The impact of post-exercise protein-leucine ingestion on subsequent performance and the systemic, metabolic and skeletal muscle molecular responses associated with recovery and regeneration

A Thesis

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TITLE: The impact of post-exercise protein-leucine ingestion on subsequent performance and the systemic, metabolic and skeletal muscle molecular responses associated with recovery and regeneration

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ABSTRACT

The objective was to determine the effect of post-exercise protein-leucine coingestion with carbohydrate and fat on subsequent endurance performance and investigate whole-body and skeletal-muscle responses hypothesised to guide adaptive-regeneration. ***Methods***. *Study-1A* Twelve trained-men ingested protein/leucine/carbohydrate/fat (20/7.5/89/22 $g \cdot h^{-1}$) or carbohydrate/fat (control, 119/22 $g \cdot h^{-1}$) supplements after intense cycling over six days. Glucose and leucine turnover, metabolomics, nitrogen balance and performance were examined. *Study-1B* Immune-function responses to supplementation were investigated via neutrophil O_2^- production, differential immune-cell count, hormones and cytokines. *Study-2A* Twelve trained-men ingested low-dose protein/leucine/carbohydrate/fat (23.3/5/180/30 g), high-dose (70/15/180/30 g) or carbohydrate/fat control (274/30 g) beverages following 100-min of intense cycling. *Vastus lateralis* biopsies were taken during recovery (30-min/4-h) to determine the effect of dose on myofibrillar protein synthesis (FSR), and mTOR-pathway activity inferred by western blot. *Study-2B* The transcriptome was interrogated to determine acute-phase biology differentially affected by protein-leucine dose. ***Results.*** Protein-leucine increased day-1 recovery leucine oxidation and synthesis, plasma and urinary branch-chain amino acids (BCAAs), products of their metabolism, and neutrophil-priming plasma metabolites versus control. Protein-leucine lowered serum creatine kinase 21-25% (±90% confidence limits 14%) and day 2-5 nitrogen balance was positive for both conditions, yet the impact on sprint power was trivial. Protein-leucine reduced day-1 neutrophil O_2- production (15-17 ±20 $mmol \cdot O_2^- \cdot cell^{-1}$) but on day-6 increased post-exercise production (33 ±20 $mmol \cdot O_2^- \cdot cell^{-1}$) having lowered pre-exercise cortisol (21% ±15%). The increase in FSR with high-dose (0.103%·h^{-1} ± 0.027%·h^{-1}) versus low-dose (0.092%·h^{-1} ± 0.017%·h^{-1}) was likely equivalent. High-dose increased serum insulin (1.44-fold ×/÷90% confidence limits 1.18), 30-min phosphorylation of mTOR (2.21-fold ×/÷1.59) and p70S6K (3.51-fold ×/÷1.93), and

240-min phosphorylation of rpS6 (4.85-fold ×/÷1.37) and 4E-BP1-α (1.99-fold ×/÷1.63) versus low-dose. Bioinformatics revealed a biphasic dose-responsive inflammatory transcriptome centred on interleukin (IL)-1β at 30-min (high-dose) and IL6 at 240-min (high-dose, low-dose) consistent with regulation of early-phase myeloid-cell associated muscle regeneration. ***Conclusions.*** Protein-leucine effects on performance during intense training may be inconsequential when in positive nitrogen balance, despite saturating BCAA metabolism, protein synthesis, and attenuating cell-membrane damage. 24 g of protein and 5 g leucine near saturated post-exercise myofibrillar FSR and simulated an early inflammatory promyogenic transcriptome common to skeletal muscle regeneration that was accentuated with 3-fold higher protein-leucine dose.

