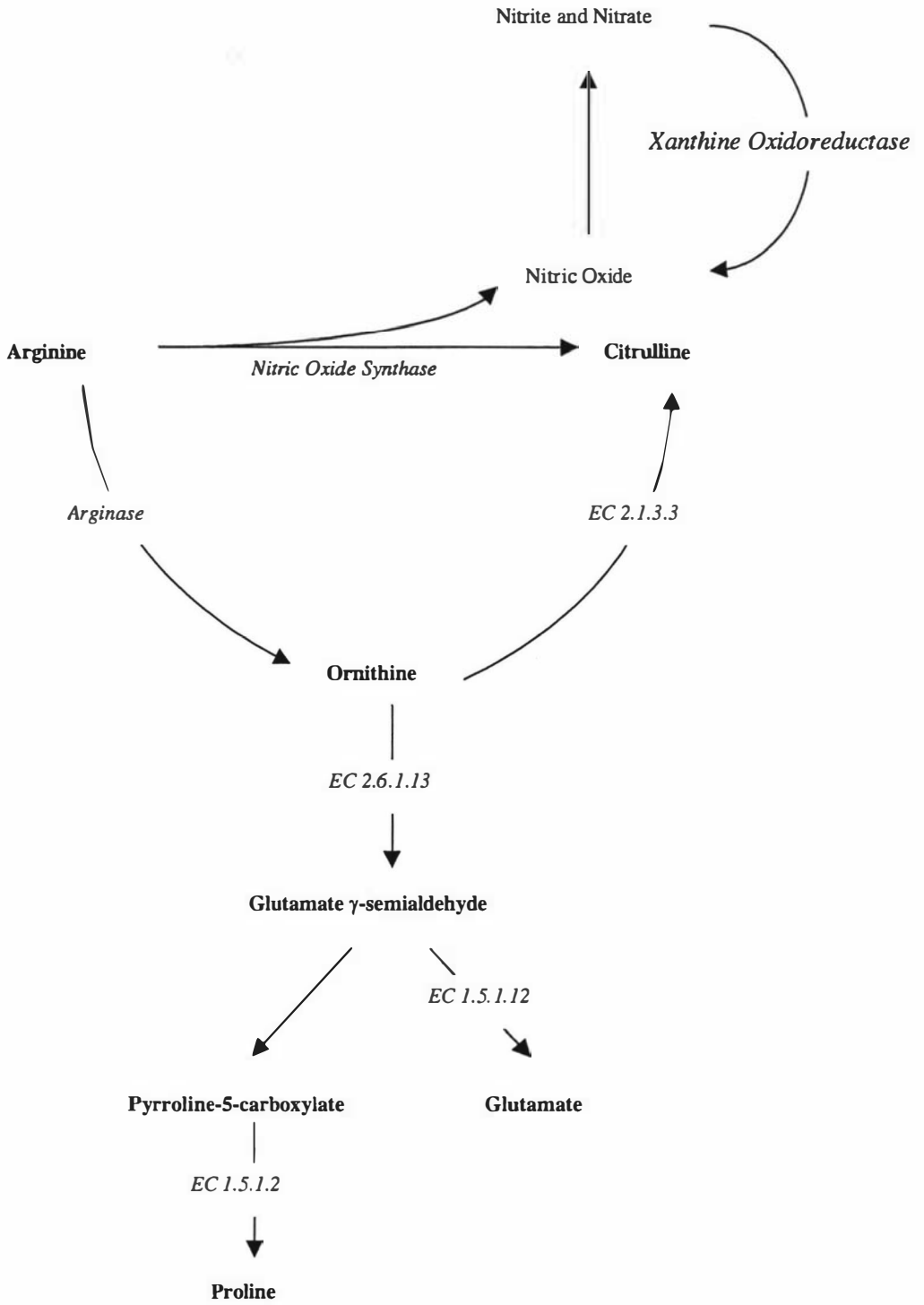

Figure 9.1: Metabolism of arginine. Possible pathways for the metabolism of arginine and utilisation of nitric oxide within the mammary gland. The enzymes responsible for each of the conversion steps are given in italics. EC 2.6.1.13, ornithine-δ-aminotransferase; EC 1.5.1.2, Pyrroline-5-carboxylate reductase; EC 1.5.1.12, 1-Pyrroline-5-carboxylate dehydrogenase; EC 2.1.3.3, Ornithine carbamyltransferase.

Whether XOR plays a role in the increased NOx in the medium of cells treated with L-NNA was considered. However, it is unlikely as the inhibition of XOR using allopurinol had no effect on either the mIFN-γ stimulated NOx from Comma-D cells (Figure 7.3), or on inhibition of mIFN-γ stimulated NOx production using AG and L-NNA (Figure 7.4).

