

## Chapter 2

# Leucine-Protein Supplemented Recovery and Exercise

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### Key Points

- Short-term (2–3 days) post-exercise protein and protein-leucine feeding relative to negligible-protein isocaloric controls substantially improved subsequent endurance performance in well-trained men under conditions of negative to neutral nitrogen balance (dietary protein insufficiency); however, effects on performance under neutral to positive balance conditions may be equivocal.
- Long-term (weeks-months) protein-carbohydrate supplementation following endurance training in deconditioned skeletal muscle improves whole-body maximal oxygen uptake (aerobic power), relative to isocaloric carbohydrate feeding.
- Protein and leucine food or supplements ingested following endurance exercise may support skeletal muscle regeneration and adaptive remodelling in trained muscle via substantially increased mTORC1 pathway activity and moderate to large (effect size) increases in skeletal muscle protein FSR, relative to non-protein controls.
- A metabolic-mitochondrial transcriptome was activated at 48 h by post-exercise protein feeding in trained human skeletal muscle. This, and evidence to show that chronic BCAA (mice) or whey protein (aged men) feeding plus exercise led to improved mitochondrial biogenesis and endurance performance supports a role for dietary BCAA and protein in the development of skeletal muscle respiratory capacity.
- New transcriptome data suggest post-exercise protein and protein-leucine supports an inflammatory, promyogenic molecular programme common to regenerative wound healing biology in trained skeletal muscle.

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## Abbreviations

ABCA5 6, 7	ATP-binding cassette sub-family A members 5–7
ACOX1	Acyl-CoA oxidase 1 palmitoyl
ACSL1	Acyl-CoA synthetase long-chain family member 1
ADAM2 12, 18, 21	A disintegrin and metalloprotease domain family
ADAMTS2 5, 8, 13	A disintegrin and metalloprotease with thrombospondin motifs family
ADFP	Adipose differentiation-related protein (perilipin-22)
ALOX5	Arachidonate 5-lipoxygenase
APOC1	Apolipoprotein C-I
ARSA and ARSB	Arylsulfatase A and B
bHLH	Basic helix-loop-helix
BTRC	Beta-transducin repeat containing (gene)
CACYBP	Calcyclin-binding protein
CASP1 3, 4, 10	Caspase 1 apoptosis-related cysteine peptidase 1, 3, 4, 10
CAV2	Caveolin 2
CD	Cluster of differentiation
CD36	Thrombospondin receptor
CD44	Cluster of differentiation factor 44
CDKN1A	Cyclin-dependent kinase inhibitor 1A (p21)
COX4I2 COX7B, COX7B2	Cytochrome c oxidase subunit IV isoform 2, VIIb, VIIb2
CPT2	Carnitine palmitoyltransferase 2
CROT	Carnitine O-octanoyltransferase
CTSC CTSH, CTSK, CTSL, CTSO, CTSZ	Cathepsin (CTS) family
CYP1B1 CYP27B1, CYP2U1, CYP46A1, CYP7A1	Cytochrome P450 (CYP) family
DGKZ	Diacylglycerol kinase zeta
DLAT	Dihydrolipoamide S-acetyltransferase
DUSP1	Dual specificity phosphatase 1
ENO1	Enolase 1
ECM	Extracellular matrix
E-box	Enhancer box
FABP1 and FABP5	Fatty acid-binding protein 1 and 5
FA-CoA	Fatty acyl coenzyme A
FBXL19	F-box and Leucine-Rich Repeat Protein 19
FBXO18	F-box protein helicase, 18
FBXO32	F-box protein 32
FBXW2	F-box and WD repeat domain containing 2
FSR	Fractional synthesis rate
GADD	Family of growth arrest- and DNA damage-inducible factors
GCK	Glucokinase (hexokinase 4)
HADH	Hydroxyacyl-CoA dehydrogenase
HK1,2	Hexokinase 1,2

HMGCS2	3-Hydroxy-3-methylglutaryl-CoA synthase 2 (mitochondrial)
HIF1 $\alpha$	Hypoxia-inducible factor alpha
IGF-1	Insulin-like growth factor 1
IGFBP3	IGF-binding protein 3
IL1 $\beta$	Interleukin 1 $\beta$
ISYNA1	Inositol-3-phosphate synthase 1
LASS4 and LASS5	LAG1 homolog ceramide synthase 4 and 5
LDHAL6A and LDHB	Lactate dehydrogenase A-like 6A and lactate dehydrogenase B
LOC15076	Hypothetical protein LOC150763
LPIN1	Lipin 1
LPL	Lipoprotein lipase
mTORC1	Mammalian target of rapamycin 1
MDH2	Malate dehydrogenase 2 NAD (mitochondrial)
MDM2	Mdm2 p53 E3 ubiquitin protein ligase homolog
ME3	Malic enzyme 3 NADP(+)-dependent mitochondrial
MMP9,13 19	Matrix metalloproteinase 9, 13, 19
mRNA	Messenger ribonucleic acid
MRPL19	Mitochondrial ribosomal protein L19
MyoD	Myogenic differentiation factor 1
NADH	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide
NDUFA4, NDUFA5, NDUFAB1, NDUF56	NADH dehydrogenase (ubiquinone) 1 family (NDUF) members
NPC1L1	Niemann-Pick disease type C1 gene-like 1
OSBP2 and OSBPL9	Oxysterol-binding protein 2 and protein-like 9
PGC-1 $\alpha$	Peroxisome proliferator-activated receptor gamma coactivator 1- $\alpha$
PPAR $\gamma$	Peroxisome proliferator-activated receptor $\gamma$
PDK4	Pyruvate dehydrogenase kinase isozyme 4
PECI	Peroxisomal D3,D2-enoyl-CoA isomerase
PFKFB3	6-Phosphofructo-2-kinase/fructose-2,6-biphosphatase 3
PKM2	Pyruvate kinase muscle isoform
PLA2G2A	Phospholipase A2 group IIA
PLIN	Perilipin
PLTP	Phospholipid transfer protein
PPM2C	Pyruvate dehydrogenase phosphatase catalytic subunit 1
RNF19	Ring finger protein 19A E3 ubiquitin protein ligase
RNF34	Ring finger protein 34 E3 ubiquitin protein ligase
RNF39	152 ring finger protein 39, 152
SCD	Stearoyl-CoA desaturase
SDHC-SDHD	Succinate dehydrogenase complex subunit C and D
SLCO2A1 and SLCO1B1	Solute carrier organic anion transporter family
SLC25A20	Solute carrier family 25 (carnitine/acylcarnitine translocase)
STARD4	StAR-related lipid transfer (START) domain containing 4
STAT1/3	Signal transducers and activators of transcription 1/3
SREBP1,2	Sterol regulatory element-binding protein 1,2
SULT1A1 and SULT1C1	Sulfotransferase family cytosolic 1A and 1C
SQSTM1	Sequestome 1
TGFB-1	Transforming growth factor beta 1
TGFBR2	Transforming growth factor beta receptor 2
TIMP1 2	Tissue inhibitor of metalloproteinases 1 and 2

