

IMPACT OF RESISTANCE AND ENDURANCE EXERCISE AND INGESTION OF  
VARYING PROTEIN SOURCES ON CHANGES IN HUMAN SKELETAL MUSCLE  
PROTEIN TURNOVER

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PROTEIN TURNOVER

By  
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TITLE: Impact of Resistance and Endurance Exercise and Ingestion of Varying Protein Sources on Changes in Human Skeletal Muscle Protein Turnover

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## **Abstract**

Both resistance and endurance exercise elicit an increase in muscle protein synthesis during recovery from exercise. Ingestion of amino acids augments the exercise-induced stimulation of muscle protein synthesis following resistance exercise. Our work showed that 8 wk of unilateral resistance training induced muscle hypertrophy only in the exercised limb. Importantly, using this unilateral model we showed that muscle hypertrophy was confined to the exercised leg and occurred without measurable changes in circulating anabolic hormones. We then went on to use the unilateral leg resistance exercise model to study how animal-derived (milk) and plant-derived (soy) proteins impacted acute post-exercise protein turnover. We observed that ingestion of soy or milk protein resulted in a positive net protein balance following resistance exercise. Moreover, milk promoted a greater net protein balance and muscle protein synthesis than soy protein. In the final study, a key finding was that acute endurance and resistance exercise differentially stimulated myofibrillar and mitochondrial protein synthesis and also differentially affected cellular signaling proteins involved in the regulation of the protein synthetic response. Specifically, the acute, untrained state response showed that resistance exercise stimulated myofibrillar and mitochondrial protein synthesis while endurance exercise stimulated mitochondrial protein synthesis. Following resistance training only myofibrillar protein synthesis increased after exercise, while mitochondrial protein synthesis was unchanged. Endurance exercise training did not affect the acute protein synthetic response and so following training mitochondrial protein synthesis was stimulated as it was acutely, prior to training. In conclusion, the studies within this thesis



provided novel insights on the impact of intact dietary proteins and differing modes of exercise on the control skeletal muscle protein metabolism.

## **Acknowledgements**

After quite a few years of doing ‘RE-search’ in the EMRG lab I have so many people to thank. First and foremost I would like to thank the participants of these studies; thank you for getting up so early, training so hard and giving up your time, blood and muscle.

Tom, thank you for putting up with me during this challenging process and for cheering me on even though you don’t quite understand what exactly it is that I do.

Thank you mom, dad and Taryn for the support you gave me during my entire education. Thanks to Carol, Bob, Sandy, Chris and Aislinn for adopting me into your family, it is so nice to have you when I am so far away from my own family.

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## **Format and Organization of the Thesis**

This thesis was prepared in the ‘sandwich format’ as outlined in the School of Graduate Studies’ Guide for the Preparation of the Thesis. This thesis is comprised of a general introduction, three original research papers (Studies 1-3), a general discussion and an appendix outlining novel methodology developed as part of this thesis. All studies are published (Studies 1& 2) or submitted (Study 3) to peer reviewed journals with Sarah Wilkinson as first author.

### **Sarah B. Wilkinson's contributions to the research presented**

Sarah implemented and managed all aspects of the studies presented in this thesis. Sarah was responsible for study design (with input from others), obtaining ethics approval, recruiting participants, collecting and analyzing most of the data. In Chapters 1 & 3, Sarah had help from other lab members in training the participants. Sarah independently wrote the first draft of each manuscript and then revised each subsequent manuscript draft based on comments from her co-authors. The contributions of the other authors are as follows:

#### *Study 1*

Emily J. Grant and Carol E. Correia: recruited and trained participants, collected and analyzed some of the data related to CT scans.

Mark A. Tarnopolsky: performed muscle biopsies and assisted in editing and preparation of the manuscript

Stuart M. Phillips: assisted with study design, data analysis, data interpretation, as well as editing and preparation/submission of the manuscript.

#### *Study 2*

Mark A. Tarnopolsky and Jay R. Macdonald performed all medical procedures (muscle biopsies, arterial and venous lines) and assisted in the editing and preparation of the manuscript.

Maureen J. Macdonald: performed the Doppler blood flow measurement and analysis and assisted in the editing and preparation of the manuscript.

David Armstrong: conducted the  $^{13}\text{CO}_2/^{12}\text{CO}_2$  breath enrichment analysis and assisted in the editing and preparation of the manuscript.

Stuart M. Phillips: assisted with study design, data analysis, data interpretation, as well as editing and preparation/submission of the manuscript.

### *Study 3*

Stuart M. Phillips: assisted with study design, data analysis, data interpretation, as well as editing and preparation/submission of the manuscript.

Philip J. Atherton and Rekha Patel: assisted with the development of the mitochondrial isolation procedure and also with some of the Western blot analyses.

Kevin E. Yarasheski: provided assistance with the determination of myofibrillar and mitochondrial isotopic enrichment of leucine and assisted in the editing and preparation of the manuscript.

Mark A. Tarnopolsky: performed muscle biopsies and assisted in editing and preparation of the manuscript

Michael J. Rennie: provided support for method development, study analysis and also assisted in the editing and preparation of the manuscript

## List of Abbreviation

1RM	single repetition maximum
4EBP-1	eukaryotic initiation factor 4E binding protein
AA	amino acids
Akt	protein kinase B
AMPK	AMP-activated protein kinase
Arg	arginine
Asn	asparagine
Asp	aspartic acid
ATP	adenosine triphosphate
AUC	area under the curve
BCAA	branched-chain amino acids
BSA	bovine serum albumin
C	cortisol
CS	citrate synthase
CSA	cross-sectional area
CT	computerized tomography
Cys	cysteine
EAA	essential amino acids
EE	endurance exercise
eIF	eukaryotic initiation factor
FAK	focal adhesion kinase
FSR	fractional synthetic rate
GbL	G protein b-subunit-like protein
GCMS	gas chromatography-mass spectrometry
GH	growth hormone
Gln	glutamine
Glu	glutamic acid
Gly	glycine
GSK	glycogen synthase kinase
GTP	guanosine triphosphate
HIF	hypoxia inducible factor
His	histidine
HPLC	high-performance liquid chromatography
IGF-1	insulin-like growth factor
Ile	isoleucine
IMF	intermyofibrillar mitochondria
KIC	ketoisocaproic acid
Leu	leucine
LH	leuteinizing hormone
Lys	lysine
m <sup>7</sup> GTP	7-methylguanosine
MBV	mean blood velocity

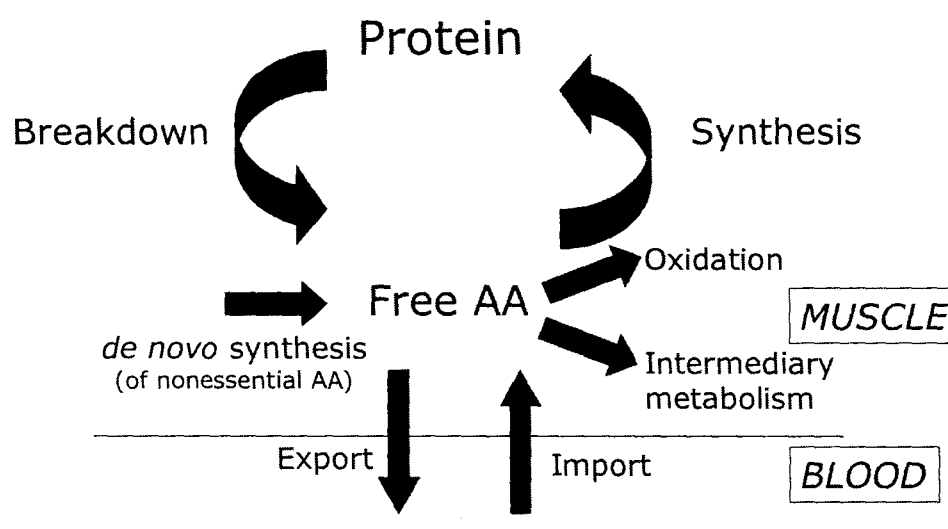
Met	methionine
met-tRNA	initiator methionyl-tRNA
MPS	muscle protein synthesis
mTOR	mammalian target of rapamycin
NB	net balance
NEAA	non-essential amino acids
NOLD	non-oxidative leucine disposal
NRF	Nuclear Respiratory Factor
p70S6k	70 kDa ribosomal protein S6 protein kinase
PKD1	3'-phosphoinositide-dependent protein kinase 1
PGC-1 $\alpha$	peroxisome proliferator-activated receptor $\gamma$ co-activator 1 $\alpha$
Phe	phenylalanine
PI3	phosphatidylinositol-3
R <sub>a</sub>	rate of appearance
R <sub>d</sub>	rate of disappearance
RE	resistance exercise
Rheb	Ras homolog enriched in the brain
RMR	resting metabolic rate
rpS6	ribosomal protein S6
Ser	serine
SHBG	sex-hormone binding globulin
SS	subsarcolemmal mitochondria
T	testosterone
TAA	total amino acids
TCA	tricarboxylic acid cycle
T <sub>f</sub>	free testosterone
T <sub>f</sub> /C	the free testosterone to cortisol ratio
Thr	threonine
TR	trained
tRNA	transfer RNA
Trp	tryptophan
Tyr	tyrosine
UT	untrained
Val	valine
VO <sub>2peak</sub>	peak oxygen consumption

## **Introduction**

Skeletal muscle is critical in locomotion. As a tissue it also plays an important role in glycemic regulation, lipid clearance, and immune function. It is often referred as a ‘plastic’ tissue because of its ability to adapt its phenotype in response to differing external stimuli, in particular to different loading schemes. Because of skeletal muscle’s importance in human health and its relative plasticity, it is important to study how skeletal muscle can undergo phenotypic adaptation. There is no doubt that phenotypic change would come about due to changes in tissue protein metabolism and turnover of particular proteins or protein pools. It is known that skeletal muscle responds robustly and changes phenotypically in response to differing patterns of exercise, showing marked and rapid changes in protein turnover in response to changes in nutritional status most notably the provision of protein (amino acids).

Skeletal muscle proteins are continually and simultaneously being synthesized and broken down. That these two opposing processes are occurring at the same time might seem counterproductive, however, this protein turnover provides constant repair and remodeling within the muscle allowing for dynamic changes in content, different protein isoform expression and removal of damaged proteins. Figure 1 depicts the concept of protein turnover within skeletal muscle schematically. Proteins are synthesized from amino acids present within the skeletal muscle free amino acid pool that must be charged to transfer RNA (tRNA) prior to being incorporated into proteins. Amino acids enter this free amino acid pool by import into the muscle from the blood, through *de novo* synthesis of non-essential amino acids, or as the product of muscle

protein breakdown. Amino acids from the free pool can also be oxidized, exported into the blood or undergo reactions in intermediary metabolism (such as the TCA cycle). The overall size of the free amino acid pool is thus a balance between influx from protein breakdown, import, and *de novo* synthesis and efflux into protein synthesis, amino acid export and use in intermediary metabolism,

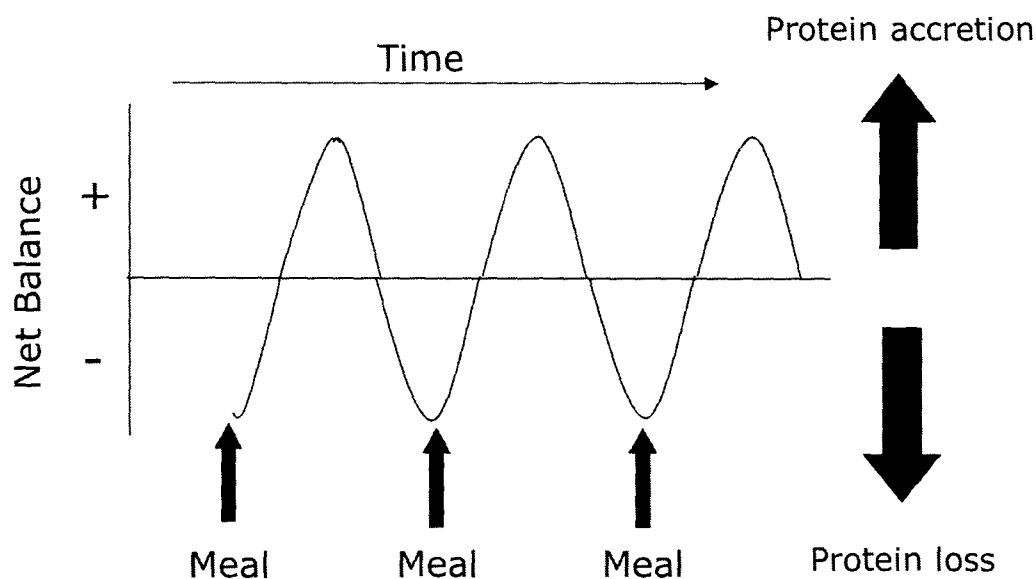


**Figure 1.** Schematic representation depicting protein turnover in skeletal muscle. Adapted from (100).

The difference between muscle protein synthesis and breakdown, often referred to as net protein balance (NB), determines whether there will be a net accumulation or loss of skeletal muscle protein. If synthesis is greater than breakdown (positive NB), the muscle is in an anabolic state and protein accretion will occur; however, if breakdown is greater than synthesis, resulting in loss of protein (negative NB), the muscle is said to be in a catabolic state. Within any given day, skeletal muscle goes through anabolic and catabolic periods depending on nutrient supply. For example, following an overnight fast,



when there are lower amounts of circulating amino acids and when catabolic hormones (i.e., cortisol) are higher than anabolic (i.e., insulin) hormones, muscle protein breakdown exceeds synthesis. When a mixed meal containing protein is consumed there is a systemic rise in insulin, in particular if the meal contains carbohydrate, and also a systemic hyperaminoacidemia. Both give rise to an increased delivery of amino acids to tissues including skeletal muscle. During the fed to post-prandial period an influx of amino acids occurs and protein synthesis is increased and protein breakdown is diminished. The reduction in proteolysis is due to a large extent to the rise in insulin, but is likely partially due to amino acids (103). The overall net outcome of these catabolic and anabolic periods over time determines whether we gain, lose or maintain, unchanged, an amount of muscle protein (Figure 2).



**Figure 2.** Illustration of skeletal muscle protein net balance cycling between anabolic and catabolic states within a day. Feeding results in a positive net balance, while fasting results in a negative net balance. The net outcome of fed-state gains in protein and fasted-states losses determines whether we gain, lose or maintain an unchanged muscle protein mass. Adapted from (92).

### **The effect of resistance exercise and feeding on skeletal muscle protein metabolism**

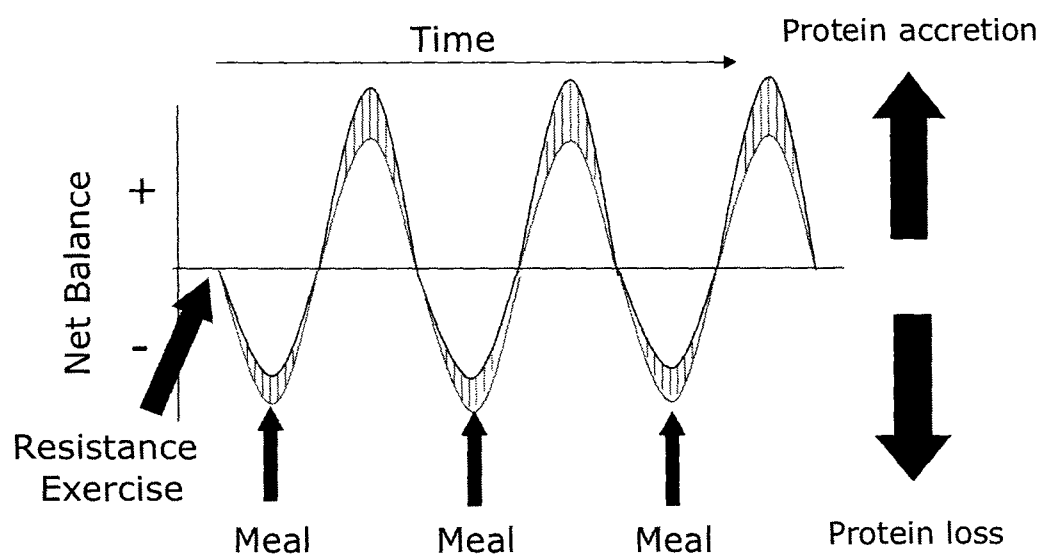
Skeletal muscle is referred to as a 'plastic' tissue because of its ability to adapt its phenotypic properties to external stimuli, such as exercise (9). Exercise, in the context of this thesis, is either low force (intensity) repetitive dynamic exercise of an 'endurance' nature or high intensity low repetition resistance exercise. While it is known that the two forms of exercise can promote dramatic genotypic and phenotypic changes the ultimate phenotypes are considered quite divergent in nature (5, 103). The focus of much of this thesis, however, is on resistance exercise, the repetition of which over a period of weeks-to-months increases muscle fibre diameter by adding new myofibrillar proteins to previously existing fibres (9, 94, 95, 116, 129). Consequently the force generating capacity of muscle increases in part due to the increments in muscle cross sectional area (34, 62).

After a single bout of resistance exercise, elevations in both protein synthesis and breakdown are observed (14, 31, 78, 94). Chesley et al. (31) found that protein synthesis increased by 50% following a resistance exercise bout and remained elevated for 24h post-exercise. In a subsequent report from the same group (78), it was observed that muscle protein synthesis returned to resting rates by 36h post-exercise. In another study, Biolo et al. (14) reported a simultaneous increase in muscle protein synthesis and breakdown following a resistance exercise bout in a single leg. It was observed that rates of protein synthesis doubled 3h post-exercise. Although increases in protein breakdown were observed, it increased by only 50% above resting value. Therefore, there was an overall increase in muscle protein NB (14). From this report it is unclear how long this

net balance remained elevated as measures were only taken at one time point post-exercise. Phillips et al. (94) examined the time course of muscle protein synthesis and breakdown in muscle after a single bout of resistance exercise. In addition to finding similar increases to Biolo et al. (14) immediately following exercise, it was reported that elevations in breakdown and synthesis persisted for up to 24h post-exercise (94). However, protein breakdown returned to resting levels by 48h post-exercise. In contrast, synthesis was still elevated at 48h post exercise resulting in an increase in fasted-state net balance at all time points post-exercise (94). A very similar pattern of not only muscle myofibrillar protein synthesis but also muscle and tendon collagen synthesis has recently been reported by Miller et al. (83) following prolonged dynamic knee extensor work. In all of these investigations (14, 31, 94), an acute rise in muscle protein synthesis was observed following a single bout of resistance exercise with a concurrent, but lesser, increase in breakdown (14, 94) leading to an overall increase in net balance. What is discrepant between these studies, however, is the size of this observed increase in protein synthesis. The rise in protein synthesis observed by Chesley and colleagues (31) was half that observed in the other studies (14, 94), and it was reported that protein synthesis remained elevated for less than 36 h (78). In contrast, Phillips et al. (94) observed that synthesis remained elevated for 48h after exercise. These divergent results might be explained by the training status of the participants and the muscle groups studied. While Chesley et al. (31) used subjects who regularly engaged in resistance training and studied the biceps muscle, both Biolo and Phillips (14, 94) used untrained subjects and took biopsies from the vastus lateralis.

*Resistance training affects muscle protein synthesis and breakdown*

As opposed to the acute changes in protein turnover seen immediately following a single bout of resistance exercise, for hypertrophy to occur, acute periods of positive net protein balance would have to accumulate to give rise to muscle fibre hypertrophy, at least that is what has been hypothesized to occur (92, 102, 103) (Figure 3).