ACKNOWLEDGEMENTS

Firstly, to Dr David Rowlands for all his enthusiasm, skill, patience and endeavour. All of this has given me a greater appreciation of what it is to be a scientist and researcher in our field, and to critically analyse and interpret – and these are useful skills for life in general. To my co-supervisors for their valuable input! Dr Alan Walmsley, only briefly involved but with different perspectives and an approach which I found useful and rewarding, and Prof. Jeroen Douwes and Dr Suzanne Broadbent for their help with manuscripts and the thesis and assistance outside of those things. To the many excellent collaborators: in particular Dr Trent Stellingwerff and Dr Daniel Moore (who were at the time of data collection with Nestec, Ltd.) for their incredibly valuable input during the study design and data analysis and for manuscript reviews; the rest of the Nestle/Nestec team (Serge Rezzi, Stephen Bruce, Isabelle Breton, Anita Thorimbert, Philippe Guy, Lionel Bovetto, Alain Fracheboud, Robert Mansourian, and Frederic Raymond); and Prof. Stuart Phillips and his group at McMaster University for their kindness in putting me up and showing me their home turf. An enormous thank you to the many support staff and students who were involved in various important aspects of data collection and analysis; Jim Clarke, Dr James Faulkner, Dr Jasmine Thomson, Andy Hollings, Marjolein Ros, Fliss Jackson, Garry Radford, David Graham, Lara Jackson, Dan Wadsworth and Wendy O'Brien at Massey University; Dr Murray Leikis, Dr Kevin Bell and Dr Sarah Beable for medical support; Tracy Rerecich, Dr Leigh Breen and Dr Nick Burd at McMaster University. Also, to my comrades in the post-grad room; Dr Bill Sukala, Wendy, Beks Bramley and Marj in particular. And a big thanks to all the participants for their blood, sweat, tears, muscle and more!Lastly, to my long-suffering family – thank you for the opportunity to finish this, and the strength to last through it and out the other side.

STATEMENT OF CONTRIBUTION

CHAPTER 3: A protein-leucine supplement increases BCAA and nitrogen turnover but not performance.

Study conception was by Dr David Rowlands and Andre Nelson, and study design by Dr David Rowlands, Dr Trent Stellingwerff, Prof. Mark Tarnopolsky and Prof. Stuart Phillips. Ethics proposal was written by Dr David Rowlands. Subjects were recruited and the study co-ordinated by Andre Nelson. Lead-in and experimental-block controlled diets were designed and co-ordinated by Andre Nelson. Supplements were produced by Lionel Bovetto and Alain Fracheboud at the Nestle Research Center, Lausanne, Switzerland. The data was collected primarily by Andre Nelson with help from Dr David Rowlands and assistance by Jim Clarke, Lara Jackson, Marjolein Ros and Jasmine Thomson. Blood and expired breath-gas collection was by Andre Nelson and Dr David Rowlands. Urine and sweat samples were collected and prepared by Andre Nelson. Blood creatine kinase and glucose and urinary and sweat urea and creatinine were analysed by Andy Hollings with help from Andre Nelson using standard kits. Blood amino acids and stable isotopes for whole-body glucose and protein turnover were analysed at McMaster University, Toronto, Canada by Tracey Rerecich. Blood and urine samples for metabolomics were analysed at the Nestle Research Center, Lausanne, Switzerland by Serge Rezzi, Stephen J. Bruce, Isabelle Breton, Anita Thorimbert and Philippe A. Guy. Statistical analyses were performed by Dr David Rowlands and Andre Nelson. The manuscript was written and prepared by Andre Nelson with guidance from Dr David Rowlands and feedback from Prof. Stuart Phillips, Dr Trent Stellingwerff, Jim Clarke, Dr Suzanne Broadbent and Dr Daniel Moore.

CHAPTER 4: Post-exercise protein-leucine feeding affects neutrophil function via immunomodulatory plasma metabolites and attenuated cortisol during a 6-day block of intense cycling.