TRIM5	Tripartite motif containing 5
UBE2C	Ubiquitin-conjugating enzyme E2C
UBE2D3	Ubiquitin-conjugating enzyme E2D 3
UBE2J2	Ubiquitin-conjugating enzyme E2 J2
UCHL1	Ubiquitin carboxyl terminal esterase L1
UGT2B28	Uridine diphosphate glucuronosyltransferase 2 family polypeptide B28
UQCRC2 and UQCRH	Ubiquinol-cytochrome c reductase core protein 2
UQCRH	Ubiquinol-cytochrome c reductase hinge protein
USP2 49, 50	Ubiquitin-specific protease 2, 49, 50
VO <sub>2</sub> max	Maximum volume of oxygen
YME1L1	Yeast mitochondrial escape 1 like 1
YY1	Yin-yang 1

## Introduction

The endurance-trained state is arguably the natural expression condition of human skeletal muscle [1]. Early humans experienced environmental selection pressure and migration out of Africa, that would have favoured a high physical endurance capacity [2, 3]. Improved endurance capabilities in early humans likely facilitated scavenging and persistence hunting and the co-emergence and increased post-exercise consumption of readily digestible protein and calorie intake [4]. Therefore, scavenging, hunting and gathering a high-protein diet [5] coupled with long-durations of endurance exercise (up to 8 h) supported not only the high energetic and tissue amino acid requirements of the musculoskeletal system, but also the metabolic demands of an increasingly larger brain mass contributing to social, cultural and technological development [5]. Animals would have been consumed within the hours following hunting, tracking or gathering, which is also when nutrient delivery to the exercised tissue is best because of transient increases in muscle blood flow, insulin sensitivity and glucose and amino acid uptake [6, 7]. Disturbances to muscle homeostasis from regular endurance exercise coupled with post-exercise hyperaminoacidaemia from a protein-rich diet might, therefore, be the normal environmental cues for adaptive remodelling in human skeletal muscle [8].

Indeed, recent evidence from experimental models in contemporary men suggests that consuming whole protein and protein plus leucine following endurance exercise plays an important role in promoting several aspects of skeletal muscle endurance-exercise adaptation, including mitochondrial biogenesis and function, ECM and microvascular plasticity [8–11]. We proposed leucine as an effective nutritive agent to promote enhanced skeletal muscle recovery from exercise owing primarily to its potent stimulatory effect on the rate of protein synthesis [12]. Enriching low-protein meals with leucine can increase post-prandial skeletal muscle mTORC1-pathway signalling, protein synthesis rates [13], and expression of some myocellular genes [14]. Leucine also enhances the secretion of insulin, which governs the expression of hundreds of skeletal muscle genes coupled to transcriptional and translational regulation, energy metabolism, intracellular signalling, the cytoskeleton, ubiquitin/proteasome pathways and the immune response [15]. Therefore, skeletal muscle plasticity common to exercise adaptation may accrue in response to post-exercise protein- and leucine-rich nutritional interventions.

In this chapter, we will discuss the role for dietary protein and leucine ingestion in supporting skeletal muscle regeneration processes following endurance exercise in trained skeletal muscle. We present the rationale for adding free-leucine to dietary protein, and evidence that protein-leucine feedings might augment the combined exercise-nutrient muscle response by boosting post-exercise regeneration processes and protein synthesis, which could be a beneficial strategy for athletes undertaking strenuous daily exercise.

## **Enhanced Skeletal Muscle Recovery from Exercise Is Unlikely to Be Associated with Improved Glycogen Storage**

Modern endurance athletes frequently engage in repeated bouts of fatiguing exercise during training or competition, and sometimes training several times per day. Glycogen is the most important fuel substrate for intense exercise. Intense and prolonged exercise substantially reduces the concentration of muscle glycogen but post-exercise carbohydrate consumption induces hyperglycaemia and hyperinsulinaemia, the latter enhancing myocellular glucose uptake and glycogen synthase activity, thereby enhancing glycogen resynthesis [16]. The post-exercise glycogen resynthesis rate can be maximized by ingesting  $>1.2$  g carbohydrate·kg body mass<sup>-1</sup>·h<sup>-1</sup> [17] but when ingesting  $<1.2$  g carbohydrate·kg<sup>-1</sup>·h<sup>-1</sup>, the addition of insulinogenic protein hydrolysates and amino acids (e.g. leucine) can also enhance resynthesis [18]. However, when protein was co-ingested with a high rate of carbohydrate ( $1.2$  g·kg<sup>-1</sup>·h<sup>-1</sup>) and compared to an isocaloric no-protein control ( $1.6$  g carbohydrate·kg<sup>-1</sup>·h<sup>-1</sup>), which was similar to the high rates of carbohydrate ingested in well-controlled performance studies [9, 11, 19], protein feeding had no effect on glycogen concentrations at 3 h or 48 h post-exercise [8]. Therefore, enhanced glycogen resynthesis is unlikely to be an important adaptive mechanism resulting from the addition of protein and amino acids to carbohydrate ingested post-exercise. Instead, dietary-protein stimulated, non-glycogen mechanisms, relating to improved cellular integrity, faster restoration of contractile function and the accrual of new and adaptive proteins, appear more likely [20, 21]. With a suspicion of the mechanisms involved, our laboratory conducted a series of performance- and mechanisms-focused studies to determine the impact of protein or protein-leucine feeding following exercise on protein synthesis and other molecular processes aligned with tissue regeneration [8, 9, 11, 19].

## **High-Protein and Carbohydrate Feeding Substantially Enhanced Subsequent Endurance Performance Relative to Isocaloric High-Carbohydrate Control**

In the first of these investigations, Rowlands et al. [19] found that high-dose protein ( $0.7$  g·kg<sup>-1</sup>·h<sup>-1</sup>) coingested with sufficient carbohydrate to ensure maximal glycogen resynthesis ( $1.4$  g·kg<sup>-1</sup>·h<sup>-1</sup>) and some fat during 4-h recovery from 2.5-h intense cycling provided no clear benefit to performance of a repeated-sprint cycling test the next day (+15 h), relative to isocaloric low-protein, high-carbohydrate ( $0.1$  and  $2.1$  g·kg<sup>-1</sup>·h<sup>-1</sup>, respectively) and fat feeding. This finding confirmed earlier work in a well-controlled isocaloric feeding model [22] suggesting that subsequent endurance performance is largely impervious to added post-exercise protein nutrition when recovery is short, relative to isocaloric controls with high ( $\geq 1.2$  g·kg<sup>-1</sup>·h<sup>-1</sup>) rates of carbohydrate intake. Therefore, adding protein to high rates of carbohydrate ingestion produced no additional benefit to glycogen restoration or performance within a 4–24 h period. However, by day 4 (+60 h) there was a substantial 4.1 % mean improvement in repeated sprint power with the high-dose feeding [19]. Furthermore, this performance effect was associated with positive nitrogen balance during day 1 recovery compared to negative nitrogen balance with the control, indicative of greater body-protein accrual, and lower blood creatine-kinase concentrations. The latter finding is common to several other short-term performance recovery investigations with protein-carbohydrate feeding [22–24] and is interesting because it suggests improved myocellular membrane stability or attenuated structural damage with post-exercise protein feeding; however, other than suspected beneficial effects on whole-body or muscle protein turnover the amino acid mediated mechanisms responsible were not forthcoming.