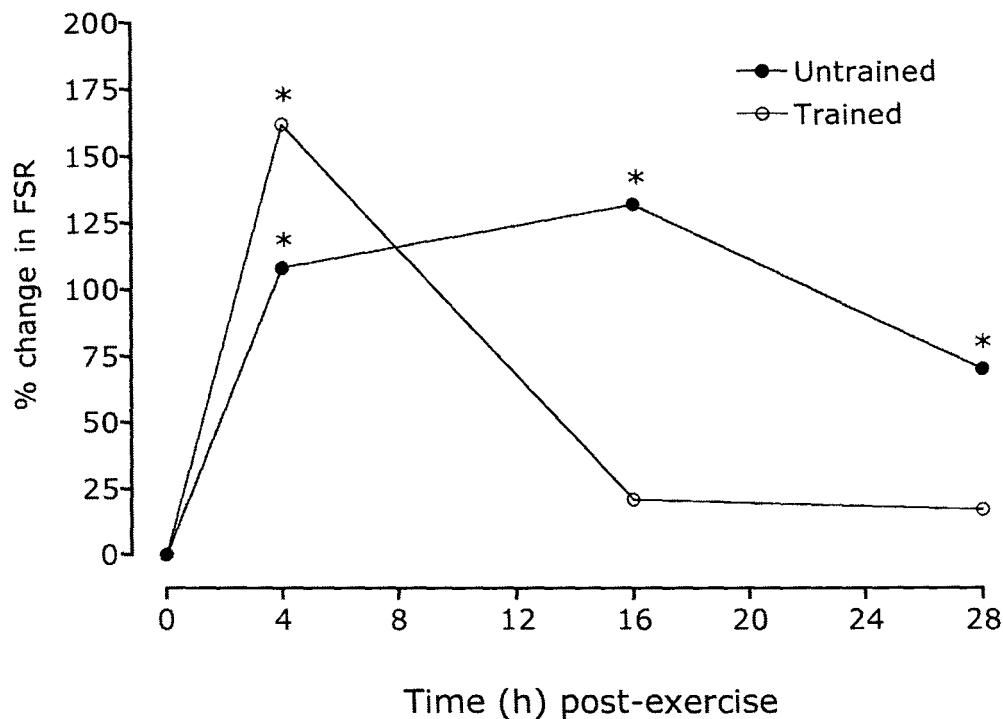


**Figure 3.** Illustration of the effect of a bout of resistance exercise on skeletal muscle protein balance. Exercise-induced protein accretion that occurs with resistance training because of a greater fed-state net balance due to the synergistic stimulation of muscle protein synthesis with feeding and exercise and a lesser fasted-state loss due to the persistent stimulation of protein synthesis in the fasted state. Adapted from (92).

Investigations into the impact of how resistance training (i.e., repeated resistance exercise) impacts the magnitude and the time-course of the post-exercise muscle protein synthesis response have been conducted (31, 78, 93-95). Using a cross-sectional study design, increases in muscle protein synthesis were reported to be attenuated at the same relative intensity (as a percentage of the subjects' single repetition maximum), and therefore a higher total load lifted, in trained as opposed to untrained subjects (95).

Furthermore, it was shown using a longitudinal design that 8 weeks of resistance exercise training attenuated the rise in mixed muscle protein synthesis following exercise at the same absolute intensity (i.e. lower relative intensity post-training), as a true evaluation of the training stimulus per se (93). Because resistance training induces gains in strength, performing exercise at the same absolute level following training would, one might hypothesize, result in a smaller ‘stimulus’ (i.e. stretch/strain or perturbation in homeostasis) for increasing protein synthesis; this would be the most straightforward explanation for the reduced rates of muscle protein synthesis observed with training (93). Again using a longitudinal design, it was shown that even at the same relative intensity of exercise that mixed muscle protein synthesis was reduced (67). What remained unchanged as a result of training was the rise in synthesis of the myofibrillar proteins indicating that the resistance exercise stimulus was perhaps more specific to the proteins that would be responsive and show an increased content as a result of resistance training (67). More recently, using the combined data from two studies, it was reported that over the 28h after resistance exercise, performed at the same relative intensity, mixed muscle protein synthesis was attenuated in the trained condition (67, 114) (Figure 4). However, muscle protein synthesis in the trained leg was higher during the first 4h after resistance exercise than in the untrained leg, showing a more rapid response to the exercise stimulus. Furthermore, muscle protein synthesis had returned to pre-exercise levels in the trained leg at 16h (67) and yet remained elevated in the untrained leg at 28h after exercise (114). Therefore, it appears that resistance exercise training ‘sensitizes’ the muscle to a resistance exercise bout, so that it responds more rapidly, but the time mixed muscle

protein synthesis remains elevated is reduced. Unresolved questions from this work (67, 114) are in what protein fractions (i.e., myofibrillar, non-myofibrillar) the synthetic response is occurring with training and what signaling events might potentially regulate these changes?



**Figure 4.** Time course of the elevation in muscle protein synthesis after a single bout of resistance exercise in the untrained and trained states. \*significantly different from rest ( $p < 0.01$ ). 4 and 28h data from Tang et al. (114) and 16h data from Kim et al. (67).

While the changes in muscle protein synthesis have been fairly well delineated following resistance exercise and resistance training the changes in protein breakdown are less well understood. Phillips et al. (95) found that muscle protein breakdown increased above resting values following exercise only in untrained participants. The decrease in protein turnover (muscle protein synthesis and breakdown) after an acute bout

of resistance exercise observed in resistance trained individuals may be explained by the training-induced reduction in muscle damage, such as Z-band disruption, seen after eccentric exercise (51). Theoretically, if less damage to the muscle protein ultrastructure occurs, then protein breakdown, measured as entrance of amino acids into the free pool (see Fig. 1), would also be reduced. However, such a hypothesis has not been directly tested. Moreover, knowing that training attenuates muscle protein damage (51, 52) then there would be less repair and regeneration, presumably reflected as a reduced rate of protein synthesis. One important consideration is that there are published reports that refute the existence of muscle damage as it is characteristically defined, which is as a mechanical phenomenon caused by high forces that literally overstretch the sarcomere and in so doing mechanically tear the Z-disk apart (97, 98). Alternatively, a series of elegant studies using electron microscopy has shown that the Z-band disruption that is a hallmark of ‘damaged’ muscle is in fact a protein remodeling process (130-132) that would presumably involve areas in which high protein turnover is occurring. A rather glaring gap in our knowledge in this area is the lack of evidence linking Z-band disruption/remodeling to any in vivo measures of protein turnover. A combination of light and electron microscopic evidence combined with established methods to measure protein synthesis and breakdown would represent an attractive way to try and shed light in this understudied area.

While there is clearly an effect of resistance exercise training on the post-exercise response in muscle protein synthesis, there is also evidence that resistance training increases muscle protein synthesis at rest (67, 93, 128, 129). The observation that resting

muscle protein synthesis is elevated following training does not appear to be due to a residual effect of the last exercise training session, as measurements at rest have consistently been made at least 72h after the last exercise bout, which is a time when available data suggest the increase in MPS has returned to baseline (78, 83, 94). Instead, it has been postulated that the increased rates of protein synthesis (and likely also degradation) represent a more rapid rate of protein turnover in muscle that is trained i.e., muscle that is chronically exposed to a remodeling stimulus such as resistance exercise.

*Increased amino acid availability stimulates muscle protein synthesis*

Amino acid availability plays an important role in regulating muscle protein turnover both at rest and following exercise. In resting skeletal muscle, amino acid infusion increases protein synthesis and appears to inhibit protein breakdown (13, 19). There seems to be a latency between the induction of hyperaminoacidemia and the stimulation of protein synthesis of ~30 min, as shown by Bohé et al. (19). After the latency period, muscle protein synthesis increased by 2.8 fold above basal rates for 1.5h. After this time, muscle protein synthesis decreased and was not significantly different from basal rates despite the same degree of hyperaminoacidemia. It is the amino acids *per se* and not a rise in insulin that is responsible for this stimulation of protein synthesis with hyperaminoacidemia (see discussion in following section). In a follow-up study, it was shown that muscle protein synthesis is more of a function of the concentration of blood rather than intramuscular amino acids (18). This is not to say that amino acids within the muscle do not stimulate muscle proteins synthesis, rather a definitive relationship between their concentration and muscle protein synthesis was not found. Any



amino acid-mediated suppression of proteolysis is, however, complicated in interpretation by the rise in insulin that accompanies amino acid infusion/ingestion since insulin markedly suppresses protein breakdown (16, 26, 32, 50).

*Increased amino acid availability and resistance exercise independently and synergistically stimulate muscle protein synthesis*

While investigators have shown that the overall net balance between synthesis and breakdown increases following a bout of resistance exercise in the fasted state, NB remains negative (14, 31, 94, 95). When amino acids are provided (ingestion as pure crystalline amino acids, intact proteins, or infusion) after exercise, net balance becomes positive, and there is a net gain of muscle protein (15, 119). Biolo et al. (15) found that the stimulatory effect of infused amino acids seen at rest was enhanced following a bout of resistance exercise. Tipton and colleagues (119) found a similar result following ingestion of amino acids. Following a bout of resistance exercise, the net protein balance when no amino acids were ingested was negative, but switched to positive when amino acids were ingested. These authors observed that the net balance became positive primarily due to an increase in protein synthesis and amino acids and resistance exercise act synergistically to stimulate protein synthesis (15, 100, 119). Amino acids markedly stimulate the rate of muscle protein synthesis at rest and following exercise in an interactive fashion (14, 15); however, the rate of muscle protein breakdown, which usually increases following performance of resistance exercise (14) does not occur when amino acids are provided (15). Thus amino acids exert a net anabolic influence on muscle protein turnover after resistance exercise by stimulating protein synthesis and suppressing

the rise in muscle protein breakdown that occurs in the fasted state. It is not known whether this suppression of proteolysis is mediated directly through the amino acids themselves or via insulin, which tends to increase in response to amino acid ingestion/infusion (15, 19) and suppresses protein breakdown (32).

*Only essential amino acids promote muscle protein synthesis following exercise*

Research has clearly demonstrated that both resistance exercise and hyperaminoacidemia induce an increase in net muscle protein balance, predominantly through an increase in protein synthesis and also through a small suppression in protein breakdown. Furthermore, when amino acids are provided in conjunction with performing resistance exercise their individual anabolic effects are synergistic. Recent evidence has suggested that the type of amino acids provided influences the response of protein turnover.

Amino acids are classified into two groups based on whether those amino acids can or cannot be endogenously synthesized. So-called essential or indispensable amino acids (there are nine amino acids generally regarded as essential for humans: isoleucine, leucine, lysine, threonine, tryptophan, methionine, histidine, valine and phenylalanine) cannot be synthesized either at all or in sufficient quantities to meet physiological needs and, hence, need to be obtained from exogenous sources (the amino acids arginine, cysteine, glycine, glutamine and tyrosine are considered conditionally essential, meaning they are not normally required in the diet, but must be supplied exogenously to specific populations, or at specific developmental stages, that do not synthesize them in adequate amounts). In contrast, amino acids that can be synthesized in the body at adequate rates to

meet physiological requirements are referred to as nonessential or dispensable amino acids. Provision of essential amino acids by infusion or ingestion stimulates muscle protein synthesis; moreover, the provision of nonessential amino acids are not necessary to stimulate muscle protein synthesis (18, 27, 119, 121).

Several reports have indicated that the provision of the essential amino acid leucine alone can have a stimulatory effect on muscle protein anabolism (68, 71, 111). Smith et al. (111) found that a large dose of leucine stimulated human muscle protein synthesis. Moreover, the addition of leucine to a protein hydrolysate led to a greater stimulation of skeletal muscle protein synthesis after a session of resistance exercise than intake of the hydrolysate without leucine (71). While more study still needs to be done the data seem to indicate that leucine alone may be sufficient in stimulating muscle protein synthesis following exercise (64). At some point, however, one would hypothesize that the leucine-induced rise in protein synthesis would presumably drain essential amino acids from the free pool and the supply would become limiting and protein synthesis would decline.

#### *Different types of protein may affect muscle protein synthesis*

It has been established that different sources of protein induce different patterns of hyperaminoacidemia because of the how they are digested (20, 38). Ingestion of most proteins, of which whey protein is the most often studied, induces a rapid and transient rise in plasma amino acid concentration and hence is referred to as a 'fast' protein. In contrast, casein promotes a slower, more moderate and longer lasting rise in aminoacidemia (20, 38). The major reason why casein is digested so much slower than

other isolated protein sources, including whey and soy protein, is that its protein structure results in the protein effectively clotting in the acid pH of the stomach (85), which slows gastric emptying. In addition, the clotted casein enters the duodenum and is digested much slower than regularly digested proteins that enter the intestine in an unfolded state and more readily digestible.

Comparing the effects of whey and casein protein ingestion, it was shown that ingestion of whey promoted whole-body protein synthesis and oxidation but had no effect on breakdown (38). By contrast, casein did not stimulate synthesis or oxidation but did suppress whole-body proteolysis. As whey and casein proteins do not have the same amino acid composition, some have argued that it is not the protein digestibility *per se*, but the amino acid composition (with whey having a slightly higher leucine content than casein) that is affecting protein kinetics. To try and resolve this, Dangin et al. (38) undertook a study looking at whole-body protein kinetics following ingestion of protein meals with identical amino acid compositions, differing only in their rate of digestion. Casein (slow) was compared to free amino acids of the same composition, acting as a fast-digested meal. A single meal of whey protein (fast) was also compared to repeated small feedings of whey (same total amount of protein but consumed in a temporal manner to mimic slow protein digestion). Stimulation of whole body protein synthesis was marked and rapid after the 'fast' protein meals, whereas it was absent after the slow meals (38). Taken together, it appears that ingestion of whey protein induces a more rapid hyperaminoacidemia, which stimulates whole-body protein synthesis (and oxidation) whereas casein has a less marked effect on whole-body protein synthesis but a greater

suppressive effect on protein breakdown (20, 38). Hence it would appear that a combination of whey and casein might be the most effective in inducing a positive protein balance, due to a simultaneous stimulation of synthesis and suppression of protein breakdown. Whether the results from whole-body studies apply to muscle is difficult to determine since muscle protein synthesis is only ~25% of whole-body protein synthesis (89). If, however, the effects of whey and casein were combined and did have an effect on muscle protein similar to that seen at the whole-body level then a combination of whey and casein would be the best option to maximize net muscle protein gains. Bovine milk contains a combination of whey and casein in a ratio of 1:4 and represents a source of other essential nutrients and as such likely represents the most attractive, and cost effective, protein source that may have the desired affect on muscle protein synthesis.

Support for the hypothesis that milk provides an optimum combination of whey to stimulate synthesis and casein to inhibit breakdown can be seen from whole-body protein turnover data (38), but the effects at the muscle level are not known. Fouillet and colleagues (45) used compartmental modeling to investigate the relative peripheral nitrogen retention (an indication of muscle protein synthesis) following ingestion of milk or soy proteins. It was found that ingestion of isolated soy protein resulted in a lower whole-body retention of dietary nitrogen compared to an isonitrogenous amount of milk protein. As opposed to milk, soy protein was more rapidly digested with a lower transit time and absorption of nitrogen from the intestine, which resulted in the amino acids from this protein being used for plasma protein synthesis by the splanchnic bed and also directed toward urea synthesis. This sequestering of amino acids by the splanchnic bed

caused a subsequent reduction in amino acid uptake in the periphery (i.e., all non-splanchnic tissues). Given the mass of muscle as a contributor to peripheral amino acid uptake, these data suggest that milk protein would promote a greater net retention of protein in muscle as opposed to soy. The effect of different types of protein on protein metabolism in muscle, particularly in combination with exercise, remains to be studied.

*Interaction of resistance exercise with protein ingestion: acute studies*

Until recently, researchers have studied the effect of amino acids on muscle protein turnover by providing pure crystalline amino acids either orally or via infusion. However, for practical purposes, it is important to study how intact dietary proteins affect muscle protein turnover. Furthermore, due to differences in the digestive properties or amino acid composition of different proteins, it is of interest to investigate how different types of dietary protein affect muscle protein turnover. To date, three studies have shown that ingestion of whole proteins following resistance exercise can support a positive muscle protein balance (41, 117, 118). All three studies have examined the effect of fluid milk (41) or its constituent protein fractions, whey and casein (117, 118) on muscle protein balance. Elliott et al. (41) found that whole milk was better able to support net muscle protein synthesis than skim milk. Surprisingly, Tipton et al. (118) found no difference in the time-course of aminoacidemia or net muscle protein balance with ingestion of whey or casein proteins following resistance exercise. No study to date has compared proteins from animal versus plant sources.

*The effect of insulin and carbohydrate on muscle protein metabolism*

The impact that insulin has in modulating muscle protein synthesis is controversial. The most widely held view is that increased systemic insulin alone cannot support a full synthetic response in the absence of a sufficient hyperaminoacidemia. Rather, insulin acts in a permissive and mildly facilitative fashion at low concentrations when there are sufficient amino acids present to stimulate muscle protein synthesis (12, 48, 82, 100, 103). For example, two recent studies have shown that muscle protein synthesis is stimulated in response to hyperinsulinemia only when sufficient amino acids are present (12, 48). While it seems that insulin's role in promoting muscle protein synthesis is minimal, there is no doubt that insulin markedly inhibits muscle protein breakdown at rest and following resistance exercise (16, 26, 50). Recent evidence from Greenhaff and co-workers (M.J. Rennie, personal communication) has shown that when insulin is clamped at  $5 \mu\text{U}\cdot\text{ml}^{-1}$  (i.e., a typical resting level in healthy persons) that a 3-fold increase in blood amino acids nearly doubled leg muscle protein synthesis (measured as direct incorporation). At the same amino acid concentration, increasing insulin to  $30 \mu\text{U}\cdot\text{ml}^{-1}$  halved leg protein breakdown but synthesis remained unchanged.

Studies, where carbohydrate is provided following resistance exercise (25, 26, 84, 101, 104), suggest that adding carbohydrate to an amino acid mixture following a bout of resistance exercise may curb protein breakdown, and improve the net protein balance beyond what is observed with amino acid ingestion alone. For example, Miller et al. (84) observed that addition of carbohydrate to an amino acid mixture did not result in a greater stimulation of muscle protein synthesis than amino acids alone, but that net protein

balance was improved when carbohydrate was added to the amino acids. The problem with this result, was that the amino acid dose used (3g of crystalline essential amino acids) was subsequently shown to be insufficient to maximally stimulate muscle protein synthesis (27), thus it may be more applicable that with insufficient amino acid/protein ingestion insulin exerts an effect on protein balance. By comparison to the effect of amino acids on muscle protein synthesis, carbohydrate-induced insulin secretion would have a comparatively minor effect in improving muscle protein balance.

### **The effect of endurance exercise and feeding on skeletal muscle protein metabolism**

The majority of studies that have examined the effect of exercise on skeletal muscle protein turnover have focused on resistance exercise due to the anabolic nature of the stimulus. There are some exceptions, in which the response of muscle protein turnover to endurance exercise has been reported (29, 83, 109, 120). Resistance exercise training results in well documented changes in skeletal muscle protein content (9, 94, 95, 116, 129); which might explain the focus of researchers on the effects of resistance exercise on skeletal muscle protein metabolism. However, endurance exercise also results in a changes in muscle proteins, including increases in mitochondrial content (43, 53, 60, 86) and, as a result, increases in oxidative enzyme content and activity (59, 76, 113). Endurance training has also been shown to induce a change in fibre type toward more oxidative type IIa, and arguably type I, fibres and a lower muscle fibre area (112). Thus, even though the net result of endurance training is not hypertrophy, the increased mitochondrial content and possibly an increase in connective tissue and other



cytostructural proteins (79, 113) would appear to indicate that even endurance exercise would likely be associated with increases in protein synthesis.