The mechanism responsible for L-NNA increasing the NOx production in unstimulated cells may also be responsible for the failure of L-NNA to fully inhibit the production of NOx from stimulated cells and explants, to a level that the production was not different from the non-treated cells and explants. For example, 1 mM L-NNA did not fully inhibit the production of NOx from Comma-D cells treated with 25 U/ml mIFN-γ (Figure 4.8 B), nor when explants from pregnant rats were treated with 250 U/ml rIFN-γ (Figure 5.11 D). In contrast, 1 mM L-NNA inhibited NOx production from Comma-D cells treated with 250 U/ml mIFN-γ (Figure 4.8 D) and explants of mammary tissue from lactating rats, treated with 250 U/ml rIFN-γ (Figure 5.12 D). However, the production of NOx by IFN-γ and L-NNA co-treated explants from lactating rats was very small and not significantly different from that produced by those explants treated with IFN-γ alone. This further highlights the low stimulatory effect of IFN-γ on mammary tissue. Full inhibition of NOx production was also apparent when explants from pregnant and lactating rats were co-treated with 10 μg/ml LPS and 1 mM L-NNA (Figure 5.11 B and Figure 5.12 B). It is not known why there is a discrepancy as to whether L-NNA inhibits the production of NOx from IFN-γ treated cells and explants to a level not different from the non-treated cells and explants. As discussed in 4.10.4 this may be related to the increased NOx production apparent when unstimulated Comma-D cells were treated with L-NNA (Figure 4.7). Whether this also occurs in explants is not known as unstimulated explants were not treated with inhibitors in this Thesis. Together, these results highlight some important issues, in that the use of L-NNA as an inhibitor of low levels of NO production must now be questioned. The results also provide a future research direction in determining how L-NNA causes the increase in NOx production.

7.2 Regulation of Nitric Oxide Production in the Mammary Gland

Following the development of the NOx assay, which allowed accurate, although indirect, measurement of NO production, the experiments went on to examine the control of NO production within the mammary gland. The regulation of both iNOS and eNOS were examined.

7.2.1 *Regulation of Nitric Oxide Production by Cytokines and Cytotoxins*

The regulation of the cytokine and cytotoxin induction of the inducible isoform of NOS was examined in the mammary gland. These studies suggest that IFN-γ induces NO production by acting directly on epithelial cells whereas LPS acts indirectly through other cell populations (Figure 9.2). For example, although there were vast differences between the responses of explants and Comma-D cells, both responded to treatment with IFN-γ with increased NOx released into the media (Chapters 4 and 5). NOx production increased approximately 370 % in IFN-γ stimulated, compared to non-treated explants, whereas production of NOx by IFN-γ stimulated Comma-D cells was approximately 6500 % of the non-treated production. This is in direct contrast to the response to LPS, where it was the explants that responded with a greater increase (1500 %) compared with the Comma-D cells (200 %). Thus, it appears that that LPS is not acting directly on mammary epithelial cells, but the response to LPS apparent in the explants is due to the other cell populations within the explants. This is supported by the findings of Buhner *et al.* (2000) who reported that LPS had no effect on the barrier function of the colorectal cell line HT-29/B6 but stimulated electrogenic secretion in tissue samples of the human distal colon. Buhner *et al.* (2000) suggested that LPS did not act directly on the enterocytes but other cells within the tissue moderated the response. Furthermore the bovine mammary epithelial cell line MAC-T secrete interleukin in response to LPS stimulation (Boudjellab *et al.*, 1998; Boudjellab *et al.*, 2000) and the epithelial cell line isolated from the bovine mammary gland (FbE cells), release NO after exposure to interleukin-1β (Boulanger *et al.*, 2001). Thus it is possible that the increased NOx apparent in the medium of mammary explants following treatment with LPS is due to

the stimulated release of cytokines and their subsequent action on the epithelial cells. Given that there was a 200 % increase in the NOx of the Comma-D cells treated with LPS, this suggests that there is also another pathway of LPS stimulation which results in the release of low levels of NOx from the epithelial cells themselves. The exact mechanism by which this occurs is currently unknown. It is also not known why the direct stimulation of the mammary epithelial cells (Comma-D cells) with IFN-γ results in such a large increase in NOx production whereas the indirect stimulation (explants) does not result in such a substantial increase. This is unlikely to be due to the different sources of IFN-γ used (explants were stimulated with rat recombinant IFN-γ and Comma-D cells were stimulated with mouse recombinant IFN-γ) as no difference in the amount of NOx produced was apparent in Comma-D cells stimulated with the two sources (Figure 4.4). Further work could determine if LPS stimulation of Comma-D cells and explants results in the release of cytokines. If these are released they could be examined to determine their effects on the production of NOx.