## **A Rationale for Adding Leucine to Protein after Exercise: Amplify Insulin Secretion and Protein Manufacture without Providing Energy and Nitrogen in Excess of What Is Necessary to Enhance Skeletal Muscle Recovery**

Long-term energy balance (chronic daily energy intake matching expenditure) is important to endurance athletes for the purpose of maintaining lean body mass and a high power-to-mass ratio. Over-consuming protein above that required to make nitrogen balance while maintaining a stable body mass inevitably reduces intake of other macronutrients; there is a risk that this may somewhat compromise glycogen availability for high-intensity training or competition, although effects may be negligible because the extra amino acids contribute to the effective carbohydrate pool via increased gluconeogenesis [25]. Planned caloric reduction and/or an increased training volume are methods periodically used to reduce body fatness and improve the power-to-mass ratio; tissue catabolism is increased during times of energy deficit, but can be minimized by adequate dietary amino acid provision [26]. Nevertheless, a criticism of the Rowlands et al. [19] design was the high protein-dose provided. Consumption of a high-protein diet ( $3.6 \text{ g}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$ ) relative to low or moderate protein ( $0.8$  and  $1.8 \text{ g}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$ , respectively) impairs post-endurance exercise muscle protein synthesis [27] and increases tissue-protein breakdown and amino acid oxidation without an increase in the resting protein synthesis rate [28], suggesting that habitual high-protein feeding might be a less-than-optimal dietary strategy if tissue protein synthesis is an important mechanism of adaptation. For these reasons, adding free-leucine and lowering the protein-dose in a meal should retain potency of anabolic signalling with a reduced overall nitrogen and caloric load, and may also support other aspects of skeletal muscle plasticity to exercise via the insulin response and other pathway-associated gene expression.

With respect to the increased protein synthesis, the stimulatory effect of dietary protein on skeletal-muscle protein turnover is mediated largely by increases in the concentration of blood and muscle leucine, stimulating translation via enhanced intracellular mTOR-pathway signalling [12, 29, 30]. Recent evidence from EAA-cultured C2C12 myotubes indicates leucine has  $\sim 3$  times the p70S6K- and rpS6-phosphorylating potency of other EAAs, whereas the effect of valine and isoleucine was insubstantial [29]. Furthermore, EAA signalling potency appeared limited to regulation of mTOR, p70S6K and rpS6 phosphorylation (translation initiation) rather than elongation via eEF2 and eIF2 $\alpha$  [29]. Leucine increases expression of mRNAs for several myofibrillar proteins (myosin heavy chain-slow and heavy chain-fast and myosin light chain-1 and -3) via increased 4E-BP1 and S6K1 phosphorylation and mTOR-independent mechanisms [14] suggesting leucine-mediated mechanisms include effects on pre-translational events. Leucine may also impact protein turnover by lowering post-exercise proteolysis [31, 32].

The insulinotropic effect of leucine may also promote post-exercise tissue recovery. For instance, adding free-leucine ( $0.1 \text{ g}\cdot\text{kg}^{-1}$ ) to whey protein and carbohydrate ( $0.3 \text{ g}\cdot\text{kg}^{-1}$  and  $0.7 \text{ g}\cdot\text{kg}^{-1}$ , respectively) further increased insulin secretion over that induced by whey protein and carbohydrate or carbohydrate ( $0.7 \text{ g}\cdot\text{kg}^{-1}$ ) alone [33]. Leucine-induced insulin secretion increases muscle blood-flow and, therefore, nutrient delivery [34]. Insulin also affects protein turnover by reducing protein breakdown but has little effect on the rate of muscle protein synthesis, with or without amino acid provision [35]. Nevertheless, insulin has widespread effects on gene transcription, regulating  $\sim 800$  genes in rested skeletal muscle [15]. In healthy humans, insulin infused at physiological concentrations acted synergistically with amino acids to increase mitochondrial ATP rate, mitochondrial-gene expression (NADH dehydrogenase subunit IV and COX subunit IV), mitochondrial protein synthesis and COX and citrate synthase enzyme activity [36, 37]. Thus, superimposition of leucine- and insulin-regulated mitochondrial biogenesis may combine to enhance some aspects of the skeletal muscle adaptive response to endurance exercise.

While leucine alone stimulates aspects of cellular growth, ingesting complete protein is still necessary to provide amino acid substrate for protein synthesis and to avoid a rebound reduction in the plasma concentration of isoleucine, valine [38] and glutamine (cellular glutamine efflux is coupled to leucine influx [39]) to support protein synthesis [13]. Whole proteins may also provide potentially beneficial bioactive peptide digestion products [40]. Next we asked whether a lower quantity of protein but with added free-leucine [19] could also elicit a worthwhile benefit to subsequent endurance performance and mechanisms associated with skeletal muscle recovery from exercise stress.

## **Post-exercise Protein-Leucine Supplementation Improved Subsequent Performance Under Nitrogen Stressed Conditions**

Thomson et al. [9] reported a worthwhile benefit of post-exercise protein-leucine feeding to subsequent high-intensity endurance cycling performance. Leucine ( $0.1 \text{ g}\cdot\text{kg}^{-1}\cdot\text{h}^{-1}$ ) was added to whole protein, carbohydrate and fat ( $0.4/1.2/0.2 \text{ g}\cdot\text{kg}^{-1}\cdot\text{h}^{-1}$ , respectively) ingested during the first 90 min of post-exercise recovery during 3 days of high-intensity cycling. A tightly controlled crossover design was utilised whereby the specific post-exercise effect of high-protein recovery feeding was isolated by providing the alternate supplement (isocaloric low-protein, carbohydrate and fat,  $0.06/1.6/0.2 \text{ g}\cdot\text{kg}^{-1}\cdot\text{h}^{-1}$ , respectively) at the opposite end of the day; this also enabled dietary protein intake to be clamped at  $1.6 \text{ g}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$  with a controlled background diet, thereby removing total daily protein intake as a variable [9]. Average nitrogen balance (total amino acid nitrogen intake minus nitrogen loss) was marginally negative during the experimental period in both protein-leucine fed and control conditions, indicating that the benefit of protein-leucine to recovery performance occurred under mild nitrogen stress. Protein-leucine feeding was also found to lower the plasma creatine-kinase concentration and reduced perceived tiredness during repeated sprint performance tests.

From analysis of the confidence interval, we noted the possibility that the true effect of protein and protein-leucine feeding on recovery of endurance performance within several days could also be trivial [9, 19]. Indeed, when a protein-leucine, carbohydrate and fat supplement ( $20/7.5/89/22 \text{ g}\cdot\text{h}^{-1}$ , respectively) or isocaloric carbohydrate/fat control ( $119/22 \text{ g}\cdot\text{h}^{-1}$ ) was administered for 1–3 h after exercise during a 6-day training block, and with the background diet comprising  $1.9 \text{ g protein}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$  (protein-leucine) and  $1.5 \text{ g protein}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$  (control) yielding a mildly positive nitrogen balance in both dietary conditions, subsequent high-intensity performance was not substantially affected by the protein-leucine recovery feeding [11]. And yet, protein-leucine feeding was still associated with reductions in creatine kinase of similar magnitude to that found earlier by our group [9, 19, 22] and others [23, 24]. These findings are important because they suggest a role for daily dietary nitrogen balance on the ergogenicity of post-exercise protein-leucine feeding in endurance athletes. The data indicate that protein-leucine supplementation in the immediate hours post-hard endurance exercise is most useful for aiding recovery when the remaining daily diet is mildly deficient in protein or amino acids, leading to a neutral-to-negative nitrogen balance physiological environment.