Several studies have shown that muscle protein synthesis is elevated following both moderate (29, 109) and intense (83) endurance exercise. However, Tipton et al. (120) found no significant change (despite a 42% increase in mixed muscle fractional synthetic rate in the swimming only condition) in muscle protein synthesis following intense swimming exercise. Using electrical stimulation in isolated rat muscle to mimic endurance exercise or resistance exercise, Atherton et al. (5) reported increased myofibrillar protein synthesis following resistance-type contractions and increased PGC-1 $\alpha$ , a transcriptional cofactor known to increase mitochondrial biogenesis, following endurance-type contractions. This study supports the contention that resistance and endurance exercise induce protein synthesis in differing fractions of muscle protein, most obviously myofibrillar versus mitochondrial proteins. However, this hypothesis remains to be tested. To date only one study has measured protein breakdown following endurance exercise (109). A small transient increase in muscle protein breakdown was observed from measurements made 10 minutes after 45 minutes of treadmill walking at 45% of  $\text{VO}_{2\text{peak}}$ ; however, it returned to resting levels by 1h after exercise (109). Further study will need to determine how intensity and duration of the exercise, training status of the participants and provision of nutrients (protein, amino acids and carbohydrate) affects muscle protein synthesis and breakdown following endurance exercise.

### **Mixed and sub-fraction skeletal muscle protein synthesis**

In most studies the measure of protein synthesis utilizes the entire muscle biopsy. Since skeletal muscle consists primarily of myofibrillar proteins (~70% of protein by weight) (8), changes in mixed muscle protein synthesis could mask changes (or a lack thereof) in the synthetic rate of proteins that are relatively smaller sub-fractions of skeletal muscle proteins such as enzyme, signaling, mitochondrial and/or structural proteins if the response in the myofibrillar proteins were much greater or less than that of the other protein fractions. In contrast, the measure of mixed muscle protein synthesis represents an average measure of synthesis rates of all proteins and their respective protein subfractions, and even changes in contractile protein synthesis may not be entirely reflected in measures of mixed muscle protein synthesis if the stimulus induces synthesis of mainly mitochondrial proteins for example.

Stimulation of muscle protein synthesis by amino acid infusion led to changes in mixed muscle protein synthesis that were closely paralleled by changes in myofibrillar, sarcoplasmic and mitochondrial protein synthesis (19). This would be the expected result since amino acid provision should stimulate a rise in the synthesis of all proteins. The same cannot be said of exercise, however, where it has been shown that chronic performance of resistance exercise (i.e., training) results in attenuated mixed but not myofibrillar protein synthesis following an acute bout of exercise (67, 124). Because different proteins within skeletal muscle may respond differently to various loading stimuli, and measures of mixed muscle protein synthesis does not reflect changes in different types of proteins, future studies will need to examine the effect of various

stimuli on particular subfractions of muscle (see appendix I for an expanded discussion of this topic).

### **Regulation of muscle protein synthesis**

Characterization of the full spectrum of control of protein synthesis and breakdown following exercise is complex and has not been fully elucidated. Highly regulated and potentially flux generating steps that could affect protein turnover include: transcriptional changes in messenger RNA (mRNA) abundance (and also mRNA stability), changes in mRNA processing following transcription, changes in translation efficiency and post-translational modifications of proteins (10).

#### *Changes in mRNA translation regulate skeletal muscle protein synthesis after acute resistance exercise*

Muscle protein synthesis has been shown to increase an hour or two after exercise (14, 29, 31, 94, 109, 114). Therefore, it has been hypothesized (100) that a change in translation of mRNAs and not an increased abundance of mRNA (transcription) most likely explains much of the acute increase in protein synthesis following exercise. The observation that mixed muscle protein synthesis was elevated following resistance exercise in humans (31, 123) and in rats (42) with no concurrent changes in total RNA levels lends some weight to the hypothesis that changes in translation represents the primary locus of control, and not transcription, in the increase in protein synthesis following acute exercise. Large changes in specific mRNAs could occur without any change in total RNA, however. Welle et al. (123) found that 23h after resistance exercise there was no change in the levels of mRNA encoding for myosin heavy chain, despite an

observed increase in myofibrillar protein synthesis compared to the resting control. Furthermore, Kubica et al. (74) found that total eIF2B $\epsilon$  protein increased, with no concomitant change in the expression of eIF2B $\epsilon$  mRNA, 3h and 24h following resistance exercise in rats. Only at 48h was there a change in eIF2B $\epsilon$  mRNA expression. While there is evidence that acutely, muscle protein synthesis following exercise is controlled through changes in translational efficiency and not increased transcription (31, 42, 74, 123), others have found an increases in mRNA transcripts within hours following an acute bout of resistance (72, 96, 127) and endurance exercise (80, 96). Without changes in the synthesis rates of individual proteins that correspond to mRNA encoding that protein the exact relationship remains difficult to discern. Within the human genome, there is the potential for ~85,000 different mRNAs to be expressed (11). Although only a small proportion of these gene transcripts and their proteins are relevant to the regulation of protein turnover following exercise and feeding within skeletal muscle, the task of determining the time course of changes of these genes and their proteins, let alone the individual protein synthetic rates, would be complex. Furthermore, the ability to correlate the changes in mRNA with protein levels is a challenge due to the transient nature of increased mRNA following exercise (72, 80) and the post-translational modification or selection of which mRNAs will be translated. Therefore, further study needs to be undertaken to fully determine how protein turnover is controlled following acute exercise.

*The effect of exercise training on translation and transcription*

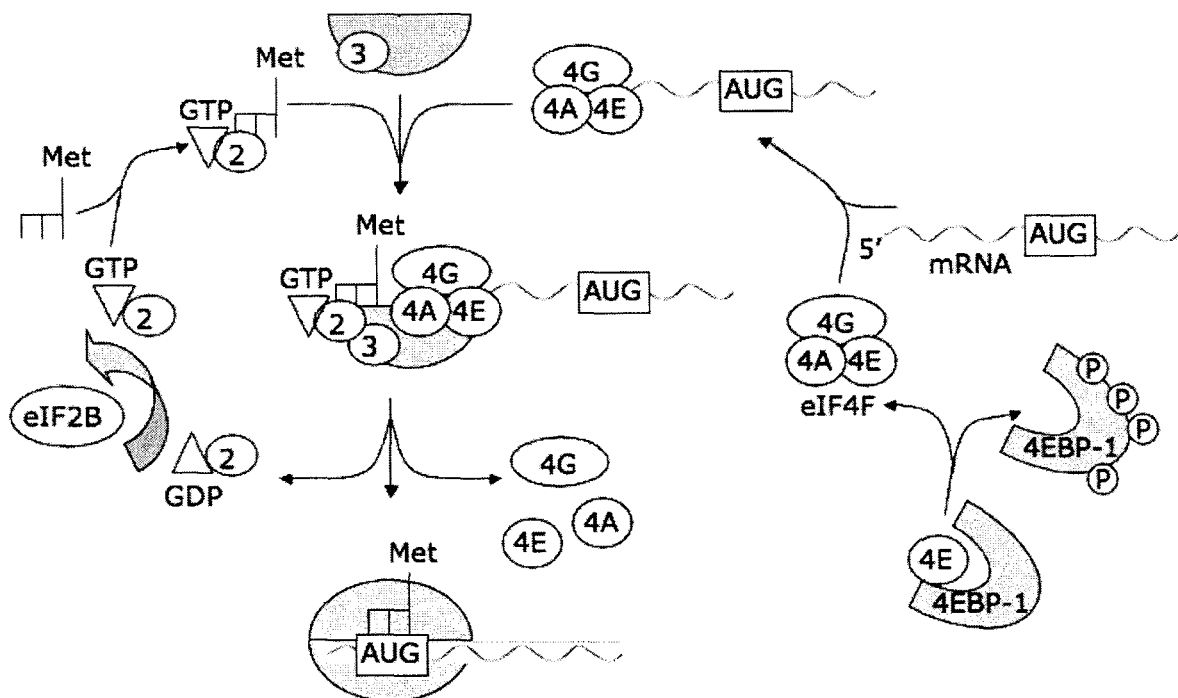
Although increased protein translation appears to be the flux generating step for muscle protein synthesis after acute resistance exercise (31, 42, 74, 123), exercise training may change the transcription of relevant genes involved in the phenotypic adaptation associated with the mode of exercise (90, 122). Some researchers have found a relationship between training induced increases in particular proteins and increases in their mRNA transcripts (90, 122) while others have not (75, 110). For example, Turnstall et al. (122) found that following a single bout of endurance exercise no changes were observed in the transcription of genes involved in skeletal muscle fatty acid metabolism. However, following endurance training, changes in lipid oxidation and abundance of proteins involved in lipid metabolism were related to changes in the expression of genes involved in skeletal muscle lipid metabolism. In contrast, Leblanc et al. (75) found that an increased pyruvate dehydrogenase protein expression following 8 weeks of endurance training with no concurrent change in mRNA levels. Because there are thousands of proteins changed in response to exercise (11), it is not surprising that some of the changes are related to increased transcription while others are due to increased translation. It may also be that far more subtle changes in the timing of increases/decreases in mRNA or protein translation/abundance are missed when only discrete time points are measured following isolated acute exercise bouts. Further, changes in nutritional status could also influence the changes in gene transcripts and also protein synthesis. Thus, mismatches in gene abundance changes and protein synthesis, or protein abundance are hardly surprising given the limited data that is available, particularly in humans. Clearly, further

study is required to determine the impact of exercise training on gene expression and protein translation.

#### *Overview of translation initiation*

There has been a sharp increase in work attempting to understand how protein translation is controlled in response to different stimuli in intact skeletal muscle. Most recent findings come from investigations using skeletal muscle cells *in vitro* and animal models of exercise (23, 99). However, recently a number of published reports with humans have reported interesting results (33, 36, 37, 39, 40, 46, 47, 63, 70, 77, 115). Translation of mRNA involves three distinct stages: initiation, elongation and termination (22, 99). Although all three of the stages are subject to regulation, translation initiation appears to be the most tightly controlled and is thought to be the flux generating step in protein synthesis (22, 88, 99). Changes in protein synthesis are controlled by changes in the activity of eukaryotic initiation factors (eIF) (23, 99), the on/off status of which is regulated by direct covalent addition or removal of phosphate groups. The process of translation initiation consists of several steps (Figure 5) (23, 99). First, eIF1A, eIF3 and eIF6 promote the dissociation of 80S ribosomes, usually present as polysomal aggregates, into individual 60S and 40S subunits. A complex consisting of guanosine triphosphate (GTP), eIF2 and the initiator methionyl-tRNA (met-tRNA), containing the anticodon for the AUG start sequence on mRNA, is formed. Once the eIF2-GTP-met-tRNA complex is formed it can bind to the 40S ribosomal subunit resulting in the 43S preinitiation complex. When the 43S preinitiation complex is formed, it is capable of binding mRNA. Although, before the 43S preinitiation complex binds with mRNA, the eIF4F complex

must form on the mRNA. When eIF4E is released from a protein-specific binding protein (4EBP-1), it binds to the 7-methylguanosine ( $m^7$ GTP) cap located at the 5'-end of the mRNA. It forms the eIF4F complex with eIF4A, a RNA helicase that unwinds the mRNA's secondary structure and eIF4G, a scaffold protein that links all three proteins and stabilizes the complex. Once the eIF4F complex is formed with the mRNA,

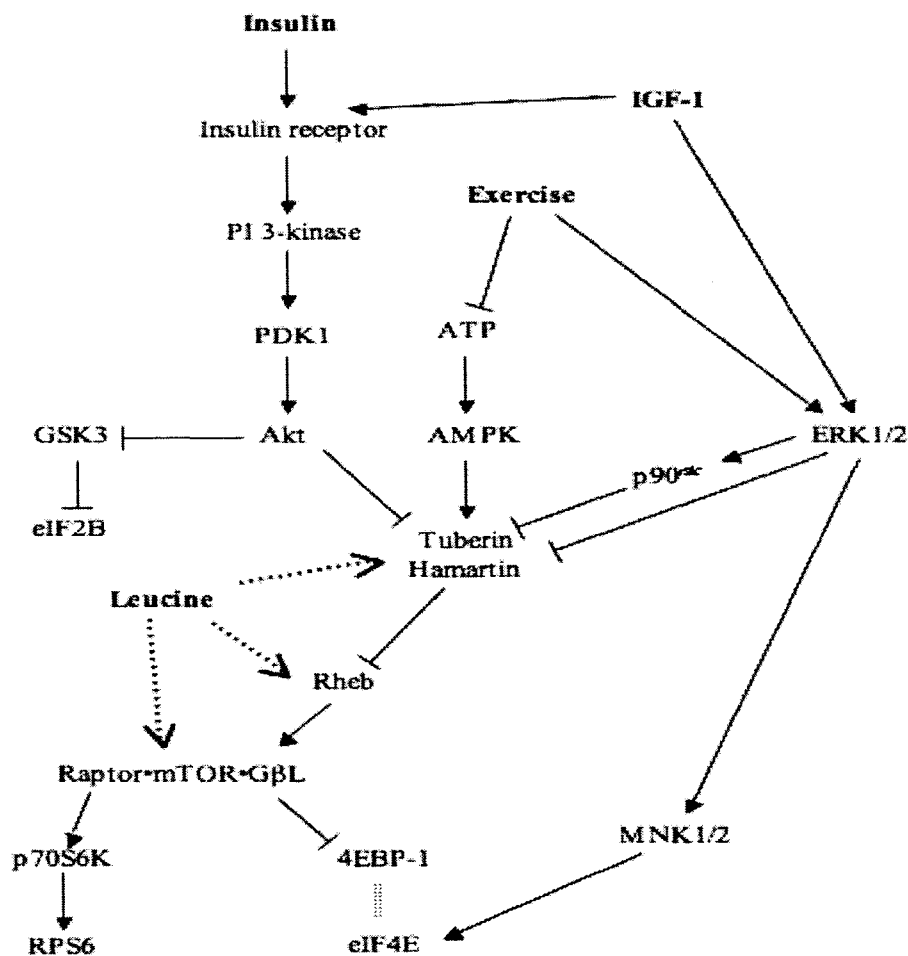


**Figure 5.** Illustration of key steps in translation initiation. The names of the eukaryotic initiation factors (eIF) are designated by number (i.e. 2 represents eIF2 and 4E represents eIF4E). Modified from (22).

it mediates the binding of the mRNA to the 43S preinitiation complex resulting in the 48S initiation complex. The mRNA is scanned to identify the 5' AUG start sequence. In the final step before elongation begins, eIF5 promotes the hydrolysis of GTP bound to eIF2 causing the eIF2-GDP complex to be released from the 48S initiation complex. The

48S initiation complex then attaches to the 60S ribosomal subunit which forms the 80S initiation complex. At this time, the other initiation factors are released.

The phosphatidylinositol-3 (PI3) kinase-Akt-mammalian target of rapamycin (mTOR) signal transduction pathway (Figure 6) has been linked to the control of two key steps in the regulation of translation initiation: the formation of the 43S preinitiation complex and the binding of the mRNA to that complex (5, 6, 17, 23, 55, 87).



**Figure 6.** Regulation of signaling proteins involved in translation initiation. Signaling is affected by insulin or IGF-1, amino acids (in particular leucine) and exercise. Lines ending in arrowheads denote activation, whereas lines ending in perpendicular lines denote inhibition. Dashed lines represent incompletely defined steps in the pathway. Adapted from (22, 69, 126).



Briefly, the binding of insulin or IGF-1 to the insulin receptor results in the activation of PI3 kinase. Through a cascade of protein-protein interactions, protein kinase B (PKB, also termed Akt) is phosphorylated. Phosphorylation of Akt regulates the phosphorylation of glycogen synthase kinase 3 (GSK-3) as well as a downstream protein, mTOR. mTOR phosphorylates 4EBP-1 and the 70 kDa ribosomal protein S6 protein kinase (p70S6k), which, in turn, phosphorylates ribosomal protein S6 (rpS6), a component of the 40S subunit. Recent studies have shown that there are multiple sites within this pathway where amino acids and exercise may exert their effects on protein synthesis, that are independent of stimulation of the insulin/IGF-I receptor (shown in Figure 6 and discussed in detail below).

One of the key regulated processes by PI3 kinase-Akt-mTOR pathway is the formation of the eIF2-GTP-met-tRNA complex which when bound to the 40S ribosomal subunit forms the 43S preinitiation complex (Figure 5). After a round of translation initiation, the GTP on eIF2 is hydrolysed to GDP. Before a new round of translation initiation can begin, eukaryotic initiation factor 2B (eIF2B) must catalyze the exchange of GDP for GTP on eIF2 so it can bind Met-tRNA. eIF2B is regulated either directly through the phosphorylation of its  $\epsilon$ -subunit or indirectly through the phosphorylation of the  $\alpha$ -subunit of eIF2, which then acts as a competitive inhibitor of the guanine nucleotide exchange reaction. eIF2B $\epsilon$  activity is inhibited by phosphorylation mediated by GSK-3 (Figure 6). Therefore, the upstream phosphorylation of Akt, in turn, phosphorylates and inhibits the activity GSK-3, which relieves the inhibition on eIF2B $\epsilon$  and results in enhanced guanine nucleotide exchange.

Another key regulated process is the binding of the mRNA to the 40S ribosomal subunit, mediated by the formation of the eIF4F complex on the mRNA. Both 4EBP-1 and eIF4G bind to the same site of eIF4E. Therefore, 4EBP-1 inhibits the formation of the eIF4F complex and the binding of the mRNA to the 40S ribosomal subunit by binding and sequestering eIF4E (Figure 5). The interaction between 4EBP-1 and eIF4E is regulated by mTOR, part of the PI3 kinase-mTOR pathway. Hyperphosphorylation of 4EBP-1, catalyzed by mTOR, releases eIF4E to associate with eIF4G and form the eIF4F complex (Figure 6).

A final mechanism of regulated processes by PI3 kinase-Akt-mTOR pathway is downstream phosphorylation of rpS6 by p70S6k, the phosphorylation of which is also controlled by mTOR (Figure 6). Until recently, it was thought that phosphorylation of rpS6 promoted the preferential translation of 5'-terminal oligopyrimidine mRNAs that encode for ribosomal proteins; however, recent evidence has suggested that this is not the case (91, 105). Instead, phosphorylation rpS6 has been linked to the regulation of cell size (91, 105), how this occurs remains unclear at this time.

The phosphorylation of eIF4E is not regulated by the PI3 kinase-Akt-mTOR pathway, rather by the ras/MAP kinase pathway (Figure 6) (10, 99). The functional significance of eIF4E phosphorylation remains unclear; however, Scheper et al. (108) have suggested that the phosphorylation of eIF4E subsequent to formation of the initiation complex may allow for the eIF4F complex to detach from the 5'-cap during scanning. This would allow for the rapid recruitment of the next initiation complex or

rendering the cap binding factors available for translation of different mRNAs, which would accelerate translation.

### **The effect of amino acids and exercise on translation initiation in skeletal muscle**

Proteins associated with translation initiation can be rapidly (within minutes) activated by a variety of stimuli including amino acids (1, 2, 4, 35) and exercise (5, 23, 87, 126). The mechanisms by which amino acids and exercise affect translation initiation pathways are not completely understood. Within the last few years, new understanding has been gained from work mostly using animal models.

#### *Amino acids are independent stimulators of protein translation in skeletal muscle*

Provision of amino acids has been shown to stimulate muscle protein synthesis in human skeletal muscle at rest (19). Recently, it has been shown that proteins involved in translation initiation are activated in response to branch-chain amino acids and, in particular, to leucine (1, 4). Leucine has no effect on eIF2B or eIF2 $\alpha$ , the two proteins that regulate binding of met-tRNA to the mRNA (1, 35). However, leucine increases phosphorylation of p70S6k, rpS6, 4EBP-1 and the association of eIF4E with eIF4G (1, 35).