Immune and inflammatory study design was by Dr David Rowlands, Dr Suzanne Broadbent and Andre Nelson. Study conception, ethics approval, data collection, and statistical analyses were as detailed for Chapter 3. The neutrophil oxidative burst assay and differential immune-cell counts were conducted by Lara Jackson with assistance from Andre Nelson. Blood cortisol, testosterone and sex-hormone binding globulin were determined by Andy Hollings, and neutrophil elastase concentration by Andy Hollings and Andre Nelson, using standard kits. Immunoglobulin-A was determined by LabPlus, Auckland City Hospital, New Zealand. Interleukin-6 and 10 were analysed via Bioplex by Fliss Jackson at Massey University, Palmerston North, New Zealand. The manuscript was written by Andre Nelson with guidance from Dr David Rowlands and Dr Suzanne Broadbent and feedback from Dr Trent Stellingwerff and Jim Clarke.

CHAPTER 5: Acute phase fractional muscle protein synthetic and signalling responses to the ingestion of low and high saturating doses of a protein-leucine-carbohydrate supplement following high-intensity endurance exercise.

Study design was by Dr David Rowlands, Andre Nelson, Dr Trent Stellingwerff, Dr Dan Moore and Prof. Stuart Phillips. Ethics approval was written by Dr David Rowlands with assistance by Andre Nelson. Subjects were recruited and the study co-ordinated by Andre Nelson. Lead-in and experimental-period controlled diets were designed and co-ordinated by Andre Nelson. Supplements were produced by Garry Radford at Massey University, Palmerston North, New Zealand. Muscle biopsies were taken by Dr Murray Leikis, Dr Kevin

Bell and Dr Sarah Beable. The data were collected primarily by Andre Nelson with help from Dr David Rowlands and assistance by Jim Clarke, Dr James Faulkner, Daniel Wadsworth and Wendy O'Brien. Blood samples were collected by Andre Nelson and Dr David Rowlands. Blood glucose and lactate were determined using an automated analyser by Wendy O'Brien with assistance by Andre Nelson. Blood amino acid concentrations and blood and muscle stable isotopes for myofibrillar and mitochondrial protein fractional synthesis rates were analysed at McMaster University, Toronto, Canada by Tracey Rerecich and Dr Nick Burd. Muscle homogenates for were made by Andy Hollings and Andre Nelson. Western blots were performed by Andy Hollings with assistance from Andre Nelson at Massey University, Wellington, New Zealand; Chandra Kirana at Wakefield Hospital, Wellington, New Zealand; and Dr Leigh Breen at McMaster University, Toronto, Canada. Statistical analyses were performed by Andre Nelson with guidance by Dr David Rowlands. The manuscript was written and prepared by Dr David Rowlands and Andre Nelson with feedback from Dr Trent Stellingwerff, Dr Daniel Moore and Professor Stuart Phillips.

CHAPTER 6: The action of protein-leucine feeding and dose on the acute-phase skeletal muscle transcriptome after endurance exercise.

Study conception and data collection were as detailed for Chapter 5. Microarray methods by Frederic Raymond and microarray data analyses were by Robert Mansourian. Bioinformatics was by Dr David Rowlands (Ingenuity Pathway Analysis) and Andre Nelson (Database for Annotation, Visualization and Integrated Discovery). The manuscript was written and prepared by Dr David Rowlands and Andre Nelson.

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RESEARCH ETHICS

Ethics approval was obtained from the Central Regional Ethics Committee for the studies conducted. The potential risks, and management of the risk involved, are detailed below:

All participants were screened via a health history questionnaire for pre-existing conditions to ensure they were physically healthy and able to take part in the studies. Participants were limited to athletes in regular training, and individuals who were neither disabled nor elderly and at increased risk of discomfort during the exercise and performance portions of the research. Fatigue during the exercise and performance trials was to be expected, however, this was anticipated to be of a similar level to that experienced by participants during their own endurance training and competition. Maximal efforts were requested of the participants during both the maximal aerobic power (VO_2max) testing and performance trials and the associated discomfort is normal for the level of athlete recruited, and in fact adaptive and beneficial to health. There may have been some discomfort and a minor risk of infection with venipuncture and cannulation procedures and muscle biopsies. This discomfort was minimised in each instance by having subjects lying prone on a hospital bed, with blood and biopsy procedures performed by trained phlebotomists and medics with experience in the procedures. It was considered that the amount of blood and muscle tissue samples taken posed no risk of adverse health effects. Risk of infection were minimised by following sterile procedure guidelines.