## **Long-Term Protein Supplementation: Do the Phenotypic Outcomes Match the Suspected Mechanistic Inference?**

Although there are few well-controlled studies, chronic consumption of protein following endurance exercise training in healthy but previously untrained participants has been found to increase aerobic power (maximal oxygen consumption,  $\text{VO}_2\text{max}$ ) indicating a benefit to endurance exercise

performance. Robinson et al. [41] found that chronic post-exercise protein-carbohydrate supplementation (20/55 g, respectively) in older men and women during 6 weeks of treadmill run training did not substantially impact long-term skeletal muscle protein synthesis rates relative to isocaloric carbohydrate (75 g), but resulted in a greater improvement in  $\text{VO}_2\text{max}$  ( $12.2\% \pm \text{SD } 6.2\%$  versus carbohydrate  $3.3 \pm 8.7\%$ ) [41]. In healthy young men and women, post-exercise chocolate milk consumption (mean intake of protein/carbohydrate:  $0.94/0.31 \text{ g}\cdot\text{kg}^{-1}$ , respectively) following 4.5 weeks of aerobic cycle training improved body composition (lean-mass:fat-mass) and resulted in moderate effect-sized increases in absolute ( $\sim 0.15 \text{ L}\cdot\text{min}^{-1}$ ) and relative ( $\sim 3 \text{ mL}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$ )  $\text{VO}_2\text{max}$  versus isocaloric carbohydrate supplementation [42]. Although improvements in microvascular [8] and mitochondrial plasticity [10] associated with improved skeletal muscle growth and development in previously deconditioned skeletal muscle could account for the improvements in  $\text{VO}_2\text{max}$ , cardiovascular adaptations, such as, increased blood volume [43], might also contribute. Furthermore, these findings may not necessarily apply to well-trained athletes who already possess chronic hematological, microvascular and mitochondrial adaptations, warranting further research in trained endurance athletes on the potential impact of chronic post-exercise protein-leucine feeding on  $\text{VO}_2\text{max}$  and endurance performance.

## Effect of Post-endurance Exercise Protein and Protein-Leucine Ingestion on Candidate Molecular Mechanisms Guiding Nutrient-Modulated Skeletal Muscle Regeneration

Accumulating new data provides insight into how elevated amino acid concentrations following post-exercise protein feeding may guide homeostatic regeneration and adaptive remodelling in skeletal muscle. Some insight has come from the study of protein turnover and candidate genes, but a greater leap forward in understanding of the post-exercise molecular programme guiding protein-leucine fed regeneration has arisen from inferences obtained from bioinformatic analysis of the transcriptome.

With regard to mixed muscle protein FSR, the mean effect size (ES) increase from adding protein to carbohydrate ingested post-endurance exercise, relative to isocaloric or carbohydrate-matched control conditions, ranges from small (ES 0.49) to large (ES 1.60) (Table 2.1). While there is good evidence to suggest that the mitochondrial fraction may contribute substantially to FSR after endurance exercise [44], Breen et al. [45] have recently reported that only myofibrillar FSR, but not the mitochondrial rate, increased in the first 3 h following endurance exercise in response to protein-carbohydrate feeding (Fig. 2.1). This suggests that either the mitochondrial fraction is less responsive in the recovery timeframe assayed, increases temporally more distal to exercise, or that the post-exercise mitochondrial protein synthesis rate is already maximally activated by endurance-exercise stimuli.

Increased blood amino acid concentrations, and in particular leucine, increase skeletal muscle FSR largely through activation of translation initiation via the mTORC1 signalling pathway [12]. mTORC1 activation has also been associated with stimulation of bHLH-motif transcription factors that bind E-box regions in the promoter regions of genes regulating ribosomal RNA synthesis via RNA polymerase gene expression [46, 47], and a number of other genes also associated with the myofibrillar adaptive response to contractile stimuli: STAT1 and STAT3 (regulating PPAR $\gamma$ ) [48], HIF1 $\alpha$ , SREBP1 and 2, and YY1 [49–51]. Transcription factor HIF1 $\alpha$  upregulates the rate-limiting enzyme in glycolysis, pyruvate kinase M2 [51] and HIF1 $\alpha$  and SREBP1/2 stimulate glycolysis and lipid biosynthesis, pentose phosphate pathway activity and anabolic cell growth and cell proliferation [50]. mTOR further controls transcription of mitochondrial components via its action on YY1-governed PGC-1 $\alpha$  expression [49]. Regular protein-leucine nutritional mediation of the expression of mRNAs associated with ribosomal, metabolic, mitochondrial and myogenic functions could therefore promote increased functional protein expression and superior tissue remodelling when coupled with increased cellular protein FSR.



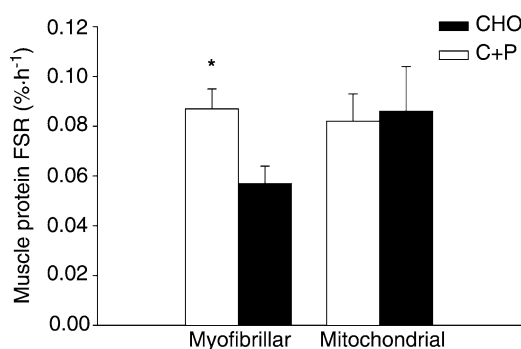
**Table 2.1** Comparison of the effects of post-endurance exercise protein-carbohydrate ingestion relative to carbohydrate-control or placebo on mixed-muscle, myofibrillar, and mitochondrial protein fractional synthesis rates (FSRs) determined via tracer-incorporation

Reference Cohort	Exercise mode Duration: Intensity $\pm$ SD (%VO <sub>2</sub> max/peak, if published)	Mean FSR $\pm$ SD % $\cdot$ h <sup>-1</sup>	FSR Comparison ES $\pm$ 90%CL <sup>a</sup> Inferences <sup>b</sup>
Howarth et al. [63]	Cycle	PRO: 0.088 $\pm$ 0.015	PRO vs L-CHO
Six healthy men	120 min: Variable intensity (50–80 %)	L-CHO: 0.066 $\pm$ 0.018 H-CHO: 0.060 $\pm$ 0.018	1.31 $\pm$ 0.65; large increase likely PRO vs H-CHO 1.60 $\pm$ 0.80; large increase very likely
Harber et al. [64]	Cycle	PRO: 0.129 $\pm$ 0.040	PRO vs Placebo
Eight trained men	60 min: 72 % $\pm$ 3 %	Placebo: 0.112 $\pm$ 0.028	0.49 $\pm$ 0.40; small increase likely
Breen et al. [45]	Cycle	Mitochondrial	Mitochondrial: PRO vs CON
Ten well-trained male cyclists	90 min: 77 $\pm$ 3 %	PRO: 0.082 $\pm$ 0.032 CON: 0.087 $\pm$ 0.051	-0.10 $\pm$ 0.07; trivial difference almost certain
		Myofibrillar	Myofibrillar: PRO vs CON
		PRO: 0.057 $\pm$ 0.024 CON: 0.057 $\pm$ 0.023	1.32 $\pm$ 0.90; large increase likely
Lunn et al. [65]	Run	MILK: 0.110 $\pm$ 0.024	MILK vs CON
Six trained male runners	45 min: 65 %	CON: 0.077 $\pm$ 0.024	0.99 $\pm$ 0.78; moderate increase very likely