Provision of leucine induces a small, transient increase in insulin concentration (2), but to a concentration lower than those seen with the provision of carbohydrate. When leucine was provided during somatostatin administration, to maintain basal fasting levels of insulin, stimulation of protein synthesis and 4EBP-1, p70S6k and rpS6 phosphorylation was attenuated (2). In diabetic rats, administration of leucine alone has no effect on 4EBP-1 and p70SK phosphorylation (2). These studies suggest that leucine

stimulates protein synthesis in insulin-dependent fashion through the activation of mTOR, p70S6k and 4EBP-1, but does not involve activation of the PI3 kinase or Akt. Further evidence for this mechanism comes from observations by Anthony et al. (4) who found that injection of rapamycin, an inhibitor of mTOR, into skeletal muscle entirely blocks the stimulation of downstream targets of mTOR and attenuates the leucine-induced rise in protein synthesis. While there is evidence that leucine stimulates protein synthesis in an insulin-dependent fashion, there is evidence of leucine stimulation via an insulin-independent mechanism to affect the association of eIF4G and eIF4E, perhaps through increased phosphorylation of eIF4G (24).

The rate of protein synthesis in the diabetic rats provided with leucine are well below that observed in non-diabetic controls (3). When insulin is infused in combination with leucine, protein synthesis is elevated above that of leucine provision alone. Furthermore, 4EBP-1 and p70SK phosphorylation is enhanced. Therefore, it appears insulin-dependent and -independent mechanisms can work separately to enhance protein synthesis.

Recently, the mechanisms involved in the leucine stimulation of signaling through mTOR have been examined; however, much still remains to be discovered (Figure 6). Two proteins that are physically linked with mTOR, regulatory associated protein of mTOR (raptor) and G protein  $\beta$ -subunit-like protein (G $\beta$ L) have been shown to be affected by amino acids and positively enhance the activity of mTOR (65, 66). Directly upstream, Rheb (Ras homolog enriched in the brain) has been shown to bind to mTOR in response to amino acid provision and enhances p70S6k, rpS6 and 4EBP-1

phosphorylation when it is overexpressed (69). Rheb activity is inhibited by two proteins, tuberlin and hamartin (also known as TSC1 and 2), that function in a complex. Tuberlin is directly phosphorylated and inhibited by Akt, suppressing the inhibitory action of the tuberlin-hamartin complex on mTOR. Evidence suggests that amino acids can also directly inhibit the activity of tuberlin and hamartin (69). Further study will need to delineate the specific mechanisms of how these proteins are affected by amino acids.

In humans, provision of exogenous EAA while glucose and insulin were kept at basal fasting levels increased muscle protein synthesis and the phosphorylation of mTOR, p70S6k and 4EBP-1 (36). Similar stimulation of muscle protein synthesis and mTOR, p70S6k and 4EBP-1 phosphorylation were seen when EAA were ingested with carbohydrate that increase circulating insulin (47). Additionally, the phosphorylation of Akt increased and AMPK $\alpha$  decreased (47). While a greater array of cellular signaling proteins need to be studied in humans, the initial results (36, 47) from studies on the effect of amino acids on translational control of protein synthesis corroborate what has been found in animal models.

#### *Resistance exercise stimulates protein translation in skeletal muscle*

Many studies have shown that resistance exercise increases the rate of muscle protein synthesis (14, 31, 78, 94, 114). Recently, activation of the PI3 kinase-Akt-mTOR pathway has been shown to increase following resistance exercise or contractions induced by electrical stimulation in association with an increase in muscle protein synthesis in animals (5, 6, 17, 23, 73, 74, 87) and humans (33, 37, 39, 40, 46, 63, 70, 77, 115). For example, Dreyer et al. (39) found an acute bout of resistance exercise in the

fasted state led to an increased phosphorylation of Akt, mTOR and p70S6k with a concurrent increase in skeletal muscle protein synthesis.

An unanswered question is how mechanical strain from resistance exercise is translated into chemical signal transduction in skeletal muscle? It has been proposed that muscle-specific IGF-1 splice isoforms production following resistance exercise could activate, in an autocrine or paracrine fashion, the PI3-kinase-Akt-mTOR pathway (10). However, PI3-kinase activity has not been shown to increase until hours after the resistance exercise bout is complete (28, 58), while changes in the phosphorylation of Akt (5, 23, 87), mTOR (5), p70S6k (5, 40), 4EBP-1 (5, 23), GSK3 $\beta$  (5), and rpS6 (23, 33, 40) are seen within minutes after exercise. This suggests that some other mechanism is activating the immediate post-exercise upregulation of translation.

It is possible that the stretch of the membrane itself might stimulate membrane-associated proteins, called integrins, to transduce the signal into a cellular response. Examples of integrins include PI 3 kinase or focal adhesion kinase (FAK) (30). FAK, a protein found at the skeletal muscle membrane, has been suggested as a possible integrator of the mechanical stimulus and integrin signaling in hypertrophying skeletal muscle because stretch has been shown to activate FAK in muscle (44). Furthermore, following ablation-induced overload in rats, there was a significant increase in total FAK and it also in its phosphorylation (54). More recently, 14d of single leg immobilization in humans, which was associated with a progressive decline in resting fasted-state muscle protein synthesis of 53%, was shown to result in a 30% reduction in phosphorylation of FAK (E. Glover, unpublished results). Thus, FAK or other integrins may provide the key

to understanding how mechanical force on the muscle is transduced to increase mRNA translation via signal transduction pathways.

*Resistance exercise combined with amino acid provision increases muscle protein synthesis by affecting intracellular signaling*

It has been well established that provision of amino acids/protein with concurrent resistance exercise synergistically enhances muscle protein synthesis (15, 25, 27, 84, 101, 118, 119). While some studies have shown that activation of the PI 3-kinase-Akt-mTOR pathway occurs following resistance exercise in the fasted state (33, 39, 40, 46), two recent studies have found that p70S6k and rpS6 were only phosphorylated following resistance exercise if large doses of BCAA (63) or protein (70) were ingested. Because hyperaminoacidemia (36, 47) and resistance exercise (33, 39, 40, 46) can independently activate translation initiation via multiple signaling proteins (69) (Figure 6), future studies should investigate the effect of resistance exercise on cellular signaling when amino acids or protein are and are not provided. For example, exercise and leucine can both directly or indirectly affect upstream regulators of mTOR. Exercise is known to stimulate ERK1/2 which, through a signaling cascade, affects the tuberin/hamartin complex, which in turn increases the activation of mTOR. At the same time, amino acids can activate these same upstream regulators of mTOR.

*Endurance exercise affects translation initiation proteins differently than resistance exercise*

Resistance exercise in combination with the provision of amino acids increases muscle protein synthesis by affecting intracellular signaling (63, 70). With repeated bouts

of resistance exercise (training), this increased muscle protein synthesis in the recovery period results in increased contractile protein deposition (hypertrophy). While it has been shown that endurance exercise can increase the rate of muscle protein synthesis during recovery (29, 83, 109), phenotypic adaptations to repeated bouts of endurance exercise differ substantially from those seen following resistance training. The activation of AMP-activated protein kinase (AMPK) in response to endurance exercise may mediate differences in cell signaling events during recovery from exercise (5, 21, 69). AMPK is activated by changes in AMP produced when ATP levels fall, and therefore, acts as an energy barometer of the cell (57). AMPK is activated during and following exercise (56) and activation of AMPK is associated with repression of mTOR signaling by activation of tuberin (21, 69, 125, 126).

Low-frequency stimulation or endurance exercise in rodents (88, 106, 107) and humans (33, 81) have consistently shown an increased activity of Akt and GSK-3 in the recovery period following exercise. Downstream targets of mTOR, such as p70S6k, rpS6 and 4EBP-1, are not activated during this time (5, 33, 49, 81, 88). It is likely that the activation of AMPK seen immediately following endurance exercise (5, 33) affects phosphorylation of tuberin and results in repression of signaling through mTOR (69) and therefore, suppresses the activation of its downstream targets.

It is still unclear what drives the increased muscle protein synthesis seen following endurance exercise. The phosphorylation of GSK-3 by Akt likely results in enhanced guanine nucleotide exchange activity by the reversal of eIF2B $\epsilon$  inhibition and therefore, could be the cause of increased muscle protein synthesis. In the absence of



changes in p70S6k and rpS6 it is difficult to imagine how this might occur. One potential mechanism is that AMPK may activate transcription factors (PGC-1, NRF, HIF-1) that are key activators of nuclear gene transcription encoding a range of mitochondrial enzymes (5, 7). This would at least provide a greater abundance of mRNAs encoding mitochondrial proteins, which, if the translational machinery were active, would then be translated. Clearly, there are differences in signaling events following an acute bout of endurance versus resistance exercise, however, the mechanism of how contraction type affects which proteins are synthesized has not been conclusively determined.

*Combining endurance exercise and amino acids*

While little research exists, one study has tested the effect of provision of a protein (and carbohydrate) beverage after endurance exercise on cell signaling in humans (61). Ivy et al (61) reported that when a carbohydrate and protein drink was provided after endurance exercise, greater phosphorylation of Akt, mTOR, rpS6 and GSK3 was observed than after exercise alone. The surprising observation that endurance exercise in the fasted state increased phosphorylation of p70S6k and rpS6 but not Akt, mTOR or GSK above resting values (61) is contrary to what others have found (33, 81). Two studies performed in the fasted state (33, 81) have reported that phosphorylation of proteins downstream of mTOR did not occur following endurance exercise, likely because of AMPK's inhibition of mTOR, while upstream proteins were activated. Further study is warranted to examine the activation of protein in the PI 3-kinase-Akt-mTOR pathway with provision of amino acids following exercise.

*The effect of exercise training on protein translational signaling proteins*

The majority of studies to date have examined intracellular signaling following an acute bout of exercise, but we know that the protein synthetic response changes in response to exercise training (67, 93, 95, 114). Resistance exercise training-induced changes in intracellular signaling proteins in humans have been reported in only a single study. From this work it was reported that resistance exercise training results in greater resting levels of Akt, GSK3 and mTOR (77). How exercise training affects the acute response in cellular signaling remains to be determined.

**Study Objectives**

The overarching aim of the studies contained in this thesis was to further our understanding of the nature of the changes in human muscle protein turnover in response to feeding (from different protein sources), exercise (both endurance- and resistance-based) and the effect of repeated exposure to endurance and resistance exercise (i.e., training). A further aim was the investigation of the underlying mechanisms of how human muscle protein turnover is controlled by feeding and exercise through the use of a human unilateral-based model of resistance/endurance exercise and resistance/endurance exercise training. Methodology employed included infusion of stable amino acid tracers to measure protein turnover, HPLC to measure changes in amino acid concentrations in blood and muscle, and Western blot techniques to measure intracellular signaling proteins abundance and phosphorylation status.

## **Specific Studies and Accompanying Hypotheses**

### **Study 1**

**Aims:** To establish whether a unilateral leg exercise model that results in phenotypic changes, measured as muscle hypertrophy, is confined to the exercised leg only. By corollary there were be little-to-no acute genotypic or phenotypic changes as acute responses to each exercise bout in the contralateral unexercised leg. To examine changes in endogenous systemic hormones to determine whether these changes play a role in the hypertrophic response and whether such changes could potentially ‘spill over’ and affect phenotypic or genotypic responses in the contralateral leg.

**Hypotheses:** We hypothesized that acute unilateral resistance exercise would not affect circulating endogenous hormone concentrations and that skeletal muscle hypertrophy would occur in the trained limb only.

### **Study 2**

**Aims:** To investigate the effect of oral ingestion of either fluid non-fat milk or an isonitrogenous and isoenergetic, and macronutrient composition matched, soy protein beverage on whole body and muscle protein turnover following an acute bout of unilateral resistance exercise in trained males.

**Hypotheses:** We hypothesized that ingestion of milk protein would stimulate muscle anabolism to a greater degree than ingestion of soy protein due to data from previously published work showing that milk proteins more effectively supported peripheral nitrogen retention whereas soy proteins were sequestered in the splanchnic bed.

### **Study 3**

**Aims:** To investigate how acute resistance and endurance exercise affected myofibrillar and mitochondrial protein synthesis prior to and following 10 weeks of training. To investigate potential cellular signaling mechanisms that may be responsible for the increased muscle protein synthesis observed following both forms of exercise.

**Hypotheses:** We hypothesized that translation initiation factors would be activated (whether by phosphorylation/dephosphorylation as appropriate) to promote a non-exercise-specific response in both myofibrillar and mitochondrial protein synthesis after acute resistance and endurance exercise in the untrained state. However, after training we expected a different response, in that training would attenuate, versus the acute response in the untrained state, the rise in protein synthesis and that each exercise mode would promote a protein synthetic response specific to the phenotypic changes that form of exercise typically induces. Alternatively stated, resistance exercise would stimulate exclusively myofibrillar and endurance exercise exclusively mitochondrial protein synthesis. An attenuation of the activation of translation initiation factors would also occur in parallel with the dampened rise in protein synthesis.

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## STUDY 1

Hypertrophy with unilateral resistance exercise occurs without  
increases in endogenous anabolic hormone concentration

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## Hypertrophy with unilateral resistance exercise occurs without increases in endogenous anabolic hormone concentration

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**Abstract** We aimed to gain insight into the role that the transitory increases in anabolic hormones play in muscle hypertrophy with unilateral resistance training. Ten healthy young male subjects ( $21.8 \pm 0.4$  years,  $1.78 \pm 0.04$  m,  $75.6 \pm 2.9$  kg; mean  $\pm$  SE) engaged in unilateral resistance training for 8 week (3 days/week). Exercises were knee extension and leg press performed at 80–90% of the subject's single repetition maximum (1RM). Blood samples were collected in the acute period before and after the first training bout and following the last training bout and analyzed for total testosterone, free-testosterone, luteinizing hormone, sex hormone binding globulin, growth hormone, cortisol, and insulin-like growth factor-1. Thigh muscle cross sectional area (CSA) and muscle fibre CSA by biopsy (*vastus lateralis*) were measured pre- and post-training. Acutely, no changes in systemic hormone concentrations were observed in the 90 min period following exercise and there was no influence of training on these results. Training-induced increases were observed in type IIX and IIa muscle fibre CSA of  $22 \pm 3$  and  $13 \pm 2\%$  (both  $P < 0.001$ ). No changes were observed in fibre CSA in the untrained leg (all  $P > 0.5$ ). Whole

muscle CSA increased by  $5.4 \pm 0.9\%$  in the trained leg ( $P < 0.001$ ) and remained unchanged in the untrained leg ( $P = 0.76$ ). Isotonic 1RM increased in the trained leg for leg press and for knee extension ( $P < 0.001$ ). No changes were seen in the untrained leg. In conclusion, unilateral training induced local muscle hypertrophy only in the exercised limb, which occurred in the absence of changes in systemic hormones that ostensibly play a role in muscle hypertrophy.

**Keywords** Testosterone · Growth hormone · Strength · Weightlifting

### Introduction

Muscle hypertrophy resulting from resistance training arises due to an accumulation of multiple post-exercise periods of positive protein balance (Phillips 2004; Phillips et al. 2005; Rennie et al. 2004; Rennie and Tipton 2000). Evidence showing that provision of exogenous anabolic hormones to humans, such as testosterone (in various forms) and growth hormone, is very strong insofar as testosterone's action as an anabolic agent is concerned (Bhasin et al. 1996; Sinha-Hikim et al. 2002, 2003b). What is also clear is that when supraphysiological doses of testosterone are administered that they synergistically interact with resistance exercise to promote lean mass and strength gains (Bhasin et al. 1996). Whether the same can be said of the rise in endogenous hormones is not clear. Versus testosterone, however, it is entirely equivocal as to whether growth hormone is a hypertrophy-promoting hormone (Lange et al. 2002; Rennie 2003). Various studies have also been conducted examining the endogenous concentration

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changes in systemic hormones that are proposed to have an active role in skeletal muscle hypertrophy and potentially also in strength gains (Ahtiainen et al. 2003, 2005; Hakkinen et al. 1998, 2001; Kraemer et al. 1998, 2001; McCall et al. 1999; Nindl et al. 2001; Raastad et al. 2001, 2003). Ultimately, however, the causative role that acute and/or chronic changes in endogenous anabolic hormone concentrations, induced as a result of resistive exercise, play in hypertrophy is difficult to determine.

The unilateral model of resistance training in humans is one that has been used to show effects of various training protocols and examine strength gains as well as cross-education effects, (i.e., strength gains in a contralateral untrained limb) (Hakkinen et al. 1996; Hubal et al. 2005; Kim et al. 2005; McCurdy et al. 2005; Munn et al. 2005). The unilateral model is convenient in that it provides for an 'untrained' contralateral control limb for comparison that is a within-subject parameter insofar as statistical analysis is concerned. This within-person comparison provides a significant advantage from the standpoint of reducing variability, with the assumption that within-person variability is less than between person, and ultimately would allow more subtle differences in muscle and muscle fibre morphology as well as molecular events to be detected (Tarnopolsky et al. 2006). The unilateral model has been shown to result in hypertrophy (Hubal et al. 2005; Kim et al. 2005; Shepstone et al. 2005) of the trained arm with no significant change, or comparatively minor changes, in the contralateral limb. Using the unilateral design, detecting strength gains is a more complicated prospect due to the cross-education effect; see Munn et al. (2004) for a recent meta analysis of this phenomenon.

We chose that the unilateral model since one can induce local hypertrophy in the ipsilateral limb and examine strength changes due the combined action of neuromuscular and hypertrophic mechanisms. The contralateral limb serves as a 'control' not for strength gains, which are contaminated due to cross-education, but for any hypertrophy due to humoral mechanisms, (i.e., hormonal 'spillover' that might potentially affect gains in strength). Thus the purpose of this study was to use unilateral resistance training to examine hypertrophy in the trained limb along with changes in the contralateral untrained limb and measure changes in endogenous systemic hormones to determine whether these changes play a role in the hypertrophic response, in either the trained or untrained limb. Our working hypothesis was that unilateral training of the quadriceps femoris would not induce a significant change in endogenous hormone concentrations but that hyper-

trophy would occur in the trained limb nonetheless. We also hypothesized that the untrained limb would not exhibit hypertrophy and would show lesser strength gains compared to the trained limb.

## Methods

### Subjects

The study was approved by the Research Ethics Board of Hamilton Health Sciences and McMaster University and conformed to the regulations laid out in the declaration of Helsinki on the use of human subjects in research. All subjects gave their written consent to all procedures prior to participating. Subjects were ten young  $21.8 \pm 0.4$  years (mean  $\pm$  SE) males ( $1.78 \pm 0.04$  m,  $75.6 \pm 2.9$  kg) who were deemed healthy based on their response to a general health questionnaire. Participants were all non-smokers and had no previous history of resistance training and participated in a minimal amount of physical activity (no more than 2 h/week).

### Testing

At least 2 week prior to beginning the training protocol subjects reported to the Exercise Metabolism Research Laboratory for orientation to all procedures. At this time preliminary measures were made to estimate isotonic single repetition maximum (1RM) as well as isometric 1RM (Biodex-System 3, Biodex Medical Systems, Shirley, NY). Subjects were also familiarized with the guided motion training equipment for leg press and knee extension (Nautilus, Vancouver, WA).