Social and psychological risks were minimised by ensuring privacy and confidentiality of participants throughout data collection and data storage periods. Initially we obtained informed consent and communicated to participants their right to discontinue or withdraw

from the studies at any time. We ensured that there were adequate change and shower facilities and we minimised the number of observers in the laboratory at any one time while the participants were being examined and/or tests conducted. Following data collection, any identifying information has been stored securely in a locked filing cabinet in a locked office with access to only those principally involved in the studies. We aimed to reduce the economic risk to participants by reimbursing them for their travel and time where necessary.

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LIST OF GENE ABBREVIATIONS AND ACRONYMS

ABCA1, ATP-binding cassette sub-family A member 1

ABCE1, ATP-binding cassette sub-family E (OABP) member 1

ACADVL, acyl-CoA dehydrogenase very long chain

ACHE, acetylcholinesterase

ACP1, acid phosphatase 1 soluble

ACTB, actin beta

ACTC1, actin cardiac muscle 1

ADAMTS9, ADAM metallopeptidase with thrombospondin type 1 motif 9

ALOX5AP, arachidonate 5-lipoxygenase-activating protein

ANGPTL2, angiopoietin-like 2

ANKRD1, ankyrin repeat domain 1

ANTXR1, arachidonate 5-lipoxygenase-activating protein

ANXA1, annexin 1

ANXA2, anthrax toxin receptor 1

APP, amyloid beta (A4) precursor protein

AQP4, aquaporin 4

ATP1B1, ATPase Na+/K+ transporting beta 1 polypeptide

ATP2A1, ATPase fast twitch 1

ATP2A2, ATPase slow twitch 2

BARX2, BARX homeobox 2

BCL6, B-cell lymphoma 6

BDNF, brain-derived neurotrophic factor

BGN, biglycan

BHLHE40, basic helix-loop-helix family, member e40

C21orf33, chromosome 21 open reading frame 33

CCND1, cyclin D1

CD2, cluster of differentiation 2

CD14, cluster of differentiation 14

CD36, cluster of differentiation 36

CD44, cluster of differentiation 44

CD86, cluster of differentiation 86

CD93, cluster of differentiation 93

CD97, cluster of differentiation 97

CD163, cluster of differentiation 163

CDKN1A, cyclin-dependent kinase inhibitor 1A

CEBPA, CCAAT/enhancer binding protein (C/EBP) alpha

CFH, complement factor H

CHRNA1, cholinergic receptor nicotinic alpha 1

CHRND, cholinergic receptor nicotinic delta

CHRNG, cholinergic receptor nicotinic gamma

CIDEC, cell death-inducing DFFA-like effector c

CLIC4, chloride intracellular channel 4

COL1A2, collagen type I alpha 2

COL3A1, collagen type III alpha 1

COL4A1, collagen type IV alpha 1

COL5A1, collagen type V alpha 1

COL5A2, collagen type V alpha 2

COL6A1, collagen type VI alpha 1

COL6A2, collagen type VI alpha 2

COL6A3, collagen type VI alpha 3

COLQ, collagen-like tail subunit of asymmetric acetylcholinesterase

CREB1, cAMP responsive element binding protein 1

CSF1R, colony stimulating factor 1 receptor

CSRP3, cysteine and glycine-rich protein 3

CTGF, connective tissue growth factor

CTSG, cathepsin G

CXCL2, chemokine (C-X-C motif) ligand 2

CYR61, cysteine-rich angiogenic inducer 61

DAB2, disabled homolog 2

DACH1, dachshund homolog 1

