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# **Nitric Oxide Production in the Mammary Gland**



A Thesis

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At

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By

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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 (NO<sub>x</sub>), the spontaneously produced metabolites of NO, was the most valid and reliable. The measurement of NO<sub>x</sub> 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 NO<sub>x</sub> 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 NO<sub>x</sub> in a dose dependent manner in response to interferon- $\gamma$  (IFN- $\gamma$ ). 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 NO<sub>x</sub> than the untreated ones, however, no dose response was apparent. The specific iNOS inhibitor, aminoguanidine (AG) and general NOS inhibitor N<sup>o</sup>-nitro-L-arginine (L-NNA) were both used to confirm that the NO<sub>x</sub> measured in the medium was produced by NOS. The production of NO<sub>x</sub> 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 NO<sub>x</sub> between the different developmental stages. The method of euthanasia of the rats also affected the amount of NO<sub>x</sub> produced. The inclusion of prolactin (PRL) also increased the production of NO<sub>x</sub> from both Comma-D cells and explants of mammary tissue. Xanthine oxidase (XO), an enzyme responsible for the conversion of NO<sub>x</sub> to NO under anaerobic conditions, does not interfere with the determination

of NO production using the NO<sub>x</sub> assay. The measurement of NO<sub>x</sub> was carried out in the milk of cows following the intramammary infusion of *Streptococcus uberis* or interleukin-1 $\beta$  (IL-1 $\beta$ ). By comparing the milk NO<sub>x</sub> concentration with the somatic cell count (SCC) and electrical conductivity (EC) of the milk, it was concluded that the source of the NO<sub>x</sub> 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( $\beta$ -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-ethanesulfonic acid      |

|                                     |  |
|-------------------------------------|--|
| HUVEC                               | human umbilical vein endothelial cells                             |
| IFN- $\gamma$                       | interferon- $\gamma$   |
| hIFN- $\gamma$                      | human recombinant interferon- $\gamma$                             |
| mIFN- $\gamma$                      | mouse recombinant interferon- $\gamma$                             |
| rIFN- $\gamma$                      | rat recombinant interferon- $\gamma$                               |
| IL-1 $\beta$                        | interleukin-1 $\beta$  |
| kb                                  | kilobases  |
| kDa                                 | kilodaltons  |
| LDL                                 | lower detection limit  |
| L-NAME                              | N <sup>G</sup> -nitro-L-arginine methyl ester                      |
| L-NMMA                              | N <sup>G</sup> -monomethyl-L-arginine                              |
| L-NNA                               | N <sup>o</sup> -nitro-L-arginine                                   |
| LPS                                 | lipopolysaccharide   |
| MBF                                 | mammary blood flow   |
| MOPS                                | 3-[N-morpholino]propane-sulphonic acid                             |
| n                                   | number of samples  |
| NADPH                               | $\beta$ -Nicotinamide adenine dinucleotide phosphate, reduced form |
| NF- $\kappa$ B                      | nuclear factor- $\kappa$ B   |
| 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                                     |
| NO <sub>x</sub>                     | nitrite plus nitrate   |
| OD                                  | optical density  |
| PAGE                                | polyacrylamide gel electrophoresis                                 |
| PBS                                 | phosphate buffered saline  |
| PBSE                                | phosphate buffered Saline & EDTA                                   |
| PGF <sub>2<math>\alpha</math></sub> | prostaglandin-F <sub>2<math>\alpha</math></sub>                    |
| PMSF                                | phenylmethylsulfonyl fluoride                                      |
| PRL                                 | prolactin  |
| PVP                                 | polyvinylpyrrolidone   |

|                |                                      |
|----------------|--------------------------------------|
| R <sup>2</sup> | 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- $\alpha$  | tumor necrosis factor- $\alpha$      |
| VSMC           | vascular smooth muscle cells         |
| x g            | times gravity                        |
| XD             | xanthine dehydrogenase               |
| XO             | xanthine oxidase                     |
| XOR            | xanthine oxidoreductase              |