To estimate isotonic 1RM for the knee extension subjects were seated with their hips at  $90^\circ$  and their back against a back rest that was at a  $30^\circ$  angle from horizontal (all tests were performed using a Nautilus guided motion machine; Tulsa, OK). The fulcrum of the machine was aligned with the lateral aspect of the midline of the subject's knee. The leg pad of the machine was positioned  $\sim 5$  cm above the subject's ankle and the rotation arm was positioned so that the subject's knee was bent to  $90^\circ$ . A full repetition was when the subject was able to move the weight through an arc of  $\sim 80^\circ$  (from  $90^\circ$  to  $\sim 170^\circ$ ). The subjects 'settings' for pin placements for seating in the machine were recorded and kept constant throughout the study. Subjects warmed up using a light weight and performed 8–10 repetitions. Subjects then performed a single best effort at a weight estimated to be the subject's 1RM based on body weight and height by an

experienced trainer. If the subject could not lift the weight, the weight was lowered and if the subject could lift the weight then it was raised. Ultimately, a 1RM was determined in this way. This estimated 1RM was checked on two subsequent occasions by simply having the subject report to the lab and asking them to once more perform a 1RM at the previously determined weight. For a 'true' 1RM to be determined the coefficient of variation (CV) between two attempts had to be less than 5%. For no subject did it require more than three attempts to determine this 1RM.

Isometric 1RM was determined in two sessions using the Biodex dynamometer. Subjects were seated in the dynamometer with their hips at 90° and their shoulders strapped with harness straps that prevented any upper-body movement. The fulcrum of the rotation arm was positioned to align with the midline of the subject's knee. The subject's knee was bent to an angle of 120° and the pad of the rotation arm was positioned 5 cm above the subject's ankle. Isometric 1RM was determined by having the subject maintain a maximal effort for knee extension and hold that effort 5 s after which subjects were allowed to rest for 2 min before attempting a second and third 1RM. The single highest peak value of any one of the three efforts was taken as the isometric 1RM. Subjects reported back to the laboratory for repeat testing using the same procedures 3–4 days after the first 1RM estimate and if the CV for 1RM determined in these two sessions was greater than 5% the procedure was repeated a third time, which was necessary for only one subject.

Leg press 1RM (Nautilus, Tulsa, OK) was determined in a seated position with the subject positioned so that their back rested against the back rest at 30° from horizontal and the angle between their torso and their legs (at their hips) was ~70°. Subjects had their knee bent at an angle of 90° and their foot placed on a foot plate so that a full 1RM resulted in full leg extension to 180°. The same procedure to determine 1RM as for the knee extension was followed for the leg press. A 'true' 1RM was determined for each subject within three visits to the lab, or less.

### Training

Subjects had their legs randomly assigned to be trained in a counterbalanced manner so that five of ten subjects trained their dominant leg and the five their non-dominant leg, based on dominance from 1RM strength. Training was conducted 3 days/week (Monday, Wednesday, and Friday) for a total of 8 week. Each subject completed all training sessions. The intensity of the training was set at 80–90% of 1RM, which meant

that subjects usually reached voluntary fatigue within 6–10 repetitions per set. Subject's recovered for 3 min between sets. In the first 4 weeks of the training subjects complete 3 sets per training session and in weeks 5–8 they completed 3 sets of 8–10 repetitions and one set to voluntary fatigue (usually 5–10 repetitions). Training was progressive in nature with subjects' voluntary isotonic 1RM being retested every 2 weeks in the trained leg. Intensity was adjusted to ensure that subjects were always lifting loads between 80 and 90% of 1RM. The untrained leg was tested in the same manner for 1RM throughout the protocol.

### Biopsies

Percutaneous muscle biopsies were obtained under local anesthesia (2% xylocaine) using a 5 mm Bergstrom biopsy needle custom modified for manual suction as detailed previously (Kim et al. 2005; Shepstone et al. 2005; Stewart et al. 2004; Yasuda et al. 2005). Muscle was mounted in optimal cutting temperature (OCT) medium (Sakura Finetechnical, Osaka, Japan) with fibres oriented in the plane perpendicular to which it was to be cut. Detailed methods and procedures yielding the descriptive variables for fibre size, type, percentage area, and myosin heavy chain composition have been described in detail previously elsewhere (Kim et al. 2005; Shepstone et al. 2005; Stewart et al. 2004; Yasuda et al. 2005).

### Muscle computerized tomographic scans

Subjects had both their trained and untrained legs scanned both pre- and post-training. Pre-training the scans were performed at least 2 days after any exercise or testing procedures and the scans were performed on both legs simultaneously. A General Electric CTI Scanner (GE, Milwaukee, WI) was used to perform computerized tomographic (CT) scans at baseline and after cessation of training. A scout scan was taken of the lower limbs to determine the femur and tibia lengths, and 5 mm slices were taken at two sites: 60% of femur length, starting from the distal end and measuring proximally. The system parameters used were as follows: slice thickness 5 mm, pixel matrix 512 × 512, and exposure factors of 120 kV, 200 mA and standard reconstruction algorithm. CT scans were analyzed using a validated software program (BonAlyse 1.3, BonAlyse Oy, Jyväskylä, Finland), according to the manufacturer's instructions. Thresholds –270 to –101 Hounsfield Units (HU) were used to identify fat, and thresholds –101 to 270 HU were used to identify muscle. BonAlyse was used to calculate muscle cross

sectional area (CSA) (mm<sup>2</sup>). We have determined that muscle CSA obtained from CT scans using our scanner can be measured with a CV of less than 1% in repeat scans with the same person across several weeks.

#### Hormone measurements

Prior to the first training workout and the last training bout subjects had a venous catheter inserted into a dorsal hand vein. The hand was kept warm using a heating blanket to maintain an 'arterialized' sample of blood on which to measure hormone concentrations. Subjects had blood samples drawn immediately prior to performing the training bout (pre), immediately after the exercise bout (post), and then again at 30, 60, 90, and 120 min post-exercise. Blood was collected in heparinized tubes as well as additive-free tubes. All tubes were kept on ice briefly (never more than 5 min for plasma and 10 min for serum) before being spun in a refrigerated centrifuge (4°C, 4,500 rpm) for 10 min to isolate plasma (heparinized tubes) or serum (additive-free tubes). Plasma and serum was stored at –80°C prior to analysis.

Plasma total testosterone (T), free testosterone (T<sub>f</sub>), luteinizing hormone (LH), sex-hormone binding globulin (SHBG), growth hormone (GH), and cortisol (C) were analyzed using commercially available radioimmunoassay kits (Diagnostics Products Corporation, Los Angeles, CA) and manufacturers instructions were closely followed. To minimize any intra-assay CV all pre- and post-training samples from each subject were analyzed in duplicate on the same day. A between sample CV (on pairs) of less than 5% was taken as good agreement and the values were averaged to yield a single concentration. On pooled plasma within-sample CVs ranged from as low as 0.3% to no higher than 4.8% on 6–8 replicates.

IGF-1 concentrations were measured in duplicate by immunoassay (Quantikine IGF-1 Immunoassay, R&D Systems, Minneapolis, MN); recombinant human IGF-1 was used to generate the standard curve. The mean

intra-assay CV, determined by assaying the IGF-1 concentration in a number of samples analyzed as replicates was less than 3.0%. The mean minimal detectable concentration of IGF-1 in this assay is 0.026 ng/ml.

#### Blood metabolite

Whole blood lactate was determined by adding heparinized plasma to 0.6M perchloric acid to deproteinize the sample. The sample was then spun (15 min, 4,500 rpm, 4°C) to pellet the proteins and to the supernatant, which was kept on ice, was added 1.25M KHCO<sub>3</sub> to neutralize the sample. Again the resulting salt was spun (15 min, 4,500 rpm, 4°C) down and the resulting supernatant was collected and stored at –80°C until analysis. Analysis of whole blood lactate concentration was performed as described previously (Phillips et al. 1995).

#### Statistics

Data were analyzed using a two-way repeated measures analysis of variance with time on multiple levels as well as training status (trained vs untrained) of the leg as factors. Significant *F* ratios were further examined using Tukey's posthoc test to isolate the differences. Significance was set at *P* < 0.05. Data are presented and means ± SE.

### Results

#### Strength

Isotonic 1RM increased as a result of training for the leg press and for knee extension (Table 1; both *P* < 0.001). In addition, a moderate cross-education effect was also observed for knee extension 1RM such that the contralateral untrained limb showed a 15 ± 3% increase (*P* < 0.05; Table 1).

**Table 1** Maximal isotonic strength for both the leg press and knee extension exercises in both the control and trained leg

	Pre	3	5	7	Post
Leg press 1RM (kg)					
Control	143 ± 21 <sup>a</sup>	142 ± 26 <sup>a</sup>	145 ± 26 <sup>a</sup>	143 ± 27 <sup>a</sup>	147 ± 25 <sup>a</sup>
Trained	141 ± 26 <sup>a</sup>	149 ± 26 <sup>b*</sup>	159 ± 30 <sup>c*</sup>	161 ± 27 <sup>cf</sup>	166 ± 25 <sup>df</sup>
Knee extension 1RM (kg)					
Control	47 ± 8 <sup>a</sup>	50 ± 9 <sup>a</sup>	51 ± 9 <sup>a</sup>	52 ± 9 <sup>b</sup>	54 ± 8 <sup>b</sup>
Trained	47 ± 7 <sup>a</sup>	52 ± 7 <sup>ab</sup>	59 ± 8 <sup>b*</sup>	63 ± 11 <sup>cf</sup>	68 ± 12 <sup>df</sup>

Values are means ± SE (*N* = 10). 1RM single repetition maximum, kg kilogram. Means with different letters are significantly different \*Significantly different from control at the same time point (*P* < 0.01), † *P* < 0.001. Means with different letters are significantly different from each other (*P* < 0.05)

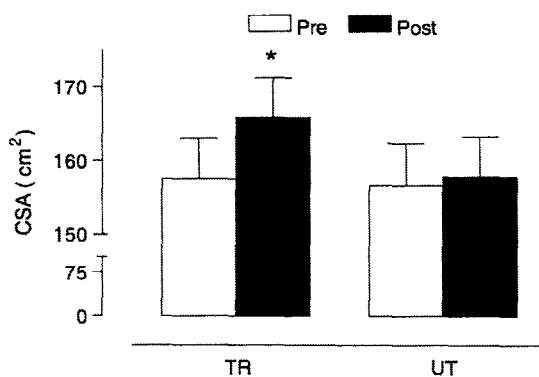
Isometric 1RM increased by  $12 \pm 2\%$  in the trained leg (PRE =  $256 \pm 46$  N m, POST =  $286 \pm 29$  N m;  $P < 0.01$ ) but remained unchanged (PRE =  $242 \pm 38$  N m, POST =  $248 \pm 28$  N m;  $P = 0.58$ ) in the untrained leg.

#### Muscle and muscle fibre cross-sectional area

Muscle CSA from CT showed an increase of  $5.4 \pm 0.9\%$  in the CSA of the trained leg (PRE =  $157.5 \pm 5.5$  cm<sup>2</sup>, POST =  $165.9 \pm 5.4$  cm<sup>2</sup>;  $P < 0.001$ ; Fig. 1), but no change in the CSA of the untrained leg  $0.0 \pm 0.5\%$  (PRE =  $157.8 \pm 6.5$  cm<sup>2</sup>, POST =  $157.7 \pm 6.4$  cm<sup>2</sup>;  $P = 0.76$ ; Fig. 1).

Type I muscle fibre CSA remained unchanged from PRE =  $4,184 \pm 426$   $\mu$ m<sup>2</sup> to POST =  $4,567 \pm 503$   $\mu$ m<sup>2</sup> ( $P = 0.06$ ) in the trained leg, and was also unchanged (PRE =  $4,291 \pm 400$   $\mu$ m<sup>2</sup>, POST =  $4,205 \pm 399$   $\mu$ m<sup>2</sup>; Fig. 2a) in the untrained leg. Type IIa fibre CSA increased by  $13 \pm 2\%$  (PRE =  $6,153 \pm 530$   $\mu$ m<sup>2</sup>, POST =  $6,952 \pm 463$ ;  $P < 0.001$ , Fig. 2b) in the trained leg and remained unchanged in the untrained leg (PRE =  $6,269 \pm 500$ , POST =  $6,144 \pm 466$ ). Type IIx fibre CSA increased by  $22 \pm 3\%$  (PRE =  $4,740 \pm 411$ , POST =  $5,769 \pm 444$ ;  $P < 0.001$ , Fig. 2c) in the trained leg and remained unchanged in the untrained leg (PRE =  $4,988 \pm 511$ , POST =  $5,014 \pm 526$ ).

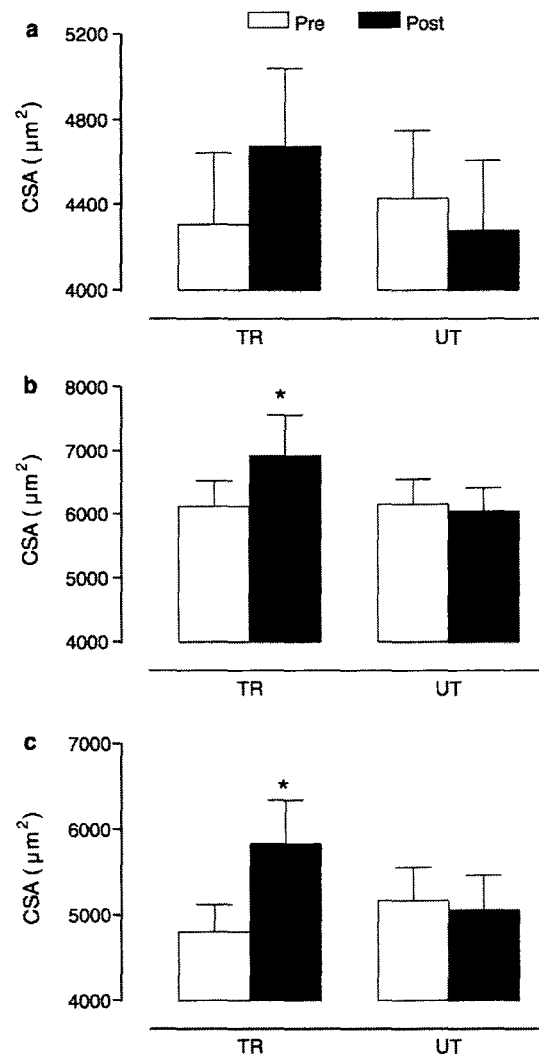
Muscle fibre percentage and percent area were calculated as previously described (Kim et al. 2005; Shephstone et al. 2005; Stewart et al. 2004; Yasuda et al. 2005) and are presented in Table 2. The percentage and percentage area of type IIa fibres was greater in the trained leg post- versus pre-training (Table 2;  $P < 0.05$ ). The shift in type IIa fibre percentage was due



**Fig. 1** Mid-thigh muscle cross-sectional area (CSA) by computerized tomography in both legs pre- and post-training; Pre and Post, respectively. TR trained leg, UT untrained leg. \*Significantly different from Pre ( $P < 0.001$ ). Values are means  $\pm$  SE ( $N = 10$ )

to a significant decline in the percentage of fibres that are type IIx, which was accompanied by a corresponding decline in the percentage of fibres that were type IIx (Table 2;  $P < 0.05$ ).

Acutely, neither the first bout of training nor the final bout of training had any effect on serum T, T<sub>f</sub>, LH, or SHBG (Fig. 3). Cortisol and the cortisol to T<sub>f</sub> ratio were also unaffected by exercise or by training (Fig. 3). IGF-1 concentration was unaffected by exercise (Fig. 4). GH concentration showed a moderate but



**Fig. 2** Muscle (vastus lateralis) fibre cross-sectional area in both legs pre- and post-training for type I fibres (a), type IIa fibres (b), and type IIx fibres (c). TR trained leg, UT untrained leg. \*Significantly different from Pre ( $P < 0.001$ ). Note that the scales on each graph are different to show differences within each fibre type. Values are means  $\pm$  SE ( $N = 10$ )

**Table 2** Muscle fibre type percentage and percentage area from vastus lateralis biopsies from both legs pre- and post-training

	Pre		Post	
	UT	T	UT	T
Fibre (%)				
I	34 ± 4	39 ± 4	36 ± 5	39 ± 3
IIa	39 ± 3	35 ± 4	39 ± 4	41 ± 2*
IIx	26 ± 2	26 ± 2	25 ± 3	20 ± 2*
Area (%)				
I	30 ± 4	34 ± 5	31 ± 3	33 ± 2
IIa	45 ± 2	42 ± 6	46 ± 4	48 ± 3*
IIx	25 ± 4	24 ± 2	23 ± 3	19 ± 5*

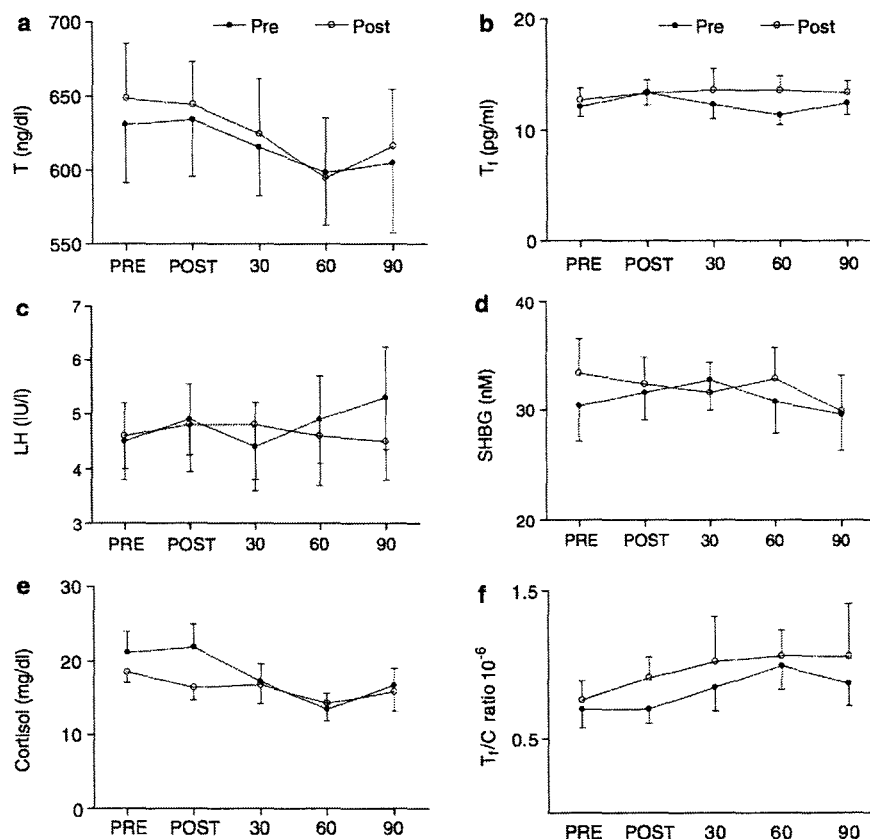
Values are means ± SE (*N* = 10)\*Significantly different from pre, within the same leg (*P* < 0.05)

statistically non-significant rise at 30 min post-exercise and a significant decline in concentration at 60 and 90 min post-exercise both pre- and post-training. Whole-blood lactate concentration was moderately elevated

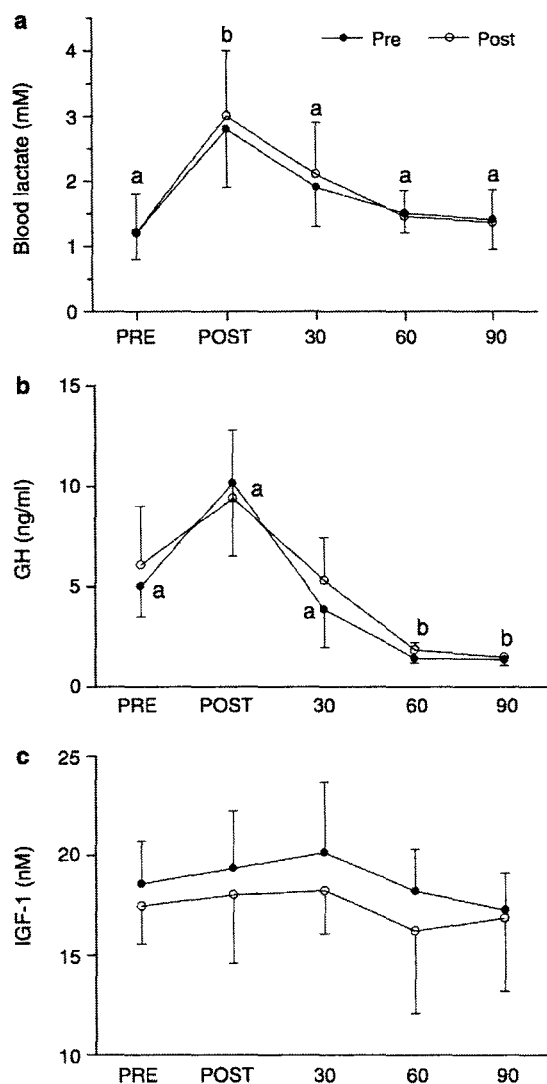
post-exercise in both the pre-trained and post-trained conditions, but only at 30 min post-exercise (Fig. 4).