All data are presented as mean  $\pm$  SD; for data published as mean  $\pm$  standard error of the mean (SEM), e.g. subject characteristics and fractional synthesis rate (FSR) [63–65]; or FSR [45], the SD was determined ( $SD = SEM \times \sqrt{n}$ ) Absolute VO<sub>2</sub>max (L $\cdot$ min<sup>-1</sup>) data [63] were converted to relative VO<sub>2</sub>max (mL $\cdot$ kg<sup>-1</sup> $\cdot$ min<sup>-1</sup>) based on the published participant mean body mass

<sup>a</sup>Add and subtract this number by the mean effect to obtain the upper and lower 90 % confidence limits

<sup>b</sup>Inferences are based on the smallest important effect, defined as the pharmacokinetic threshold criteria of a 20 % difference. ES thresholds: <0.2 trivial, <0.6 small, <1.2 moderate, <2.0 large, <4.0 very large, >4.0 extremely large. Thresholds for assigning qualitative terms to chances of substantial effects: <0.5 %, almost certainly not; <5.0 %, very unlikely; <25 %, unlikely; <75 %, possible; >75 %, likely; >95 %, very likely; >99.5 %, almost certain



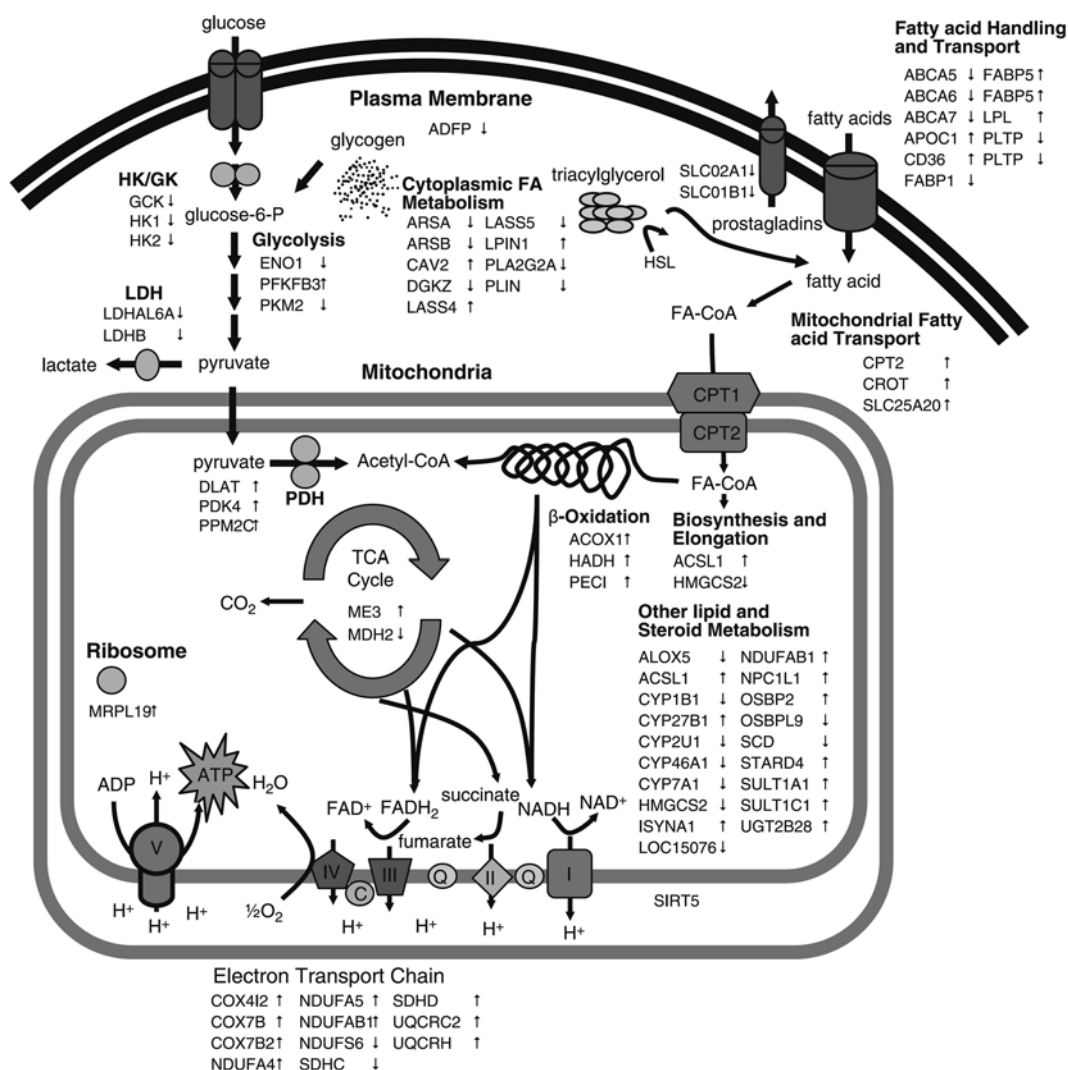
**Fig. 2.1** Myofibrillar ( $n=10$ ) and mitochondrial ( $n=8$ ) fractional synthetic rate following protein plus carbohydrate (C+P; 25 g carbohydrate, 10 g whey protein) or carbohydrate only (CHO, 25 g) feeding immediately and at 30 min following 90 min of endurance exercise at 77 % maximal oxygen uptake in trained men [45]. Values are means  $\pm$  SEM. Image obtained by permission from John Wiley and Sons, Boston, MA

## Protein-Fed Post-Endurance Exercise Skeletal Muscle Transcriptome Describes Metabolic and Mitochondrial Gene Expression Occurring after Inflammatory Promyogenic Regeneration Responses