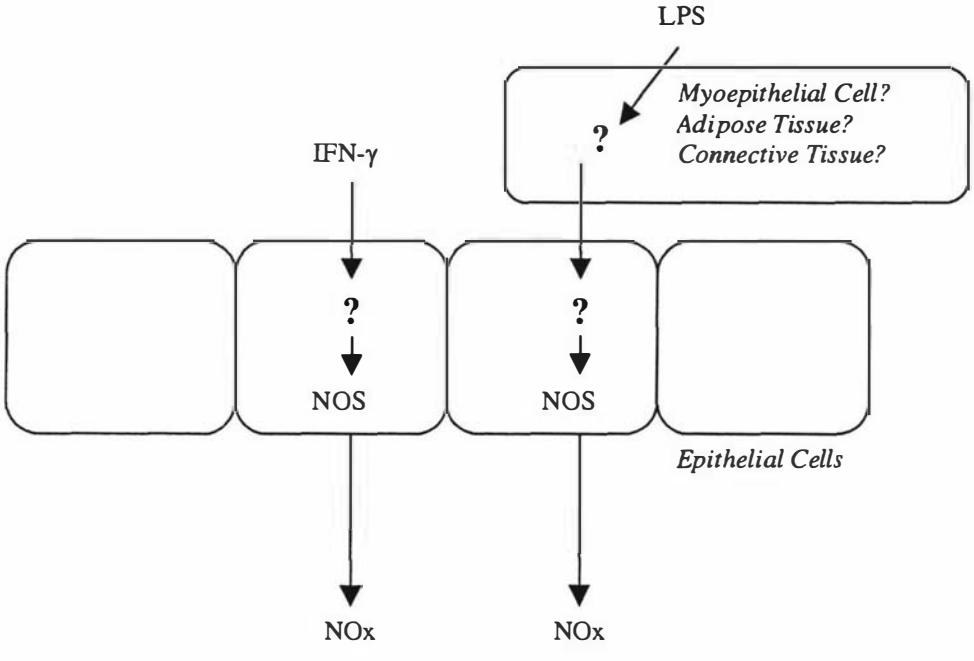

Figure 9.2: Regulation of mammary NOS by cytokines and cytotoxins. IFN-γ appears to have a direct effect on mammary epithelial cells whereas the action of LPS may occur indirectly through stimulation of other cell populations within the mammary gland.

7.2.2 *Regulation of Nitric Oxide Production by Hormones - Pregnancy versus Lactation*

Differences in production of NOx by mammary explants from pregnant versus lactating rats were also apparent, with explants from pregnant rats producing significantly more NOx in the basal state than explants from rats in mid-lactation. In turn, the production from the explants collected from rats in mid-lactation is significantly greater than that of those explants collected in late-lactation (Figure 5.5). However, no differences were apparent in the responses of the explants from pregnant and lactating rats to rIFN-γ (Figure 5.10) or to LPS (Figure 5.8). Several theories were postulated for these differences.

Differences in mRNA

The results presented in Chapter 5 indicate that the concentrations of iNOS mRNA in mammary tissue collected from pregnant and lactating rats were similar (Figure 5.2 and Figure 5.3), however, the eNOS mRNA expressed in the lactating rat differs from that of the pregnant rat (Figure 5.4). The tissues from lactating rats appeared to express lesser amounts of the expected 4 kb band and greater amounts of a 2 kb band. This second band for eNOS has not previously been reported in mammary tissue. However, alternative transcript splicing of the eNOS gene has been reported in the suprachiasmatic nuclei (Caillol *et al.*, 2000) and may also occur within the mammary gland. The apparent decreased amounts of the 4 kb eNOS band could be the reason for the lower NOx production present in explants from lactating rats. Reproductive state of the rats from which the mammary tissue is collected does not appear to influence the regulation of iNOS, as Northern analysis showed similar amounts of iNOS mRNA in the tissues from pregnant and lactating rats. Furthermore, the amounts of NOx produced following stimulation with either LPS or rIFN-γ (Figure 5.8 and 5.10) were similar. Future experiments should include Northern analysis on mRNA collected from mammary explants following various treatments, and comparisons made with the amount of NOx in the medium.

Effect of Prolactin

The studies presented in this Thesis indicate that PRL plays a role in the stimulation of NO production in the mammary gland. Further, the results presented suggest that the increased production of NO is due to full length PRL, and not the 16K PRL fragment. Comma-D cells (Figure 6.1) and explants of mammary tissue from pregnant (Figure 6.6) and lactating rats (Figure 6.11) all respond to treatment with 5 μg/ml PRL with increased release of NOx into the medium. Explants from pregnant rats cultured with PRL produced 5-fold more NOx than those explants cultured without PRL. The production by explants from lactating rats treated with PRL was 2.2-fold higher than that from the untreated explants. The claim that PRL is having a stimulatory effect on NOx production is supported by the work of Bolander (2001) and Raso *et al.* (1999) who reported that PRL transiently increases NOx production in primary cultures of mouse mammary epithelial cells, and stimulates nitrite production and iNOS expression in C6 cells (rat glioma cell line). The results presented in this Thesis are in contrast to those of Corbacho *et al.* (2000) who reported that a 16 kDa N-terminal proteolytic fragment of PRL (16 K PRL) stimulated NOx production in fibroblasts and type II alveolar cells.