## Discussion

Skeletal muscle hypertrophy as a result of resistance training in humans is due to an expansion of protein content of a pre-existing muscle fibre. This expansion occurs because of the synergistic interaction of feeding and resistance exercise (Phillips 2004; Phillips et al. 2005; Rennie et al. 2004; Rennie and Tipton 2000). The greater protein content is observed as a fibre with an increased cross-sectional area, but ultimately one with a constant nuclei to cytoplasmic ratio implying that new nuclei have been added to the fibre. The metabolic and molecular sequence of events that lead to hypertrophy are complex; however, one theory that has been put forward is that the endogenous rise in concentrations of anabolic hormones such as T, GH, and/or IGF-1

**Fig. 3** Acute concentration changes in **a** Testosterone (T), **b** Free testosterone ( $T_f$ ), **c** Luteinizing hormone (LH), **d** sex-hormone binding globulin (SHBG), **e** cortisol (C), and **f** the free testosterone-

one to cortisol ratio ( $T_f/C$ ), following a single isolated bout of resistance exercise both pre- and post-training. Values are means ± SE (*N* = 10)



**Fig. 4** Acute concentration changes in plasma lactate (**a**), growth hormone (**b**), and insulin-like growth factor following a single isolated bout of resistance exercise both pre- and post-training. Means with different letters are significantly different from each other ( $P < 0.05$ ). Values are means  $\pm$  SE ( $N = 10$ )

may be playing a role in the hypertrophic process (Ahtiainen et al. 2003, 2005; Hakkinen et al. 1998; Hansen et al. 2001; Kraemer et al. 1998, 2001; McCall et al. 1999; Nindl et al. 2001; Raastad et al. 2001, 2003). Ultimately, much of the evidence to support this thesis comes from longitudinal studies in which changes in concentrations of hormones such as testosterone are retrospectively correlated with hypertrophy (Ahtiainen et al. 2003; Hakkinen et al. 2001; McCall et al. 1999). Hansen et al. (2001) used arm only versus

arm preceded by leg exercise to test whether endogenous hormonal increases, which were observed to be greater in arm plus leg exercise group, affected strength gains in each, respective, arm. Strength gains in the arm only training group were lower than the arm plus leg group, however, the change was not statistically significant. Due to differences in the mean initial strength of the groups (higher in the arm only), however, it is difficult to interpret these findings (Hansen et al. 2001). In addition, no measures of muscle cross-sectional area, lean mass, or fibre size were made by Hansen et al. (2001) so it is difficult to ascribe the higher endogenous hormonal changes seen in the arm plus leg group as truly causative in the strength gains let alone whether greater hypertrophy occurred. Currently, to the authors' best knowledge, an investigation that highlights a causative role that exercise-induced rises in endogenous anabolic hormones play in hypertrophy does not exist.

There is little question that exogenous administration of testosterone to supraphysiological levels results in hypertrophy when given to non-exercising persons, and also augments exercise-induced hypertrophy when combined with resistance exercise (Bhasin et al. 1996; Sinha-Hikim et al. 2002, 2003b). Good evidence exists to show that testosterone administration enhances muscle protein synthesis (Ferrando et al. 1998) and increases satellite cell number (Sinha-Hikim et al. 2003a); hence it is with good basis that exogenous testosterone would enhance resistance exercise-induced muscle hypertrophy and strength gains (Bhasin et al. 1996). By comparison, changes in endogenous testosterone that occur with resistance exercise (Ahtiainen et al. 2003, 2005; Hakkinen et al. 1998; Hansen et al. 2001; Kraemer et al. 1998, 2001; McCall et al. 1999; Nindl et al. 2001; Raastad et al. 2001, 2003) are orders of magnitude less than those seen with exogenous testosterone administration (Bhasin et al. 1996; Sinha-Hikim et al. 2002, 2003a) and are often within the normal daily diurnal variability in testosterone [for review see Byrne and Nieschlag 2003]. In addition, the changes in testosterone concentration that are seen with resistance exercise are transient, normally not lasting any longer than 60–90 min after exercise (Ahtiainen et al. 2003, 2005; Hakkinen et al. 1998; Hansen et al. 2001; Kraemer et al. 1998, 2001; McCall et al. 1999; Nindl et al. 2001; Raastad et al. 2001, 2003). Furthermore, these changes in testosterone have been shown to track changes blood hematocrit implying that it is not, in fact, an increase in endogenous testosterone production that occurs with resistance training, but simply a hemoconcentration. In the current study we observed no change in either total or free testosterone or in the free testosterone to cortisol ratio either acutely or with training.

Changes in growth hormone concentration following resistance exercise have been shown to be due to varying isoforms of the hormone (Nindl et al. 2000) and a very consistent response to heavy resistance and high intensity anaerobic exercise [for review see Kraemer and Ratamess (2005)]. A number of clinical trials with human growth hormone administration have been performed in young and old men and women and evidence for a true anabolic effect is difficult to discern (Lange et al. 2002; Rennie 2003). We observed no elevation in growth hormone, but instead observed a significant reduction in concentration, admittedly only the 22 kDa isoform was measured by the immunoassay we utilized (see Methods for details). This finding is most likely due to the relatively small muscle mass utilized for the resistance exercise with an accompanying small rise in blood lactate (Fig. 4); activation of large muscle masses with accompanying lactate responses, via either high intensity aerobic or resistive exercise, result in substantial increases in plasma GH (Kraemer and Ratamess 2005).

Along with no change in GH concentration we, not surprisingly, saw no change in plasma IGF-I concentration. Insofar as supplemental IGF-I treatment is concerned, the longest trial to date saw no significant effect of IGF-I treatment in elderly women on lean or bone mass after 1 year (Friedlander et al. 2001). While there is no question that IGF-I does play a role in the hypertrophic response of skeletal muscle that is synergistic with resistance exercise (Lee et al. 2004), the effects of this hormone are more likely autocrine or paracrine in nature (Adams 2002; Lee et al. 2004) and the changes in circulating IGF-I may not accurately reflect the local-acting hormonal concentration.

Our results show that a single leg, (i.e., unilateral) model of resistance training results in hypertrophy in the absence of changes in systemic hormones that are proposed to play a role in the hypertrophic process (Ahtiainen et al. 2003, 2005; Hakkinen et al. 1998; Hansen et al. 2001; Kraemer et al. 1998, 2001; McCall et al. 1999; Nindl et al. 2001; Raastad et al. 2001, 2003). As such, the unilateral training model we describe here appears to be a good model for induction of local fibre hypertrophy that does not induce any measurable change in muscle or fibre phenotype in the contralateral limb, at least not as far as we could detect. Recently, Hubal et al. (2005) reported that unilateral limb training of the arm did result in hypertrophy of the contralateral untrained arm of  $1.4 \pm 0.3\%$ , this increase in size, while statistically significant, represented a hypertrophy of only  $\sim 7\%$  of that seen in the trained arm. Clearly, such a small gain in size could only be detected when a large sample size was exam-

ined as it was by Hubal et al. [(2005);  $N = 585$  total]. Such small changes in hypertrophy of the contralateral limb would, however, suggest that it could still serve as a valid internal control for most measures.

The degree of hypertrophy we observed ( $5.4 \pm 0.9\%$  increase in mid thigh CSA; Fig. 1) with only 8 weeks of resistance training (24 total sessions) is comparable to that seen with other unilateral training studies of 4–7% varying in duration from 8 to 10 weeks (Higbie et al. 1996; Housh et al. 1992; Kim et al. 2005), but is markedly less than that seen with a longer duration unilateral training (24 weeks) study in which an increase in thigh muscle CSA of 13% was observed (Narici et al. 1996). As such, our results represent relatively early events in the hypertrophic process. Nonetheless, we also observed a hallmark fibre type change in the trained limb; that is a reduction in type IIx fibre type at the expense of an increase in type IIa fibre type (Staron et al. 1989, 1994; Williamson et al. 2001). Hence, from a model perspective we propose that this unilateral resistance training regime induces 'typical' hypertrophic changes in the trained limb.

We observed the expected results in terms of strength gains with the unilateral resistance training model (Munn et al. 2004). Namely, we observed comparatively minor changes in strength in the contralateral limb compared to the trained limb (Table 1). The 15% relative change in isotonic knee extension strength of the contralateral limb is in the upper end of the range of that reported by Munn et al. (2004) in their meta-analysis. We propose that this increased contralateral leg strength may be larger due to the relative frequency of 1RM testing we employed in the contralateral untrained leg, which may itself have enhanced neuromuscular strength changes. When leg press isotonic 1RM was examined, however, we observed no change in strength in the contralateral limb (Table 1). However, strength was a secondary outcome in this study and is used here only as a variable that illustrates the greater hypertrophy seen in the trained limb. Moreover, as an argument that gains in strength seen in the untrained limb were neurologically mediated we observed no change in maximal isometric torque, a non-specific mode of strength testing in comparison to the mode of training, in this limb compared to trained limb.

Our interpretation of the current data is that a minimal testosterone concentration is required for basal functioning of the regulatory processes underlying hypertrophy, (i.e., protein synthesis, gene expression changes, and satellite cell activation), as demonstrated by the reduced lean mass seen in hypogonadal men (Ferrando et al. 2002). However, hypertrophy can occur in the absence of changes in endogenous



testosterone as our results demonstrate. Our results do not rule out the possibility that exercise-induced increases in endogenous testosterone concentration, known to occur with large muscle mass and/or whole-body resistance exercise, may augment hypertrophy, as is clearly seen with large supraphysiological doses of exogenous testosterone (Bhasin et al. 1996). In summary, the evidence we present here suggests that as little as 8 weeks of unilateral limb training involving knee extension and leg press induces local hypertrophy, greater strength changes, and hallmark fibre type shifts only in the trained limb whereas, the contralateral untrained limb appears to be a valid control for the trained limb, at least from a phenotypic perspective. All of these changes occurred in the absence of changes in systemic anabolic hormones meaning that the unilateral model is one of hypertrophy that is not augmented by systemic hormones.

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## STUDY 2

Consumption of fluid skim milk promotes greater muscle protein accretion after resistance exercise than does consumption of an isonitrogenous and isoenergetic soy-protein beverage

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## Consumption of fluid skim milk promotes greater muscle protein accretion after resistance exercise than does consumption of an isonitrogenous and isoenergetic soy-protein beverage<sup>1–3</sup>

Sarah B Wilkinson, Mark A Tarnopolsky, Maureen J MacDonald, Jay R MacDonald, David Armstrong, and Stuart M Phillips

### ABSTRACT

**Background:** Resistance exercise leads to net muscle protein accretion through a synergistic interaction of exercise and feeding. Proteins from different sources may differ in their ability to support muscle protein accretion because of different patterns of postprandial hyperaminoacidemia.

**Objective:** We examined the effect of consuming isonitrogenous, isoenergetic, and macronutrient-matched soy or milk beverages (18 g protein, 750 kJ) on protein kinetics and net muscle protein balance after resistance exercise in healthy young men. Our hypothesis was that soy ingestion would result in larger but transient hyperaminoacidemia compared with milk and that milk would promote a greater net balance because of lower but prolonged hyperaminoacidemia.

**Design:** Arterial-venous amino acid balance and muscle fractional synthesis rates were measured in young men who consumed fluid milk or a soy-protein beverage in a crossover design after a bout of resistance exercise.

**Results:** Ingestion of both soy and milk resulted in a positive net protein balance. Analysis of area under the net balance curves indicated an overall greater net balance after milk ingestion ( $P < 0.05$ ). The fractional synthesis rate in muscle was also greater after milk consumption ( $0.10 \pm 0.01\%/h$ ) than after soy consumption ( $0.07 \pm 0.01\%/h$ ;  $P = 0.05$ ).

**Conclusions:** Milk-based proteins promote muscle protein accretion to a greater extent than do soy-based proteins when consumed after resistance exercise. The consumption of either milk or soy protein with resistance training promotes muscle mass maintenance and gains, but chronic consumption of milk proteins after resistance exercise likely supports a more rapid lean mass accrual. *Am J Clin Nutr* 2007;85:1031–40.

**KEY WORDS** Skeletal muscle, protein synthesis, dietary protein, feeding, hypertrophy

### INTRODUCTION

Both hyperaminoacidemia (1–3) and resistance exercise (4–8) independently stimulate muscle protein synthesis. Furthermore, there is an additive effect of combining resistance exercise with feeding (3, 9–12), which leads to an enhanced anabolic environment. The gain in muscle protein mass induced by resistance training is due to the summation of the series of acute responses of muscle protein synthesis and

breakdown caused by the combined stimulus of exercise and feeding (13, 14).

It is currently unclear whether proteins from different sources induce a greater anabolic response after resistance exercise. Different milk proteins result in a different time course of hyperaminoacidemia (15, 16). Proteins, such as soy and whey, which are digested rapidly, lead to a large but transient rise in aminoacidemia, stimulate protein synthesis, and are referred to as “fast” proteins. By contrast, casein protein is considered a “slow” protein because it promotes a slower, more moderate, and longer lasting rise in plasma amino acids and does not stimulate protein synthesis, at least at the whole body level, but suppresses proteolysis (15). Our hypothesis was that, to promote an anabolic environment for muscle protein synthesis after resistive exercise, a supply of both fast dietary proteins, which stimulate protein synthesis, and slow dietary proteins, which suppress muscle protein breakdown, are advantageous (15, 16). Such a combination of fast and slow proteins is available in fluid bovine milk, which contains  $\approx 80\%$  casein and  $\approx 20\%$  whey protein by mass. Whole-body protein turnover data support the hypothesis that milk provides a combination of whey to stimulate synthesis and casein to inhibit breakdown (16). Using a modeling approach, Fouillet et al (17) estimated that ingestion of soy protein resulted in a lower whole-body retention of dietary nitrogen than did milk protein. Furthermore, soy protein induced a more rapid digestion, transit time, and absorption of nitrogen from the intestine, which was more readily retained by the splanchnic bed. This sequestering of amino acids by the splanchnic bed caused a subsequent reduction in amino acid uptake by peripheral tissues, including skeletal muscle (17). Data from previous studies suggest that the digestibility of a protein source differentially affects whole-body protein turnover at rest;

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however, it has yet to be fully elucidated what effect the protein source has on whole-body and muscle protein turnover after resistance exercise.

Given our knowledge of the effect of dietary protein ingestion at rest on whole-body protein turnover, we aimed to investigate the effect of oral ingestion of either fluid nonfat milk or an isonitrogenous and isoenergetic macronutrient-matched soy-protein beverage on whole-body and muscle protein turnover after an acute bout of resistance exercise in trained men. We hypothesized that the ingestion of milk protein would stimulate muscle anabolism to a greater degree than would the ingestion of soy protein, because of the differences in postprandial amino-acidemia.

## SUBJECTS AND METHODS

### Subjects

Eight healthy men with a mean ( $\pm$ SE) age of  $21.6 \pm 0.3$  y, body mass of  $81.7 \pm 5.9$  kg, and height of  $177.6 \pm 4.1$  cm who regularly engaged in resistance training ( $\geq 4$  d/wk) were recruited for the study. Each participant was advised of the purposes of the study and its associated risks. The participants were required to complete a health questionnaire and were deemed healthy on the basis of the responses. All subjects were nonsmokers, did not use any medication chronically, and gave their written informed consent before participation. The Hamilton Health Sciences Research Ethics Board approved the project, which complies with all standards set by the Declaration of Helsinki.

### Experimental protocol

The subjects performed 2 trials in random order separated by  $\geq 1$  wk. On each trial day, the participants received either a soy or milk beverage after a unilateral resistance exercise bout. A unilateral bout was used to isolate the effect of protein ingestion, after resistance exercise, to a single muscle mass with ample postexercise hyperemia and amino acid supply. On each trial day, the samples were taken only from the exercised leg. The drink order and leg that was tested, in terms of dominance based on strength, were randomized in a counterbalanced manner.

Each subject's single repetition maximum (1 RM, ie, the maximal amount of weight lifted at one time) for each leg was tested on 2 separate occasions  $\geq 2$  wk before the trials began ( $\bar{x} \pm$  SE: seated leg press,  $122 \pm 7$  kg; prone hamstring curl,  $51 \pm 3$  kg; and seated leg extension,  $69 \pm 4$  kg). The mean ( $\pm$ SE) leg volume was  $12.7 \pm 0.7$  L, which was determined by using an anthropometric approach (18).

The participants were asked to refrain from participating in strenuous exercise and from consuming alcohol for 2 d before each trial day. On each trial day, the subjects consumed a beverage with a defined formula (2170 kJ, 67% of energy as carbohydrate, 17% of energy as protein, and 16% of energy as fat; Boost, Novartis Nutrition Corporation, Fremont, MI) in the morning (0600) after an overnight fast (no food after 2000 the previous night). After 2.5 h (postabsorptive), the subjects reported to the exercise metabolism laboratory at McMaster University. A baseline breath sample was collected into a 100-L Douglas bag before being injected into a 10-mL evacuated tube for subsequent analysis of baseline  $^{13}\text{CO}_2/^{12}\text{CO}_2$ . Breath enrichment was analyzed by using an automated  $^{13}\text{CO}_2$  isotope ratio mass spectrometry breath-analysis system (BreathMat plus;

Thermo Finnigan, San Jose, CA) per previously described methods (19). Breath-by-breath carbon dioxide production was measured for 5 min. with an online gas collection system (Moxus; AEI Technologies, Pittsburg, PA).

A polyethylene catheter was then inserted into a forearm vein, from which a baseline blood sample was taken to determine background amino acid enrichment. After the baseline blood sample was drawn, the bicarbonate pool was primed with  $\text{Na}^{13}\text{CO}_3$  ( $3.5 \mu\text{mol/kg}$ ), and primed constant infusions of L-[1- $^{13}\text{C}$ ]leucine (prime:  $7.6 \mu\text{mol/kg}$ ; infusion rate:  $7.6 \mu\text{mol} \cdot \text{kg}^{-1} \cdot \text{h}^{-1}$ ) and L-[ring- $^2\text{H}_5$ ]phenylalanine (prime:  $2 \mu\text{mol/kg}$ ; infusion rate:  $2.4 \mu\text{mol} \cdot \text{kg}^{-1} \cdot \text{h}^{-1}$ ) were initiated (Figure 1). All isotopes were purchased from Cambridge Isotopes (Andover, MA), dissolved in 0.9% saline, filtered through a 0.2- $\mu\text{m}$  filter, and infused with the use of a calibrated syringe pump (KD Scientific, Holliston, MA). The infusion protocol was designed so that steady state was achieved within 1.5 h in both the intramuscular and plasma pools. After baseline sampling, the subjects rested for 1.5 h, during which time a 20-gauge polyethylene catheter was inserted into the radial artery for blood sampling (Figure 1). The catheter was kept patent by using periodic flushes of 0.9% saline containing 1 IU heparin/mL, which was maintained at a pressure above systolic pressure. At  $\approx 1$ –2 cm distal to the inguinal crease, a 3 French 10-cm polyethylene catheter was inserted into the femoral vein in an antero-grade orientation.