DCN, decorin

DDB1, damage-specific DNA binding protein 1

DDIT3, DNA-damage-inducible transcript 3

DDIT4, DNA-damage-inducible transcript 4

DIO2, deiodinase iodothyronine type II

DMD, dystrophin

DNMT3L, DNA (cytosine-5-)-methyltransferase 3-like

DUSP1, dual specificity phosphatase 1

DUSP10, dual specificity phosphatise 10

DYRK1A, dual-specificity tyrosine-(Y)-phosphorylation regulated kinase 1A

EFEMP1, EGF containing fibulin-like extracellular matrix protein 1

EGR1, early growth response 1

EIF4G2, eukaryotic translation initiation factor 4 gamma 2

F2RL1, coagulation factor II receptor-like 1

FBXO32, F-box protein 32

FMOD, fibromodulin

FOS, FBJ murine osteosarcoma viral oncogene homolog

FSTL1, follistatin-like 1

GADD45A, growth arrest and DNA-damage-inducible alpha

GADD45B, growth arrest and DNA-damage-inducible beta

GADD45G, growth arrest and DNA-damage-inducible gamma

GEM, GTP binding protein overexpressed in skeletal muscle

GJA1, gap junction protein alpha 1

H19, imprinted maternally expressed transcript

H6PD, hexose-6-phosphate dehydrogenase

HBP1, high mobility group box transcription factor 1

HK2, hexokinase 2

HLA-DQA1, major histocompatibility complex, class II, DQ alpha 1

HMGA1, high mobility group AT-hook 1

ID2, inhibitor of DNA binding 2

IFIT3, interferon-induced protein with tetratricopeptide repeats 3

IGF1, insulin-like growth factor 1

IGFBP1, insulin-like growth factor binding protein 1

IGFBP3, insulin-like growth factor binding protein 3

IL10RB, interleukin 10 receptor beta

IL1B, interleukin 1-beta

IL6, interleukin-6

ING5, inhibitor of growth family member 5

IRAK1, interleukin-1 receptor-associated kinase 1

IRF1, interferon regulatory factor 1

ITGB1, integrin beta 1

JUN, jun proto-oncogene

KLF10, Kruppel-like factor 10

KLF2, Kruppel-like factor 2

KLF4, Kruppel-like factor 4

LCP1, lymphocyte cytosolic protein 1

LDLR, low density lipoprotein receptor

LUM, lumican

LYVE1, lymphatic vessel endothelial hyaluronan receptor 1

MAP2K2, mitogen-activated protein kinase kinase 2

MAP4, microtubule-associated protein 4

MB, myoglobin

MGP, matrix Gla protein

MLYCD, malonyl-CoA decarboxylase

MRAS, muscle RAS oncogene homolog

MRVI1, murine retrovirus integration site 1 homolog

MT2A, metallothionein 2A

MTPN, myotrophin; SMAD1, mothers against decapentaplegic homolog 1

MYBPH, myosin binding protein H

MYC, v-myc myelocytomatosis viral oncogene homolog

MYCN, v-myc myelocytomatosis viral related oncogene, neuroblastoma derived

MYH1, myosin heavy chain 1

MYH11, myosin heavy chain 11

MYL4, myosin light chain 4

MYOD1, myogenic differentiation

MYOG, myogenin

NAMPT, nicotinamide phosphoribosyltransferase

NDRG2, N-myc downstream regulator 2

NFIC, nuclear factor 1 C-type

NFKB1, nuclear factor of kappa light polypeptide gene enhancer in B-cells 1

NFKBIA, nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor alpha

NR4A3, nuclear receptor subfamily 4 group A member 3

OGT, O-linked N-acetylglucosamine transferase

PDE4D, phosphodiesterase 4D

PELI3, pellino 3

PHGDH, phosphoglycerate dehydrogenase

PHLDA1, pleckstrin homology-like domain family A member 1

PI1, glutathione S-transferase pi 1