However, little evidence for the proteolytic cleavage of PRL by the mammary gland was found from the studies presented in this Thesis. Western analysis of conditioned medium from both Comma-D cells (Figure 6.16) and explants of mammary tissue (Figure 6.17) did not reveal a 16K PRL fragment. The mammary gland is capable of cleaving PRL into various sized proteolytic fragments, including 16K PRL (Clapp, 1987; Baldocchi *et al.*, 1992; Baldocchi *et al.*, 1994), however, the work presented here suggests that this did not occur. These conclusions could be further supported by Western analysis using purified 16K PRL as a positive control thereby confirming that the antibody used is capable of recognition of the 16 K PRL fragment.

Effect of Arginase

The studies presented in this Thesis do not support a hypothesis that NOx production is inversely related to arginase activity. Mammary arginase activity increases by 300% at mid-lactation (Jenkinson & Grigor, 1994). Thus it was suggested that the increased arginase activity may result in decreased NOx in the medium of the

explants, due to competition for arginine by NOS and arginase (Figure 9.1). The results presented in Figure 5.5 clearly support this, with the concentrations of NOx produced by explants collected from rats in mid-lactation significantly lower than that produced by explants from pregnant rats. However, the activity of arginase in the rat mammary gland during late lactation is considerably higher than that detected during pregnancy, and lower than that reported for mid-lactation (Yip & Knox, 1972). If the activity of arginase was decreasing the production of NOx, one would expect the stages with the highest arginase activity to have the lowest NOx concentrations and vice versa. This was not the case.

Competition between NOS and arginase does not appear to occur following stimulation of NOS either. No difference in the amount of NOx produced was apparent when explants from either pregnant or lactating rats were stimulated with LPS (Figure 5.8). Competition between NOS and arginase for arginine has been reported in LPS activated murine macrophages (J774A.1 cells; Chang *et al.*, 1998) and in murine macrophages (RAW 264.7 cells) where treatment with IFN-γ increased iNOS but nearly abolished the AII induction (Wang *et al.*, 1995; Gotoh *et al.*, 1996). In direct contrast, both iNOS and non-hepatic arginase (AII) increase in murine macrophages (RAW 264.7 cells) following activation with LPS. However, the results of Chang *et al.* (1998) suggest that the competition between NOS and arginase is more pronounced when arginine is limiting. Treatment of the cells with an arginase inhibitor increased the production of NOx from RAW 264.7 cells, although when the arginine concentration exceeded 0.5 mM, the effect of the arginase inhibitor was abolished. The concentration of L-arginine in the medium (DMEM:F12) used to culture the cells and explants in the experiments reported in this Thesis was 0.7 mM. Therefore the concentrations of arginine within the cultures should well exceed the concentration required to abolish the arginase effect. Together, these results suggest that even though arginase activity increases during lactation, arginase is unlikely to be responsible for the differences in basal production by the explants.

Effect of Xanthine Oxidase

Whether XO activity affects the production or measurement of NO in mammary explants was not determined. Xanthine oxidase (XO) has been reported to convert nitrite and nitrate to NO (Millar *et al.*, 1998; Zhang *et al.*, 1998; Godber *et al.*, 2000). In mammary tissue of mice the activity of XOR is greatest during mid-lactation (Ringo & Rocha, 1983; Hayden *et al.*, 1991; Kurosaki *et al.*, 1996; McManaman *et al.*, 1999). However, experiments using Comma-D cells suggest little influence of XO on the production of NO within the mammary gland (Figures 7.2, 7.3 and 7.4). It must be noted that the generation of NO from NOx by XOR generally occurs during hypoxic conditions, and in these experiments, both Comma-D cells and mammary explants are cultured in a humidified 5 % CO_2:95 % air atmosphere. Further experiments could be conducted to determine if unlike in Comma-D cells, XO plays a role in the production and measurement of NOx in mammary explants.

7.3 Origin of Nitric Oxide in Milk

The experiments investigating the presence of NOx in milk provided an opportunity to further investigate the origin, role and regulation of NO production in the intact mammary gland. There are several possible sources for NOx in milk, the mammary epithelium, the somatic cells within milk and that transferred into the milk from the blood were all considered (Figure 9.3). Many studies, including those with Comma-D cells (Chapter 4) and mammary explants (Chapter 5) show that the mammary epithelium responds to stimulators of NO production in a well-defined manner. However, the work presented in these chapters ignore the effect of the somatic cells within the milk. Unlike the production of NOx by the mammary epithelium, the role of somatic cells is less defined.