After 1.5 h, blood samples were taken from the radial artery and femoral vein. Femoral artery blood flow was determined by using pulsed-wave Doppler ultrasonography, and a percutaneous muscle biopsy sample was obtained. The subjects then performed a standardized leg workout, ie, leg press, hamstring curl, and knee extension with a single leg. The subjects performed 4 sets of each exercise, with 10 repetitions per set for the first 3 sets, and the last set to exhaustion. Exercise intensity was set at 80% of 1 RM with an interset rest period of 2 min. After the resistance exercise protocol was completed, blood samples and muscle biopsy samples were obtained. The subjects then ingested (in a randomized single-blinded fashion) a 500-mL drink that contained either fluid nonfat milk or an isonitrogenous, isoenergetic, and macronutrient-matched soy-protein beverage (745 kJ, 18.2 g protein, 1.5 g fat, and 23 g carbohydrate as lactose for milk and as maltodextrin for the soy beverage). The drinks were made from commercially available isolated soy protein (GeniSoy, Fairfield, CA) or skim milk powder. After drink consumption, femoral artery blood flow, breath samples, arterial and venous blood samples, and muscle biopsy samples were obtained every hour thereafter for 3 h (Figure 1). The biopsy samples were taken only from the exercised leg within a given experimental condition. On a second day,  $\geq 1$  wk after the initial trial, the subjects followed the same protocol, except that the contralateral leg was tested and they received the alternative beverage after exercise.

### Analytic methods

#### Blood flow

Femoral artery mean blood velocity (MBV) was measured by using pulsed-Doppler ultrasonography (model system 5; GE Medical Systems, Horten, Norway). Data were acquired continuously with a 10-MHz probe, corrected for insonation angle, placed on the skin surface 2–3 cm proximal to bifurcation of the femoral artery into the superficial and profundus segments. The

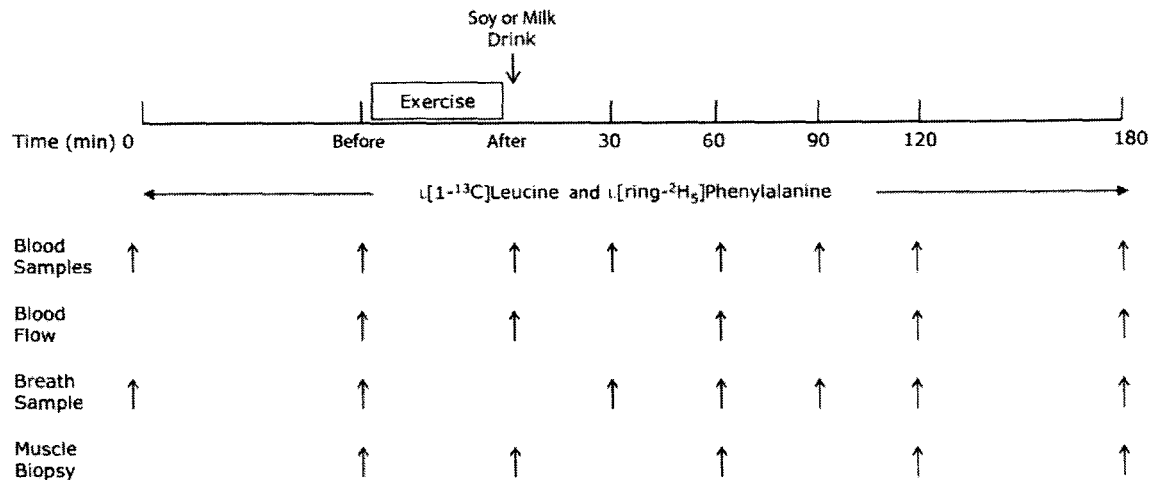


FIGURE 1. Experimental protocol.

ultrasound gate was maintained at full width to ensure complete insonation of the entire vessel cross-section with constant intensity (20). MBV data were recorded at 200 Hz and stored on a computer for subsequent analysis. Average MBV was calculated by integrating the total area under the MBV profile for 15 subsequent heart cycles at each time point. Femoral artery diameter was measured simultaneously by using 2-dimensional echo-Doppler ultrasound (10-MHz probe) and stored to videotape for subsequent analysis. Arterial diameter was determined in triplicate before and immediately after exercise and 1, 2, and 3 h after drink ingestion. At each time point, 3 measures of systolic and diastolic diameters were used to determine mean diameter.

Mean leg blood flow (mL/min) =

$$\text{MBV}(\text{cm/s}) \times r^2 \times 60 \text{ s/min} \quad (I)$$

#### Blood samples

Blood samples were collected into heparinized evacuated containers. Whole blood (100  $\mu\text{L}$ ) was added to ice-cold perchloric acid (PCA; 0.6 mol/L, 500  $\mu\text{L}$ ); the solution was mixed and allowed to sit on ice for 10 min to precipitate all proteins. This mixture was then centrifuged at  $4000 \times g$  (15 000 rpm) for 2 min at 4  $^{\circ}\text{C}$ . The PCA was neutralized with 250  $\mu\text{L}$  of 1.25 mol  $\text{KHCO}_3/\text{L}$ , and the reaction was allowed to proceed on ice for 10 min. The samples were then centrifuged at  $4000 \times g$  (15 000 rpm) for 2 min at 4  $^{\circ}\text{C}$ . The supernatant fluid was stored at  $-50^{\circ}\text{C}$  until analyzed further (blood amino acid concentrations and blood phenylalanine enrichment). Blood plasma was obtained by centrifuging the evacuated tube at 4  $^{\circ}\text{C}$  for 10 min at  $4000 \times g$  (4500 rpm). The plasma was stored at  $-50^{\circ}\text{C}$  for the measurement of plasma insulin and glucose concentrations and plasma  $\alpha$ -ketoisocaproic acid enrichment as described below.

#### Muscle biopsy samples

Needle biopsy samples from the vastus lateralis were obtained under local anesthesia (1% xylocaine). A 5-mm Bergström biopsy needle modified for manual suction was used to obtain  $\approx 100$  mg of muscle tissue from each biopsy. Biopsies were obtained from separate incisions from the same leg during each trial and from the contralateral leg during the following trial. The

muscle was dissected free of any visible fat and connective tissue and was immediately frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$  before analysis.

#### Blood analysis

Plasma was assayed for insulin by using a commercially available radioimmunoassay kit from Diagnostic Products Corporation (Los Angeles, CA). Neutralized blood PCA extract was assayed for glucose by using a standard enzymatic method (21). Plasma  $\alpha$ -ketoisocaproic acid enrichment was determined by using methods described previously (22, 23).

#### Muscle sample analysis

Muscle samples were lyophilized to dryness while being incubated on dry ice (Savant, Rockville, MD). Samples were manually powdered and weighed. To determine intracellular amino acid concentration and phenylalanine enrichments, a portion of the muscle sample was extracted with 0.5 mol PCA/L and neutralized with 2.2 mol  $\text{KHCO}_3/\text{L}$ . The PCA extract was removed and stored at  $-50^{\circ}\text{C}$  until analyzed further. Subsequently, to determine protein-bound phenylalanine enrichment, the remaining muscle pellet was washed with distilled water, dried, and then hydrolyzed in 6 mol  $\text{HCl}/\text{L}$  at  $100^{\circ}\text{C}$  for 24 h. The protein hydrolysate was neutralized and passed over a PepClean  $\text{C}_{18}$  Spin Column (Pierce, Rockford, IL) for purification. Desorption of amino acids from the column was accomplished with a 70% acetonitrile solution, and the eluate was collected and dried under nitrogen gas.

#### HPLC amino acid analysis

To determine whole blood and muscle intracellular amino acid concentrations, the whole-blood and muscle PCA extract was derivatized by using a Waters AccQ-Fluor reagent kit (Milford, MA) by heating for 30 min at  $55^{\circ}\text{C}$  to form the 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate derivative of all physiologic amino acids. Samples and standards (Sigma, St Louis, MO) were run on an HPLC (HPLC: Waters model 2695; column: Waters Nova-Pak  $\text{C}_{18}$ , 4  $\mu\text{m}$ ; detector: Waters 474 scanning fluorescence detector). The amino acids were detected by using a scanning fluorescence detector with excitation and emission wavelengths of 250 and 395 nm, respectively. Amino acid peak areas

were integrated and compared with known standards and analyzed by using a Waters Millenium<sup>32</sup> software package (Milford, MA). This method achieved separation of 19 of the 20 physiologic amino acids, with the exception of tryptophan, which was not included in the analysis.

#### Protein amino acid content analysis

To determine the amino acid content of the milk and soy proteins ingested by the participants, 5 aliquots of each protein were hydrolyzed in 6N HCl for 24 h at 100 °C. The samples were then neutralized with 6N NaOH and filtered through a 0.2- $\mu$ m filter. A small portion of the sample was then derivatized in the same manner as were the blood and muscle samples and run on the HPLC to determine the percentage of each individual amino acid (mg amino acid/mg protein). The milk protein was composed of 43% essential and 23% branched-chain amino acids (7.6% Lys, 2.6% Met, 4.3% Phe, 5.5% Thr, 5.6% Ile, 10.5% Leu, and 7.0% Val). Analysis of the soy-protein amino acid content showed that it was made up of 41% and 21% essential and branched-chain amino acids, respectively (7.0% Lys, 1.4% Met, 5.0% Phe, 5.7% Thr, 5.4% Ile, 9.6% Leu, and 6.4% Val).

#### Phenylalanine enrichment

To determine the enrichment of phenylalanine in blood and muscle, a *tert*-butyl dimethylsilyl (*t*-BMDS) derivative was prepared. The blood and intracellular muscle PCA extracts were transferred into threaded borosilicate tubes and lyophilized in a SpeedVac rotary evaporator (Savant Instruments, Farmingdale, NY). To derivatize the dried eluent from the column clean-up for the bound sample and dried PCA extract, 50  $\mu$ L HPLC grade acetonitrile and 50  $\mu$ L *N*-methyl-*N*-(*tert*-butyldimethylsilyl) trifluoro-acetamide + 1% *tert*-butyldimethylchlorosilane (MTBSTFA and 1% TBDMCS; Regis, Morton Grove, IL) were added to the sample. Phenylalanine enrichment was analyzed by electron-impact ionization capillary gas chromatography–mass spectrometry (GC Hewlett-Packard 6890; Palo Alto, CA; MSD Agilent 5973; Palo Alto, CA) in electron ionization mode (23). The enrichment of phenylalanine in the PCA blood and muscle intracellular extracts was analyzed at mass-to-charge ( $m/z$ ) ratios of 234 ( $m + 0$ –baseline) and 239 ( $m + 5$ ). For the protein-bound phenylalanine enrichment, a standard curve was used and  $m/z$  ratios of 234, 237, and 239 were used (24, 25).

#### Calculations

The fractional synthetic rate (FSR) of muscle proteins was calculated as the rate of tracer incorporation into mixed muscle proteins by using the enrichment of intracellular free phenylalanine as the precursor, according to a previously published equation (6).

Chemical phenylalanine and total amino acid (TAA) net balance (NB) across the leg was calculated, as described elsewhere (9–12), from the difference between arterial and venous concentrations multiplied by femoral artery blood flow:

$$NB = (C_a - C_v) \times BF \quad (2)$$

where  $C_a$  is the arterial amino acid concentration,  $C_v$  is the venous amino acid concentration, and BF is femoral artery blood flow. Because phenylalanine is not metabolized in muscle, a positive net balance signifies net uptake and muscle protein anabolism and a negative net value indicates net release of amino acids and

muscle protein catabolism. Nitrogen NB was calculated by multiplying the concentration of each amino acid by nitrogen content per amino acid.

Area under the NB curve was calculated by using the PRISM software package (GraphPad Software Inc, San Diego, CA). A baseline of 0 was used to determine the total positive area under the curve for the time points after drink consumption (30, 60, 90, 120, and 180 min).

In the 2-pool model, muscle protein synthesis and breakdown is estimated by using the rate of appearance ( $R_a$ ) and disappearance ( $R_d$ ), respectively, of L-[ring<sup>2</sup>H<sub>5</sub>]phenylalanine in the blood (23):

$$R_a = (E_a/E_v - 1) \times C_a \times BF \quad (3)$$

$$R_d = NB + R_a \quad (4)$$

where  $E_a$  is the arterial enrichment of L-[ring<sup>2</sup>H<sub>5</sub>] phenylalanine,  $E_v$  is the venous enrichment of L-[ring<sup>2</sup>H<sub>5</sub>] phenylalanine, and  $C_a$  is the arterial amino acid concentration.

Leucine flux ( $Q$ ), oxidation, and nonoxidative leucine disposal (NOLD) were calculated by using previously published equations (26). Exercise and feeding is known to effect the retention of carbon dioxide in the body (23); therefore, values of 0.81 (26) and 0.83 (22) were used for calculations before exercise and during the recovery period, respectively. NOLD was used as an index of whole-body protein synthesis,  $Q$  was used as an index of whole-body protein breakdown, and oxidation was used as an index of whole-body protein oxidation.

#### Statistics

Sample size estimates were based on the ability to detect a 25% difference between groups in mixed muscle fractional synthetic rate using an  $\alpha$  value of 0.05 and a  $\beta$  value of 0.2, with an estimated population variance in the measure based on past studies from our lab and from literature values. To protect power, we added 2 subjects to the final calculated sample size estimate. Data were analyzed by using STATISTICA (version 6.0; Statsoft, Tulsa, OK) with a repeated-measures analysis of variance. Area under the curve measures were analyzed by using paired *t* tests. When a significant *F* ratio was observed, a post hoc analysis with Tukey's honestly significant difference test was used to determine differences. Significance was set at  $P < 0.05$ . Data are presented as means  $\pm$  SEMs.

#### RESULTS

All subjects completed the exercise protocols. The number of repetitions and sets were evenly matched so that the exercise stimulus was similar in each trial. Plasma insulin and glucose concentration increased above concentrations before exercise 60 min after drink consumption, with no differences observed between the drinks (Table 1). By 120 min after drink consumption, blood glucose and insulin concentrations were no different from those observed before exercise.

Femoral artery blood flow was significantly elevated immediately after the resistance exercise bout and returned to concentrations not different from those before exercise by 60 min after drink consumption (Table 2).

The sum of TAA concentration showed a time-by-beverage interaction such that concentration was elevated after both soy- and milk-protein consumption (Figure 2) 30, 60, and 90 min

**TABLE 1**Effect of milk- and soy-protein consumption on plasma glucose and insulin concentration<sup>1</sup>

	Before exercise	After exercise	60 min	120 min	180 min
Glucose (mmol/L)					
Milk					
Artery	4.5 ± 0.2	4.7 ± 0.2	5.1 ± 0.3 <sup>2</sup>	4.9 ± 0.1	4.7 ± 0.1
Vein	4.0 ± 0.1	4.5 ± 0.2	4.7 ± 0.3 <sup>2</sup>	4.4 ± 0.2	4.4 ± 0.1
Soy					
Artery	4.5 ± 0.1	4.8 ± 0.2	5.4 ± 0.2 <sup>2</sup>	4.7 ± 0.1	4.6 ± 0.1
Vein	4.0 ± 0.1	4.5 ± 0.2	4.9 ± 0.3 <sup>2</sup>	4.4 ± 0.1	4.4 ± 0.1
Insulin (μIU/mL)					
Milk	3.0 ± 0.2	4.1 ± 0.5	14.0 ± 3.2 <sup>2</sup>	5.0 ± 0.8	3.6 ± 0.4
Soy	3.4 ± 0.3	4.3 ± 0.5	16.5 ± 4.9 <sup>2</sup>	4.1 ± 0.6	3.3 ± 0.4

<sup>1</sup> All values are  $\bar{x} \pm \text{SEM}$ ;  $n = 8$ . A 3-factor ANOVA was performed to test for main effects of time, beverage, and site (artery or vein) on the glucose data. No 3-factor interaction was significant. A 2-factor ANOVA was performed to test for main effects of time and beverage. There was a significant time-by-beverage interaction ( $P < 0.05$ ). A 2-factor ANOVA was performed on the insulin data to test for main effects of time and beverage. A main effect of time was observed. When a significant  $F$  ratio was observed, post hoc analysis with Tukey's honestly significant difference test was used to determine differences.

<sup>2</sup> Significantly different from before exercise with both beverage groups combined,  $P < 0.05$ .

after each drink; however, by 120 min after drink consumption, AA concentrations were no different from those observed before or immediately after exercise. The sum of TAA was significantly greater in the soy trial than in the milk trial 30 min after drink consumption ( $P = 0.05$ ).

Intramuscular lysine and phenylalanine concentrations were significantly elevated above concentrations before exercise by 60 min after both drinks were consumed, but returned to concentrations no different from those before exercise by 120 min after drink consumption (Table 3). The intramuscular concentrations of Ile, Leu, Lys, Phe, and Val and the sum of essential amino acids were all significantly reduced at 180 min after the drink was consumed compared with concentrations observed 60 min after the proteins were consumed (Table 3).

Leucine oxidation did not change significantly over the entire protocol (Table 4). NOLD, a measure of whole-body protein synthesis, was significantly elevated 1 h after both milk- and soy-protein consumption compared with values before beverage consumption (Table 4). In observations made 120 and 180 min after drink consumption, NOLD was not significantly different from values observed before exercise. Leucine flux, an indication of whole-body protein breakdown, was significantly elevated 60 min after protein-drink consumption compared with values observed before exercise (Table 4). However, by 120 min after exercise, leucine flux was not significantly different from that before exercise. There were no differences between soy and

milk in any variables measured by whole-body protein oxidation or turnover.

FSR showed a significant time-by-beverage interaction such that FSR was significantly greater during the 3 h of recovery from exercise after both soy and milk drink consumption than after the time period when resistance exercise was performed (Figure 3). There was no difference in muscle FSR between the soy and milk trials during the exercise time period; however, muscle FSR observed after milk consumption was 34% greater than that after soy consumption ( $P < 0.05$ ).

There was no effect of either protein or time on the  $R_a$  of phenylalanine (Figure 4A). The  $R_a$  of phenylalanine showed a main effect of time and was elevated 30 min after protein consumption, when both beverages were combined (Figure 4B). Net phenylalanine balance showed a significant time-by-beverage interaction such that values were negative before exercise (Figure 4C); however, 30 and 60 min after both soy- and milk-protein consumption, NB became positive and remained significantly elevated above concentrations seen before consumption. In the soy trial, NB was again negative by 120 min after drink consumption, whereas NB remained positive in the milk condition and different from that in the soy condition at the 90- and 120-min time points. By 180 min after drink consumption, NB was negative in both the soy and milk trial. Positive area under the milk NB curve was significantly greater than that in the soy trial

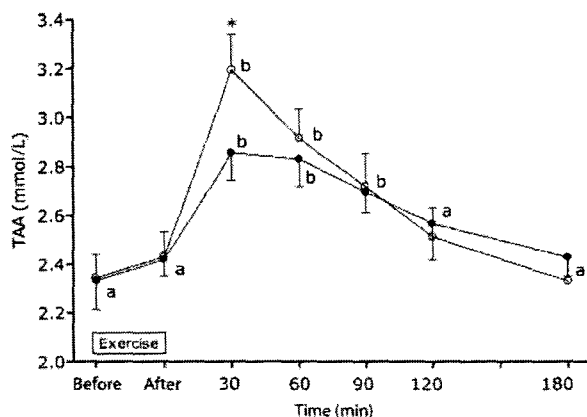
**TABLE 2**Effect of milk- and soy-protein consumption on femoral artery blood flow<sup>1</sup>

	Before exercise	After exercise	60 min	120 min	180 min
Blood flow (mL · min <sup>-1</sup> · 100 mL leg <sup>-1</sup> )					
Milk	1.46 ± 0.19	6.44 ± 1.02 <sup>2</sup>	1.88 ± 0.30	1.55 ± 0.29	1.42 ± 0.24
Soy	1.51 ± 0.23	6.88 ± 0.98 <sup>2</sup>	1.80 ± 0.27	1.49 ± 0.23	1.45 ± 0.25
Blood flow (L/min)					
Milk	0.19 ± 0.03	0.82 ± 0.13 <sup>2</sup>	0.25 ± 0.05	0.20 ± 0.04	0.19 ± 0.04
Soy	0.19 ± 0.03	0.84 ± 0.12 <sup>2</sup>	0.23 ± 0.04	0.19 ± 0.03	0.18 ± 0.03

<sup>1</sup> All values are  $\bar{x} \pm \text{SEM}$ ;  $n = 8$ . A 2-factor ANOVA was performed to test for main effects of time and beverage. No time-by-beverage interaction was observed. A main effect of time was observed, and post hoc analysis with Tukey's honestly significant difference test was used to determine differences.