Recent transcriptome data [8] suggests no impact of protein nutrition on the metabolic-mitochondrial transcriptome until ~48 h post-exercise in men, at which time, PGC-1 $\alpha$  and other mitochondrial gene expression were elevated in response to post-exercise protein feeding 2-day prior (Fig. 2.2). Mitochondrial respiratory capacity and endurance performance increased in endurance training mice chronic fed on branched chain amino acids [10]. Given the high mitochondrial protein content in muscle (~30 % [52]) and association of total mitochondrial volume with skeletal muscle respiratory capacity and endurance [53], boosting mitochondrial biogenesis by increasing BCAA availability might be a key functional adaptation to protein feeding coupled to endurance exercise. To provide an unbiased global picture of the impact of exercise on gene expression guiding remodelling, Mahoney et al. [54] used high-throughput gene microarray to show that a single bout of endurance exercise in lean healthy men stimulates the expression of genes involved in the oxidative stress response, electrolyte shuttling, transcription, proteolysis, cell growth and death and metabolism. Taking a similar novel discovery approach, our group determined the skeletal muscle transcriptome specifically induced in response to milk protein feeding following endurance exercise [8]. At 3 h following exercise, the gene expression profile was consistent with increased ECM protein expression and turnover, attenuated stress response and cell stability, activation of myocellular growth and development, modulation of cellular inflammatory and immunity and defence mechanisms [8]. With respect to immunity and defence, evidence for nutrition-modulated inflammatory leukocyte activity is interesting because neutrophils and macrophages are crucial for phagocytosis of damaged tissue and stimulation of successful skeletal muscle regeneration and myogenesis after exercise [55, 56]. Upregulated ECM protein turnover and regulated expression of matrix metalloproteinases and inhibitors (increased MMP9, MMP13, MMP19; decreased TIMP1, TIMP2; Fig. 2.3) suggested post-exercise protein feeding may also influence control over basement membrane degradation and ECM remodelling, facilitating recruitment of myogenic, myeloid, vascular and fibroblastic cells to damaged muscle [55], and open the interface for leukocytes, cytokines and myogenic growth factors [57].

In our 6-day performance study we found some evidence for modulated circulating-neutrophil function during recovery [58] and greater ECM protein turnover [11] lending further support for a role for protein-leucine ingesting during recovery in physiological support for athletes. Employing mass spectrometry-based metabolomics, we studied blood and urinary metabolites associated with whole-body amino acid and lipid turnover. Post-exercise protein-leucine feeding increased the mean 3-h recovery concentration of plasma amino acids (glycine, arginine, glutamine, leucine) and myristic acid metabolites (C14, myristoylcarnitine; and C14:1-OH, hydroxymyristoylcarnitine) with neutrophil priming capacity, and reduced neutrophil superoxide production (NADPH oxidase activated respiratory burst). By day 6, however, the protein-leucine supplement reduced pre-exercise cortisol and acylcarnitine C16 (palmitoylcarnitine) during exercise, and increased post-exercise neutrophil superoxide production, relative to control. Although it is only one measure of neutrophil function, activation of the superoxide-generating NADPH oxidase complex is critical to neutrophil microbicidal capacity [59] and increased release of superoxide might benefit neutrophil cytotoxic activity, especially during intense exercise training.

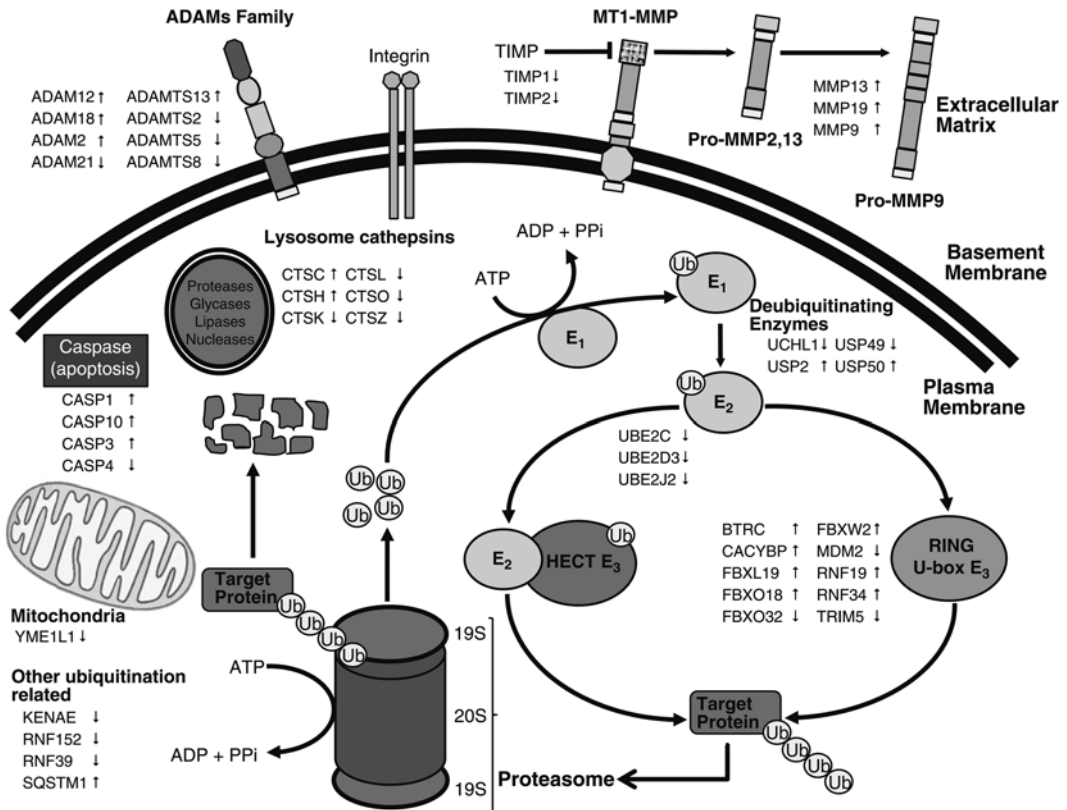
Protein-leucine supplementation also increased urinary excretion of proline metabolites, including L-proline, hydroxyproline-derived proline, and glycylproline (Fig. 2.4). Hydroxyproline is a major component of collagen and plays a key role in collagen stability [4] and increased excretion is suggestive of greater ECM protein turnover. We provided 130 mg leucine·kg<sup>-1</sup>·h<sup>-1</sup> (free and protein bound) over 3 h following cycling. Increased post-exercise plasma C3 and C5 acylcarnitine concentrations indicate that this rate of leucine ingestion exceeds leucine degradation pathway enzymatic



**Fig. 2.2** Effect of post-endurance exercise protein versus control nutrition on expression of genes involved in the regulation of carbohydrate (glycolysis) and fatty acid transport, lipid and steroid metabolism in the skeletal muscle at 48 h post-exercise [8]. Gene expression up arrow/down arrow. Image obtained by permission from The American Physiological Society

activity after an acute bout of endurance exercise. Reduced urinary losses of BCAAs, proline, methionine and  $\alpha$ -aminoisobutyrate (a product of both valine and pyrimidine metabolism) during subsequent exercise suggested reduced turnover or retention of these amino acids and metabolites, and more widespread alterations to concentrations of plasma and urinary amino acids and their metabolites at rest and during exercise by day 6 suggests adaptive processes in response to higher dietary intakes of leucine and protein.