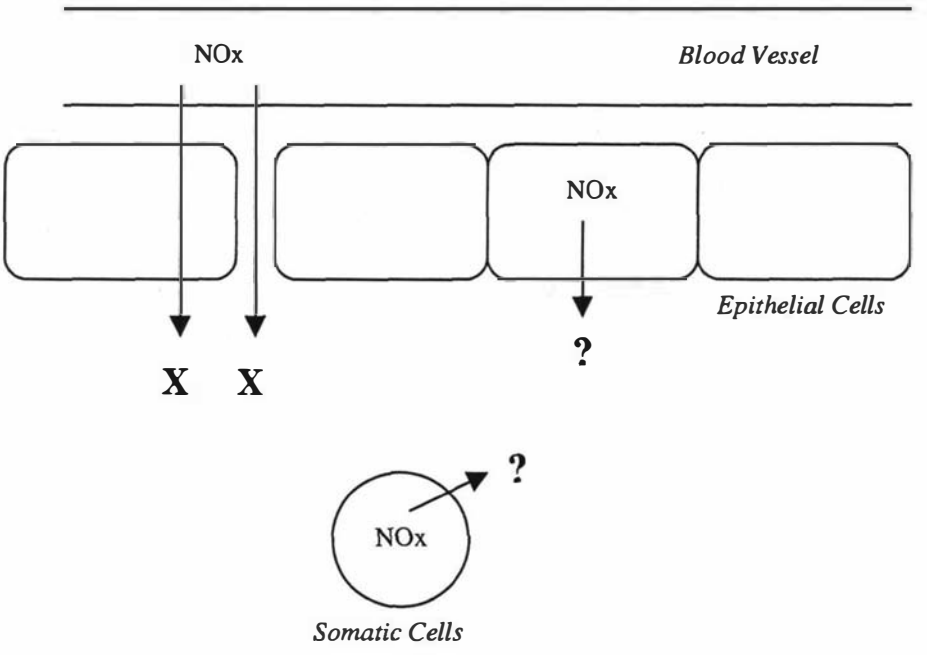

Figure 9.3: NOx in bovine milk. Three possible sources were considered. NOx transfer from the blood is not considered to be a likely source (X), whereas results presented suggest that the mammary epithelium and somatic cells (?) may play a role.

There was no association between NOx concentrations in the milk and the SCC of cows treated with an intramammary infusion of either IL-1β (Figure 8.9) or *S. uberis* (Figure 8.6). Although there are increases in the milk nitrite and NOx following intramammary infusion of *Streptococcus uberis*, (Lacasse *et al.*, 1997) or LPS (Bouchard *et al.*, 1999), this does not provide evidence that the cells in the milk are producing the NOx. Cultured somatic cells from the infected and non-infected quarters of cows with spontaneous mastitis release NOx into the medium. Treatment with LPS further enhances the production from cells from both the infected and non-infected quarters (Lacasse *et al.*, 1997). The healthy mammary gland produces milk that contains some somatic cells ($< 10^5$ cells/ml) of which most are macrophages and lymphocytes. A few epithelial cells and neutrophils make up the remainder (Harmon, 1994; Sordillo *et al.*, 1997). However, during infection there is a rapid increase in the SCC and an accompanied change in the composition of the population, to an extent that during mastitis, approximately 95 % of the somatic cells are neutrophils (Kehrli & Shuster, 1994). As bovine neturophils in culture do not appear to respond to

stimulation with cytokines and cytotoxins (Goff *et al.*, 1996; Boulanger *et al.*, 2001), it is possible that the production of NOx in the milk was from just a small population of cell types, thus explaining the lack of relationship between NOx concentration and somatic cell number (SCC).

The electrical conductivity of milk (EC) is often measured as a means of detecting mastitis. Increases in EC are associated with increases in the paracellular movement of sodium and chloride ions, and milk and blood proteins (Wheelock *et al.*, 1966). The measurement of EC also allows conclusions to be drawn about the integrity of the mammary epithelium. A relationship between NOx and EC was only apparent in the milk collected from the six cows that showed signs of clinical mastitis following intramammary infusion of *S. uberis* (Figure 8.5 B). There was no apparent relationship between NOx and EC in milk from control quarters or in quarters of cows that did not show signs of clinical mastitis (Figure 8.5 A and C). Nor was there any apparent relationship between NOx and EC in those cows that received intramammary infusions of IL-1β (Figure 8.8), even though most of the samples had SCC in excess of the limits of measurement showing that the glands were responding to the infusion (Figure 8.9 B and C). This raises the possibility that the mammary gland requires the presence of bacterial infection to elicit the production of NOx, a theory that is discussed further below. Further experiments would have to investigate this further to determine if the apparent lack of relationship was correct, or due to the lack of samples for analysis (each of the cows in the IL-1β trial was only analysed for EC at two milkings).