PIM1, pim-1 oncogene

PKM2, pyruvate kinase muscle 2

PLA2G7, phospholipase A2 group VII

PLEC, plectin

PLEKHO1, pleckstrin homology domain containing, family O member 1

PLIN2, perilipin 2

PLTP, phospholipid transfer protein

PMP22, peripheral myelin protein 22

POSTN, periostin

PPARD, peroxisome proliferator-activated receptor delta

PPARG, peroxisome proliferator-activated receptor gamma

PPP1R15A, protein phosphatase 1 regulatory (inhibitor) subunit 15A

PRDX2, peroxiredoxin

PRLR, prolactin receptor; cytoplasm

PTGER4, prostaglandin-endoperoxide synthase 2

PTGS2, prostaglandin-endoperoxide synthase 2

PTP4A3, protein tyrosine phosphatase type IVA member 3

RASA1, RAS p21 protein activator

RELA, v-rel reticuloendotheliosis viral oncogene homolog A

RGS2, regulator of G-protein signaling 2

RORA, RAR-related orphan receptor A

RPLP1, ribosomal protein large P1

RRM2B, ribonucleotide reductase M2 B

RRS1, ribosome biogenesis regulator homolog

RTN4, reticulon 4

S100A6, S100 calcium binding protein A6

S100A8, S100 calcium binding protein A8

S100A9, S100 calcium binding protein A9

S100A10, S100 calcium binding protein A10

SCD, stearoyl-CoA desaturase

SCN4A, sodium channel voltage-gated type IV alpha subunit

SETD3, SET domain containing 3

SGCA, sarcoglycan alpha

SGK1, serum/glucocorticoid regulated kinase 1

SLC37A4, solute carrier family 37 (glucose-6-phosphate transporter), member 4

SMAD3, mothers against decapentaplegic homolog 3

SMAD4, mothers against decapentaplegic homolog 4

SMAD7, mothers against decapentaplegic homolog 7

SOCS3, suppressor of cytokine signaling 3

SP4, Sp4 transcription factor

SPARC, secreted protein acidic cysteine-rich

SPI1, spleen focus forming virus (SFFV) proviral integration oncogene

SPRR2A, small proline-rich protein 2A;

SREBF1, sterol regulatory element binding transcription factor 1

STAT3, signal transducer and activator of transcription 3

TAGLN, transgelin

TAP1, transporter associated with antigen processing 1

TFRC, transferrin receptor

TGFBI, transforming growth factor, beta-induced

TGFBR2, transforming growth factor beta receptor 2

THBD, thrombomodulin

THBS2, thrombospondin 2

THBS4, thrombospondin 4

THRB, thyroid hormone receptor, beta

TIMP2, tissue inhibitor of metalloproteinase 2

TNC, tenascin C

TNFAIP6, tumor necrosis factor alpha-induced protein 6

TNFRSF12A, tumor necrosis factor receptor superfamily member 12A

TNRC6A, trinucleotide repeat containing 6A

TOM1, target of myb1

TP63, tumor protein p63

TPM3, tropomyosin 3

TRDN, triadin

TRIM29, tripartite motif containing 29

TXN2, thioredoxin 2

TXNIP, thioredoxin interacting protein

TYROBP, TYRO protein tyrosine kinase binding protein

UBC, ubiquitin C

UCP2, uncoupling protein 2

UCP3, uncoupling protein 3

USF2, upstream transcription factor 2 c-fos interacting

VCAM1, vascular cell adhesion molecule 1

VCAN, versican

VEGFA, vascular endothelial growth factor alpha

VIM, vimentin; ACTG2, actin gamma 2

WASF2, WAS protein family, member 2

WISP2, WNT1 inducible signaling pathway protein 2

ZBTB16, zinc finger and BTB domain containing 16

ZFAND6, zinc finger AN1-type domain 6

ZFHX3, zinc finger homeobox 3