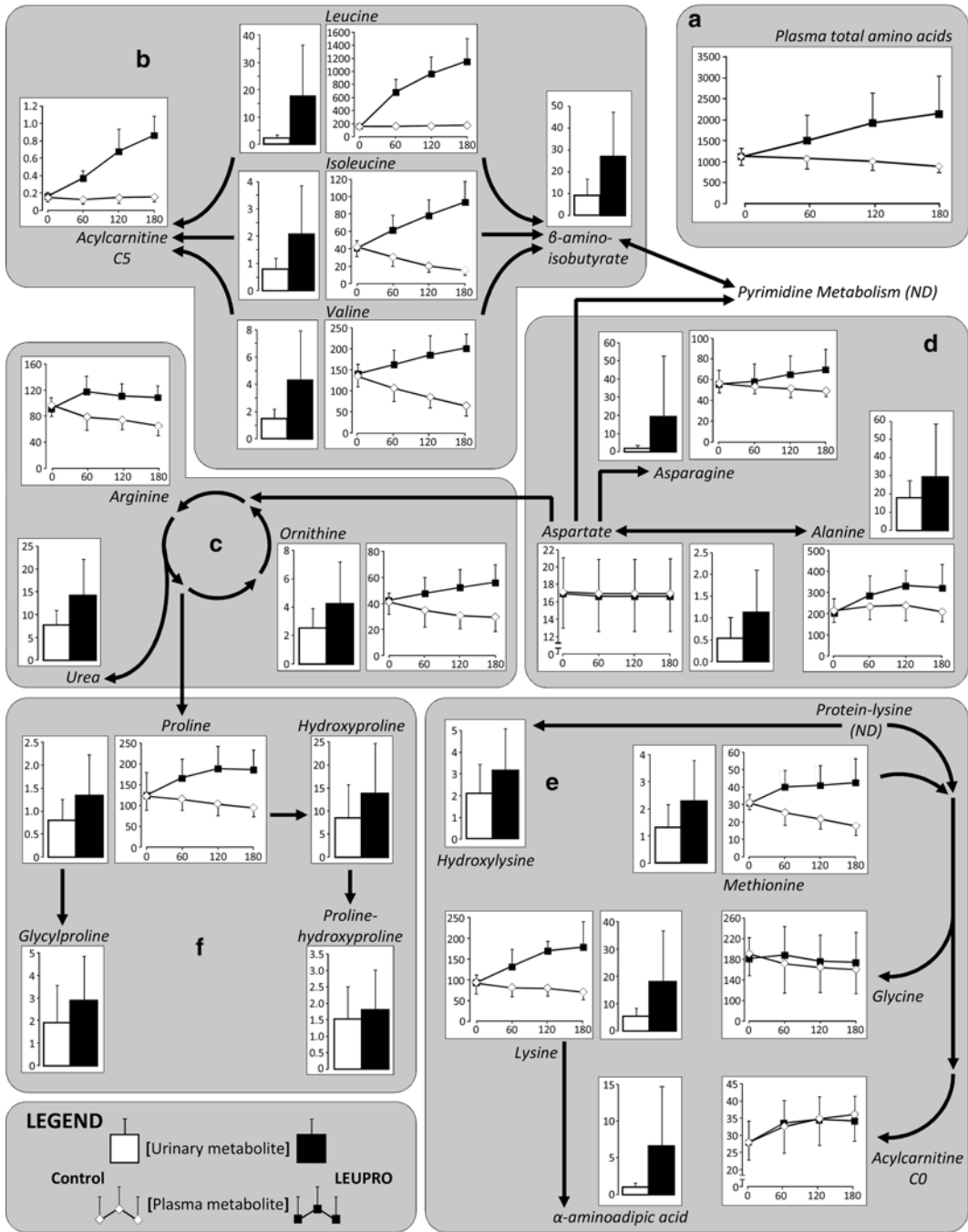
With building evidence to show that post-exercise dietary protein and leucine intake modulates skeletal muscle gene expression and increases protein synthesis in a dose-sensitive fashion [13, 14, 60], we next studied the effect of three quantities of protein-leucine (control, zero protein-leucine; high dose, 70 g whey protein/15 g leucine; low dose, 23 g whey protein, 5 g leucine) co-ingested with carbohydrate and fat over the first 90 min following intense cycling on the skeletal muscle



**Fig. 2.3** Effect of post-endurance exercise protein versus control nutrition on expression of genes involved in protein modification and breakdown at 3 h post-exercise [8]. Summarized are genes classified under the molecular function ontologies: cysteine peptidase, metallopeptidase, and ubiquitin-specific protease activity. Gene expression up arrow/down arrow. Image obtained by permission from The American Physiological Society

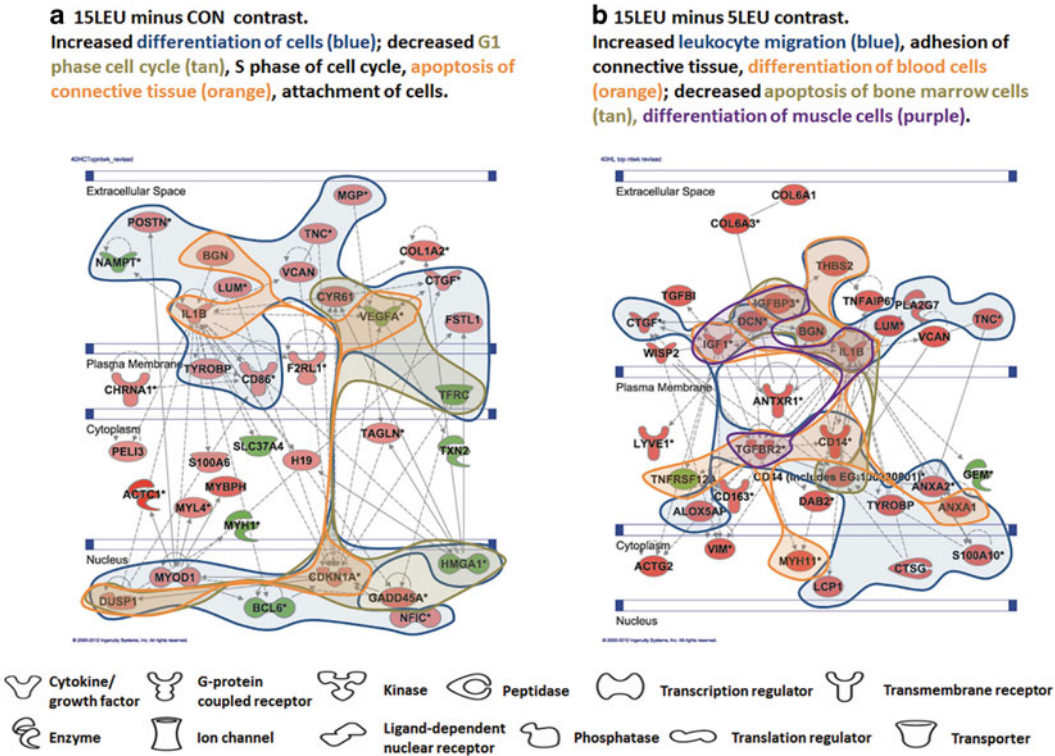
transcriptome at 30 and 240 min post-exercise [61]. Bioinformatic interrogation of high-throughput gene microarray revealed a proinflammatory transcriptome associated with increased leukocyte migration most evident with the high-dose protein-leucine at 30 min into recovery, and reverting by 240 min to an IL-6-centered antiinflammatory promyogenic molecular programme with both protein-leucine quantities, relative to control [61]. The central hubs regulating increased leukocyte migration were IL1 $\beta$  and CD44 and connected immune-cell differentiation and connective tissue remodelling factors to construct a cell-growth regulatory network that included IGF-1 and IGFBP3, TGF $\beta$ 1 and TGF $\beta$ 2, ECM function, remodelling, adhesion genes (e.g. decorin, biglycan, versican, tenascin, lumican, connective-tissue growth factor) and others involved in macrophage activation and adhesion (CD86, CD44, CD163, CD14, CD68) (Fig. 2.5). Meanwhile, other modular hub gene regulation was consistent with myogenic or satellite cell activation (MyoD, myogenin) and cell cycle control consistent with cell cycle arrest and increased cell stability via CDKN1A, GADD45A/B/G and DUSP1. The transcriptome suggests protein-leucine feeding upregulated an early-phase myeloid-cell associated regeneration response reflecting wound-healing biology [56].