Although mammary epithelial cells respond to direct simulation with IFN-γ (Section 4.8.3; Low *et al.*, 1997), there was no apparent dose response when the cells were treated with LPS (Section 4.11.3). In direct contrast, explants of mammary tissue respond to LPS in a dose dependent manner (Onoda & Inano, 1998). Mammary explants also respond to treatment with IFN-γ, albeit at a much lower level (Chapter 5). These differences between the mammary explants and Comma-D may explain why there was only a small increase in NOx in the milk of those cows infused with IL-1β. As mammary explants respond to LPS with such a significant increase in NOx in the medium and Comma-D cells do not, it suggests that the response to LPS

within the mammary gland is either due to stimulation of cells other than epithelial cells, or due to the indirect stimulation of the epithelium via these other cells. Intramammary treatment of cows with LPS increases IL-1, IL-6 (Shuster *et al.*, 1993; Shuster & Kehrli, 1995) and TNF-α (Shuster & Kehrli, 1995; Blum *et al.*, 2000) concentrations in milk. Whether these are produced by the mammary epithelium or cells within the milk is not known, however, TNF-α has a direct effect on stimulating NOx production from Comma-D cells when in the presence of IFN-γ, whereas IL-1β had no effect even in the presence of other cytokines (Low *et al.*, 1997). This lack of effect of IL-1β on Comma-D cells may in part explain why there was only a small increase in the NOx concentrations in the milk of those cows infused with IL-1β and suggests that the NOx in the milk of those cows may not be due to production by the mammary epithelium. However, as there was also no apparent relationship between NOx and SCC, this raises the possibility of another source of NOx production, or the production by only a subpopulation of the somatic cell types. Thus, further experiments are needed to determine the source of NOx in milk. This would be quite complicated as it is highly likely that there is an interrelationship between the epithelium and the somatic cells, with cytokines being produced within the gland/milk also stimulating the production of NOx.

7.4 General Comments

One of the more intriguing findings of this Thesis was that the method of euthanasia of the rats prior to excising explants of mammary tissue for culture had an effect on the basal production of NOx. Although the method of euthanasia did not effect the interpretation of the experiments reported here, it may affect the interpretation in other studies. The results also raise the question as to how the different methods of euthanasia affect the activity of NOS, the production of NO and the accumulation of NOx.

Mammary explants from rats were used to determine the interactions of different cell types in response to cytokines and cytotoxins with the production of NOx. Both

methods of euthanasia used in this study had the potential of exposing the rats and thus their mammary glands to conditions, which usually would not occur *in vivo*. Thus, the results of these studies show that it is vital that the method of euthanasia used is consistent between experiments. The method used should also be indicated so that accurate comparisons can be made between studies.

The experiments presented in this Thesis show that both Comma-D cells and explants of mammary tissue provide a useful model with which to investigate the production of NO in the mammary gland. However, there were differences in response to LPS by the explants and Comma-D cells, with the Comma-D cells showing very little response to the stimulation. Differences were also apparent between the responses to IFN-γ. This shows that although they can provide valuable information, there are limitations with the use of isolated systems such as cell and explant culture. I addressed this by also examining NO production within the lactating bovine mammary gland, a situation in which several sources of NO were possible and interactions between many different cell types were likely to occur. It must also be noted that the work presented covers experiments conducted in three different species (mouse, rat and bovine). It is likely that some of the conclusions drawn, which were done based upon the data from a particular species (or model), may not be valid for the other species or models examined. However, due to the lack of information on NO production within the mammary gland it was necessary to do so. This highlights the need for further work in this area.

In conclusion, the experiments presented in this Thesis demonstrate the ability of the mammary gland to respond to a variety of cytokines and cytotoxins with the production of NO. Several investigations were made to determine the regulation of this production and show that this is a complex system that can be influenced by many different factors. However, it does appear that the production of NO from the mammary gland is used as a defence mechanism, whereby there is an up-regulation of NO production following challenge with cytokines or cytotoxins. This Thesis also raises some exciting new areas of research, which could further explain the role NO plays within the mammary gland.

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APPENDIX

The effect of intramammary infusion of *Streptococcus uberis* on milk conductivity, somatic cell count and NOx concentrations was investigated and the results are presented in Figures 8.1, 8.2 and 8.3. This appendix contains the raw data for the 10 cows with both the control right back (RB) quarter and infused left back (LB) quarters graphed independently. Graphs are grouped into those animals classified as sub-clinical (n=3; Figure A1), clinical (n=6; Figure A2) and non-responder (n=1; Figure A3).

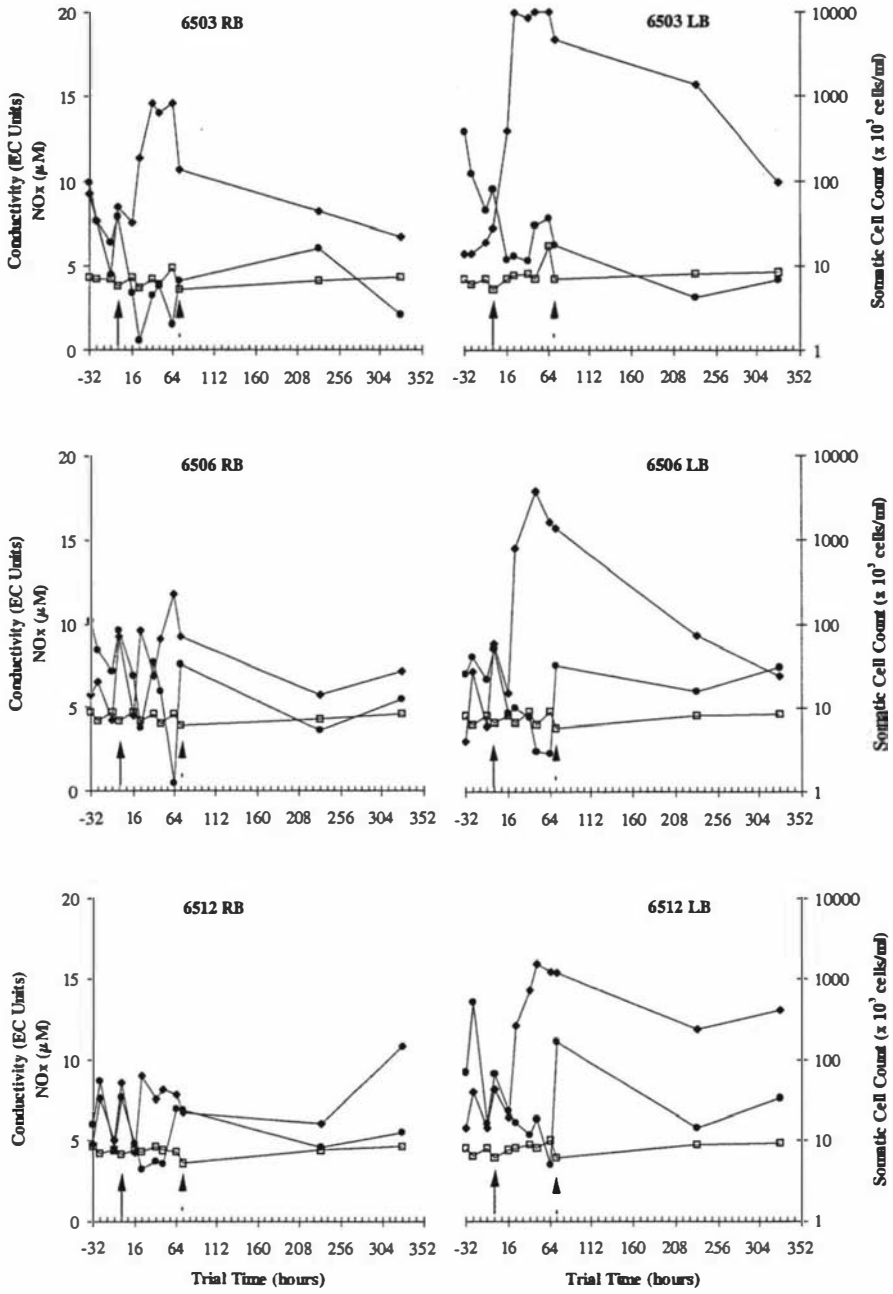

Figure A1: Cows classified as having sub-clinical mastitis following the intramammary infusion of *Streptococcus uberis*. Effect on milk conductivity, somatic cell count and NOx concentrations. The EC (–□–), SCC (–♦–) and NOx concentrations (–●–) of milk was measured in samples taken from both the right hind quarter (RB) and infused left hind quarter (LB). *S. uberis* (1000 cfu) infusion followed the afternoon milking on the 2nd day (solid arrow), treatment with cloxacillin occurred either when the animals showed clinical symptoms of mastitis, or 72 hours after infusion (striped arrow). Raw data are presented individually for each cow.