Inflammation may contribute to muscle repair and subsequent remodeling by stimulating resident and infiltrating immune cells (monocytes, macrophages and neutrophils) to cleanup cellular debris in muscle, stabilize membrane structure and the release of soluble factors to promote myogenesis and inflammatory resolution [56, 62]. Protein-leucine mediated restorative remodelling of muscle ECM and membrane stability may explain attenuated blood concentrations of muscle-membrane damage



**Fig. 2.4** Effect of 3-h protein-leucine or isocaloric control post-endurance exercise feeding on plasma and urinary metabolite concentrations during recovery from exercise on day 1 of a 6-day intense cycling protocol [11]. Shown are the responses of plasma essential and total amino acid concentration (a), and plasma and urinary concentrations of substrates and metabolites relating to the branched chain amino acids (b), the urea cycle (c), the metabolism of alanine and aspartate (d), the degradation of lysine (e) or the metabolism of arginine and proline (f). Data are means  $\pm$  SD. Concentrations of plasma metabolites are in micromoles per litre and urinary metabolites in nanomoles per litre. ND, not determined. Image obtained by permission from Wolters Kluwer Health



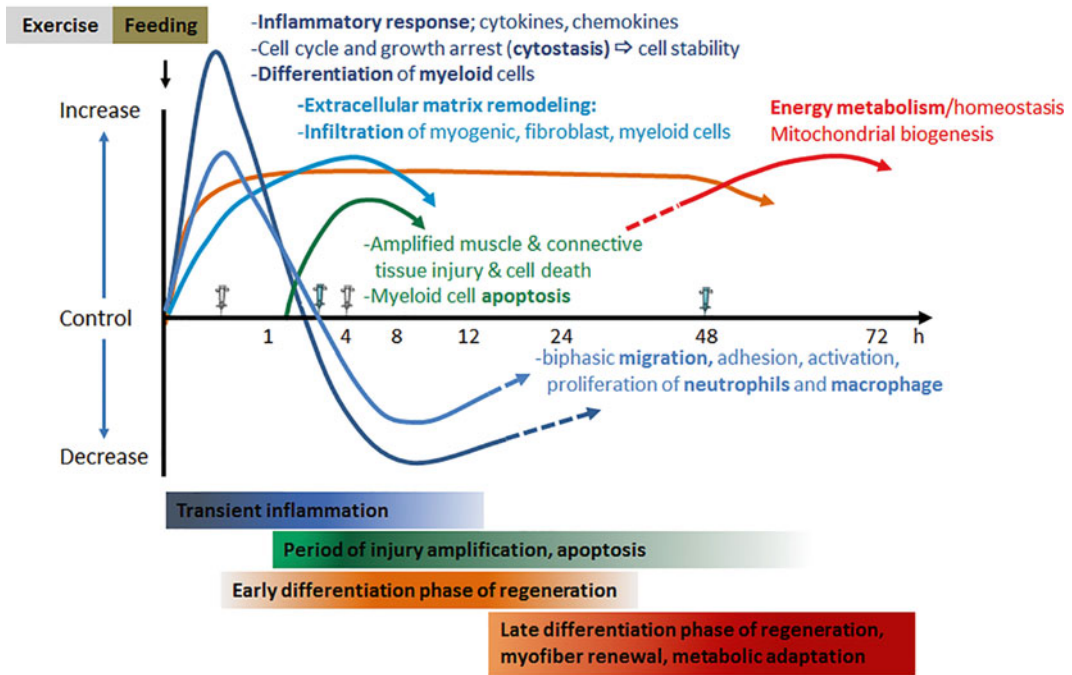


**Fig. 2.5** Two top ranked Ingenuity Pathway Analysis ([www.ingenuity.com](http://www.ingenuity.com)) networks representing the transcriptome responding to protein-leucine quantity at 30 min into recovery from cycling exercise: (a) the high-dose (15LEU) vs control dose (CON), and (b) the 15LEU vs the low-dose (5LEU) contrast. Selected molecular function modules (coloured shading and labelling) are representative of the top-ranked within-network biology. Symbol colour indicates the direction of gene regulation: *green*, down-regulated; *red*, up-regulated. For brevity, full gene names and abbreviations are not included

marker in the blood (creatine kinase) previously observed in the days following post-exercise protein-leucine feeding [9, 11]. Combined with other evidence for increased protein synthesis [11, 13] and a myogenic transcriptome, these mechanisms could, in part, explain the reported improvement in performance following 3-days of ingesting similar quantities of protein and leucine after intense endurance cycling [9]. A summary of the working evidence for the principle molecular programme activated by protein and protein-leucine feeding following intense endurance exercise is shown in Fig. 2.6.

## Conclusions

From early-human hunter-gatherers through to modern athletes, endurance exercise and post-exercise hyperaminoacidaemia has likely played an important role in human skeletal muscle health and performance. Protein-mediated mechanisms are likely to involve modulation of skeletal muscle protein turnover and gene expression to confer a functional benefit to endurance-exercise induced plasticity. Although high-dose protein feeding conferred a benefit to performance several days after initial exercise, long-term high-dose protein might overload nitrogen metabolism and negatively impact protein turnover; however, adding leucine to protein feeding amplifies insulin secretion, mTOR-signalling and protein synthesis with a reduced nitrogen load, relative to the same signalling potency with a



**Fig. 2.6** Model for the effect of protein and protein-leucine feeding on modulation of the molecular programme regulating skeletal muscle regeneration in response to intense endurance exercise, constructed by inference from molecular functions obtained from the analysis of the transcriptome; needles indicate biopsy samples, blue from [8] and clear from [61]

higher dose of whole protein. The accumulated evidence from our recent investigations in multiday high-intensity cycling suggests that subsequent performance is enhanced with post-exercise protein and protein-leucine feeding when athletes are under mild nitrogen stress, but unaffected when in positive balance. Isotope and multiomic data analysis suggests that protein-leucine feeding modulates a proinflammatory, promyogenic skeletal muscle cellular response, and later may activate and support cellular growth and mitochondrial biogenesis. Long-term studies in sedentary populations suggest increased aerobic power with peri-training chronic protein feeding, but further research is required in trained athletes.

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