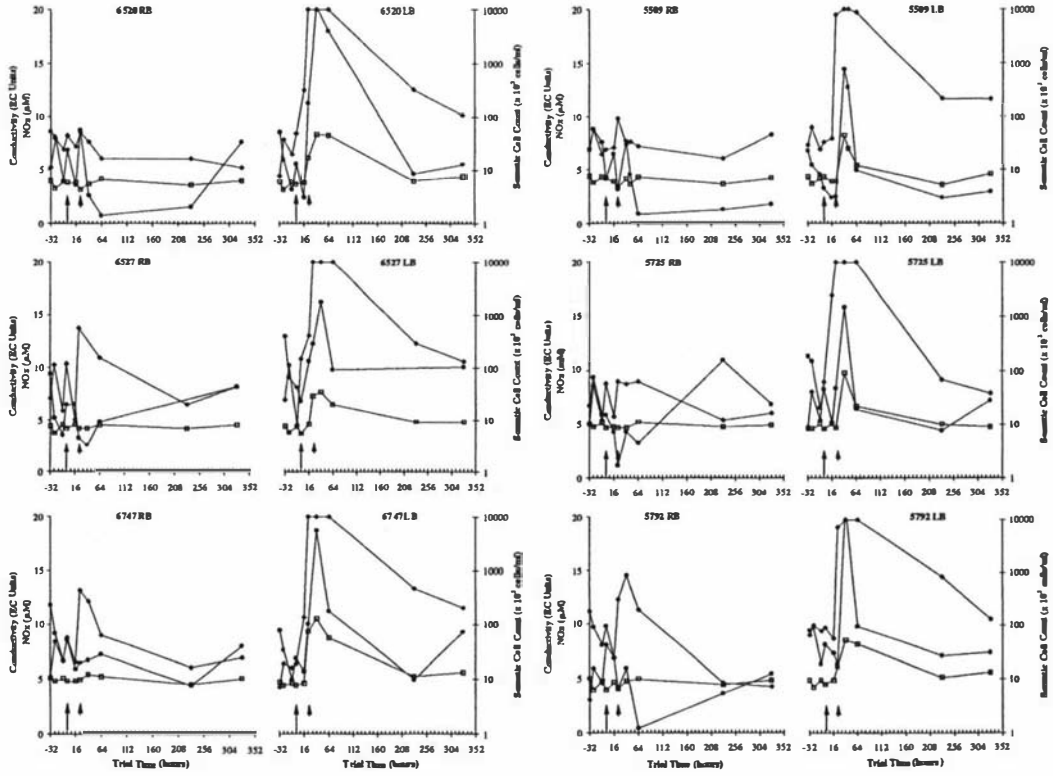

Figure A2: Cows classified as having clinical mastitis following the intramammary infusion of *Streptococcus uberis*. Effect on milk conductivity, somatic cell count and NOx concentrations. The EC (-⊟-), SCC (-✦-) and NOx concentrations (-●-) of milk was measured in samples taken from both the right hind quarter (RB) and infused left hind quarter (LB). *S. uberis* (1000 cfu) infusion followed the afternoon milking on the 2nd day (solid arrow), treatment with cloxacillin occurred either when the animals showed clinical symptoms of mastitis, or 72 hours after infusion (striped arrow). Raw data are presented individually for each cow.

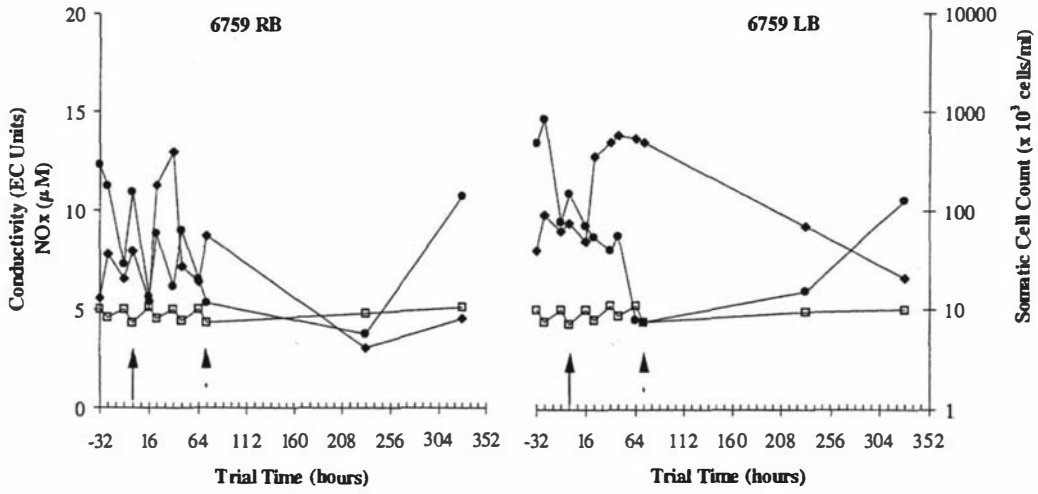

Figure A3: Cow classified as not responding following the intramammary infusion of *Streptococcus uberis*. Effect on milk conductivity, somatic cell count and NOx concentrations. The EC (-⊟-), SCC (-✦-) and NOx concentrations (-●-) of milk was measured in samples taken from both the right hind quarter (RB) and infused left hind quarter (LB). *S. uberis* (1000 cfu) infusion followed the afternoon milking on the 2nd day (solid arrow), treatment with cloxacillin occurred either when the animals showed clinical symptoms of mastitis, or 72 hours after infusion (striped arrow).