<sup>2</sup> Significantly different from before exercise with both beverage groups combined,  $P < 0.05$ .





**FIGURE 2.** Mean ( $\pm$ SEM) whole-blood total amino acid (TAA) concentrations after the consumption of a nonfat milk-protein beverage (●) or an isonitrogenous, isoenergetic, macronutrient-matched (750 kJ, 18.2 g protein, 1.5 g fat, and 23 g carbohydrate) soy-protein beverage (○). A 2-factor ANOVA was performed to test for main effects of time and beverage. A main effect of time and a time-by-beverage interaction was observed, and a post hoc analysis was conducted with Tukey's honestly significant difference test to determine differences. \*Significantly different from the milk group at the same time point,  $P < 0.05$ . Main effect of time: values with different lowercase letters are significantly different,  $P < 0.05$ .  $n = 8$ .

(Figure 4D), which indicated a more sustained net positive balance over the course of the trial.

The TAA arterial-venous NB showed a significant time-by-beverage interaction. The TAA balance was negative before

exercise and protein consumption (Figure 5). After protein consumption, the balance became positive and remained significantly elevated above concentrations before consumption at 30 and 60 min after both soy- and milk-protein consumption. The TAA NB was still positive at 90 and 120 min after drink consumption and was significantly greater than the values at the same times in the soy trial. At 180 min after beverage consumption, the values for TAA balance for both milk and soy were not different from those after consumption. When the area under the TAA NB curve (Figure 5) after drink consumption was analyzed, milk was significantly greater than soy ( $P < 0.05$ ). Nitrogen balance across the leg followed the same pattern as did TAA NB (data not shown). The area under the curve analysis indicated that milk consumption provided a more positive nitrogen balance over the 3 h after milk-protein consumption ( $53\,887 \pm 16\,524$  mmol N/100 mL leg) than after soy-protein consumption ( $19\,485 \pm 4820$  mmol N/100 mL leg) ( $P < 0.01$ ).

## DISCUSSION

The primary finding of the current study was that intact dietary proteins can support an anabolic environment for muscle protein accretion. We observed a significantly greater uptake of amino acids across the leg and a greater rate of muscle protein synthesis in the 3 h after exercise and milk-protein consumption than after soy-protein ingestion. There were no differences in blood flow or in insulin and blood glucose concentrations in response to the drinks. Additionally, the measured essential amino acid content of both proteins was not significantly different.

**TABLE 3**

Effect of milk- and soy-protein consumption on muscle intracellular indispensable amino acid concentrations<sup>1</sup>

	Before exercise	After exercise	60 min	120 min	180 min
	mmol/kg dry wt				
Ile					
Milk	0.51 $\pm$ 0.08	0.51 $\pm$ 0.08	0.64 $\pm$ 0.15 <sup>2</sup>	0.47 $\pm$ 0.08	0.45 $\pm$ 0.06
Soy	0.43 $\pm$ 0.08	0.49 $\pm$ 0.09	0.51 $\pm$ 0.09 <sup>2</sup>	0.45 $\pm$ 0.08	0.46 $\pm$ 0.11
Leu					
Milk	0.56 $\pm$ 0.04	0.57 $\pm$ 0.05	0.69 $\pm$ 0.06 <sup>2</sup>	0.55 $\pm$ 0.03	0.54 $\pm$ 0.04
Soy	0.50 $\pm$ 0.03	0.54 $\pm$ 0.03	0.59 $\pm$ 0.04 <sup>2</sup>	0.54 $\pm$ 0.04	0.44 $\pm$ 0.02
Lys					
Milk	1.38 $\pm$ 0.11	1.41 $\pm$ 0.15	1.64 $\pm$ 0.09 <sup>2,3</sup>	1.39 $\pm$ 0.14	1.38 $\pm$ 0.13
Soy	1.19 $\pm$ 0.10	1.28 $\pm$ 0.16	1.53 $\pm$ 0.12 <sup>2,3</sup>	1.40 $\pm$ 0.08	1.29 $\pm$ 0.08
Met					
Milk	0.21 $\pm$ 0.03	0.20 $\pm$ 0.02	0.23 $\pm$ 0.03	0.19 $\pm$ 0.01	0.18 $\pm$ 0.02
Soy	0.19 $\pm$ 0.01	0.21 $\pm$ 0.01	0.20 $\pm$ 0.01	0.19 $\pm$ 0.01	0.17 $\pm$ 0.02
Phe					
Milk	0.24 $\pm$ 0.02	0.25 $\pm$ 0.03	0.30 $\pm$ 0.03 <sup>2,3</sup>	0.24 $\pm$ 0.02	0.24 $\pm$ 0.02
Soy	0.23 $\pm$ 0.01	0.26 $\pm$ 0.02	0.28 $\pm$ 0.01 <sup>2,3</sup>	0.27 $\pm$ 0.01	0.23 $\pm$ 0.02
Thr					
Milk	3.97 $\pm$ 0.35	3.87 $\pm$ 0.48	4.16 $\pm$ 0.28	3.65 $\pm$ 0.44	3.50 $\pm$ 0.45
Soy	3.78 $\pm$ 0.53	4.06 $\pm$ 0.42	3.64 $\pm$ 0.43	3.68 $\pm$ 0.43	3.30 $\pm$ 0.49
Val					
Milk	0.93 $\pm$ 0.10	0.98 $\pm$ 0.10	1.05 $\pm$ 0.07 <sup>2</sup>	0.87 $\pm$ 0.06	0.85 $\pm$ 0.06
Soy	0.80 $\pm$ 0.03	0.85 $\pm$ 0.05	0.85 $\pm$ 0.04 <sup>2</sup>	0.83 $\pm$ 0.05	0.72 $\pm$ 0.02
$\Sigma$ EAA					
Milk	7.80 $\pm$ 0.62	7.80 $\pm$ 0.80	8.72 $\pm$ 0.49 <sup>2</sup>	7.37 $\pm$ 0.67	7.15 $\pm$ 0.67
Soy	7.13 $\pm$ 0.59	7.68 $\pm$ 0.60	7.77 $\pm$ 0.60 <sup>2</sup>	7.36 $\pm$ 0.48	6.60 $\pm$ 0.50

<sup>1</sup> All values are  $\bar{x} \pm$  SEM;  $n = 8$ . EAA, essential amino acids. A 2-factor ANOVA was performed to test for main effects of time and beverage. No time-by-beverage interaction was observed. A main effect of time was observed, and post hoc analysis with Tukey's honestly significant difference test was conducted to determine differences.

<sup>2</sup> Significantly different from 180 min with both beverages combined,  $P < 0.05$ .

<sup>3</sup> Significantly different from before exercise with both beverage groups combined,  $P < 0.05$ .

TABLE 4

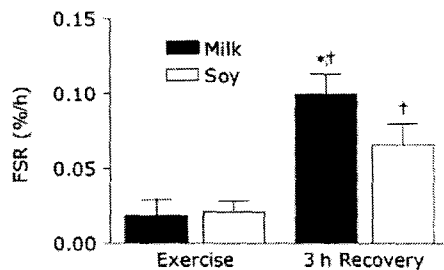
Effect of milk- and soy-protein consumption on leucine oxidation, nonoxidative leucine disposal (NOLD), and leucine flux<sup>1</sup>

	Before exercise	60 min	120 min	180 min
Leucine oxidation ( $\mu\text{mol} \cdot \text{kg}^{-1} \cdot \text{h}^{-1}$ )				
Milk	23 $\pm$ 2	25 $\pm$ 1	28 $\pm$ 1	23 $\pm$ 2
Soy	25 $\pm$ 2	26 $\pm$ 2	26 $\pm$ 2	22 $\pm$ 2
NOLD ( $\mu\text{mol} \cdot \text{kg}^{-1} \cdot \text{h}^{-1}$ )				
Milk	108 $\pm$ 2	139 $\pm$ 10 <sup>2</sup>	119 $\pm$ 6	104 $\pm$ 4
Soy	109 $\pm$ 4	133 $\pm$ 5 <sup>2</sup>	120 $\pm$ 4	101 $\pm$ 4
Leucine flux ( $\mu\text{mol} \cdot \text{kg}^{-1} \cdot \text{h}^{-1}$ )				
Milk	132 $\pm$ 4	163 $\pm$ 10 <sup>2</sup>	147 $\pm$ 6	127 $\pm$ 5
Soy	139 $\pm$ 5	164 $\pm$ 7 <sup>2</sup>	152 $\pm$ 5	126 $\pm$ 6

<sup>1</sup> All values are  $\bar{x} \pm \text{SEM}$ ;  $n = 8$ . A 2-factor ANOVA was performed to test for main effects of time and beverage. No time-by-beverage interaction was observed. A main effect of time was observed, and post hoc analysis with Tukey's honestly significant difference test was used to determine differences.

<sup>2</sup> Significantly different from before exercise with both beverage groups combined,  $P < 0.05$ .

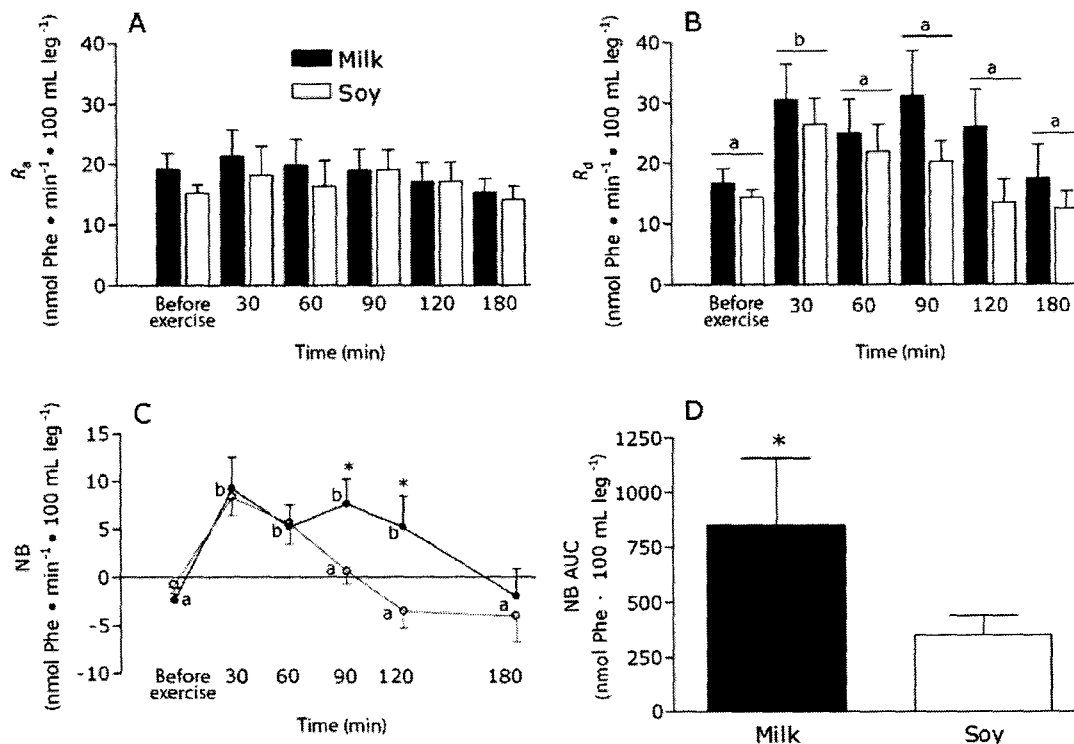
To date, 2 studies have shown that the ingestion of whole proteins after resistance exercise can support positive muscle protein balance (27, 28). Both studies examined the effect of fluid milk (27) or its constituent protein fractions, whey and casein (28), on muscle protein balance. Ours, however, is the first study to show that the source of intact dietary protein (ie, milk compared with soy) is important for determining the degree of post-exercise anabolism. We found, using arterial-venous balance, that milk protein promoted a more sustained net positive protein balance after resistance exercise than did soy protein. On the basis of our analysis of the amino acid content of the proteins, which showed that milk and soy proteins provide equal amounts of essential amino acids, it is unlikely that the differences in muscle protein synthesis and net protein balance seen in the present study are related to the amino acid content of the respective proteins. Alternatively, because of differences in digestion rates, milk proteins may provide a slower pattern of amino acid delivery to the muscle than soy protein. Therefore, we propose that a difference in the digestion rate of milk and soy protein affects the pattern of amino acid appearance, which ultimately leads to differences in the net amino acid uptake and muscle protein synthesis after resistance exercise.



**FIGURE 3.** Mean ( $\pm \text{SEM}$ ) fractional synthetic rate (FSR) of muscle proteins during the resistance exercise time period (Exercise) and 3 h after exercise and the consumption of a nonfat milk-protein beverage or an isonitrogenous, isoenergetic, macronutrient-matched (750 kJ, 18.2 g protein, 1.5 g fat, and 23 g carbohydrate) soy-protein beverage (3 h Recovery). A 2-factor ANOVA was performed to test for main effects of time and beverage. A main effect of time and a time-by-beverage interaction was observed, and a post hoc analysis with Tukey's honestly significant difference test was conducted to determine differences. \*Significantly different from the soy group at the same time point,  $P < 0.05$ . †Significantly different from Exercise,  $P < 0.05$ .  $n = 8$ .

Hyperaminoacidemia resulting from the ingestion of protein or amino acids after resistance exercise provides a potent stimulus for muscle protein accretion. In particular, essential amino acids appear crucial, and are perhaps all that are necessary, for this process (29). Both soy and milk are high-quality proteins (30). Analysis of the proteins yielded an essential amino acid composition of the milk and soy proteins of 43% and 41% of TAAs, respectively. Analysis of the individual amino acid content of the milk and soy showed that ingestion of 18.2 g protein provided  $\geq 70\%$  of the Recommended Dietary Allowance for all of the individual essential amino acids, except methionine (31). The content of methionine in the soy protein (1.4%) was lower than that in milk protein (2.6%); hence, 18.2 g protein provided 30% and 50% of the Recommended Dietary Allowance for methionine with consumption of soy and milk, respectively. In a series of nitrogen balance studies, Young (30) confirmed that the quality of soy protein is comparable with that of good-quality animal-protein sources, such as milk, and that methionine supplementation was not needed to maintain nitrogen balance. In agreement, our data suggest that the essential amino acid content is likely not the underlying reason why there were no differences between the milk and soy proteins, because no differences in the intramuscular concentration of any of the essential amino acids were detected. This suggests that the availability of essential amino acids, and thus the availability of the amino acids to charge transfer RNA in the muscle for protein synthesis, was not different between the trials. We propose that the rapid digestion of soy protein, and therefore the faster and greater increase in delivery of amino acids from the gut to the liver, may have resulted in an increased utilization of these amino acids for the synthesis of serum proteins and urea, as seen by Bos et al (32), rather than for muscle protein synthesis.

Ingestion of soy protein results in a rapid rise and fall in blood TAA concentrations, whereas milk protein ingestion produces a more moderate rise and a sustained elevation in blood amino acid concentrations (32). In support, our data show that the postexercise consumption of soy protein resulted in a rate of increase in blood TAA concentrations, between the time of ingestion and the first 30 min after exercise, of  $25 \mu\text{mol} \cdot \text{L}^{-1} \cdot \text{min}^{-1}$  that was followed by a rate of decline of  $9 \mu\text{mol} \cdot \text{L}^{-1} \cdot \text{min}^{-1}$  in the following 30 min. In contrast, with postexercise milk consumption, we saw a more modest rise in TAA concentration of  $14 \mu\text{mol} \cdot \text{L}^{-1} \cdot \text{min}^{-1}$  that was followed by a much less rapid



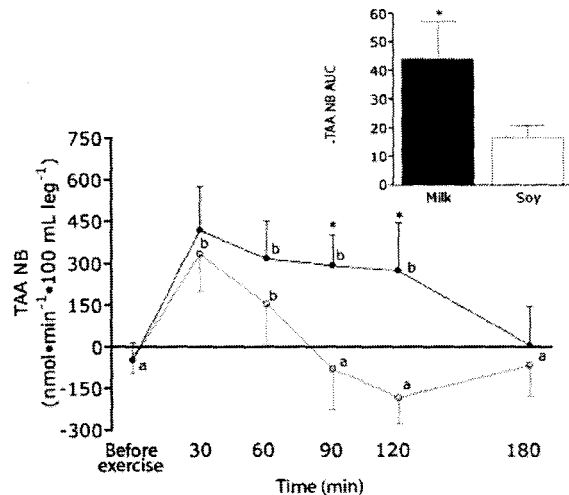
**FIGURE 4.** Two-pool model-derived mean ( $\pm$ SEM) values for rate of appearance ( $R_a$ ; A) and rate of disappearance ( $R_d$ ; B) of phenylalanine, the chemical net balance (NB) of phenylalanine across the leg (C:  $\bullet$ , milk;  $\circ$ , soy), and the positive area under the curve (AUC) for chemical NB of phenylalanine across the leg after consumption of a nonfat milk-protein beverage or an isonitrogenous, isoenergetic, macronutrient-matched (750 kJ, 18.2 g protein, 1.5 g fat, and 23 g carbohydrate) soy-protein beverage (D). A 2-factor ANOVA was performed on the  $R_a$ ,  $R_d$ , and NB data to test for main effects of time and beverage. Main effects were analyzed with Tukey's honestly significant difference test to determine differences. The NB AUC was analyzed by using a paired *t* test. A significant time-by-beverage interaction was found for the chemical NB of phenylalanine ( $P < 0.05$ ). \*Significantly different from the soy group,  $P < 0.05$ . Significant differences across time are represented by lowercase letters; means with different lowercase letters are significantly different,  $P < 0.05$ . The data in panel B were analyzed with both beverage groups combined.  $n = 8$ .

decline of  $0.8 \mu\text{mol} \cdot \text{L}^{-1} \cdot \text{min}^{-1}$ . The only statistically significant difference in TAA concentration between the soy and milk periods was that at 30 min after consumption. The peak in amino acid concentration that we observed occurred earlier than that observed by Bos et al (32), who found that the amino acid concentration peaked between 1 and 2 h after protein consumption. The test meals consumed by participants in this study (32) had 30% of total energy from fat, which would likely have slowed digestion and, thus, the rate appearance of amino acids into general circulation. We propose that the digestion rate and, therefore, the ensuing hyperaminoacidemia that differed between the milk and soy groups after exercise is what affected the net uptake of amino acids in the exercised leg. Bohé et al (1) have reported that extracellular, not intracellular, amino acid concentrations are regulators of the rise in muscle protein synthesis. Miller et al (10) showed that 2 drinks containing  $\approx 6$  g crystalline amino acids given 1 h after resistance exercise transiently and independently stimulated amino acid uptake. These data also showed, despite sustained elevations in amino acid concentration in the hour after the second drink, that amino acid uptake fell sharply. In light of previous data (1, 10), one possibility is that a rise in the extracellular amino acid concentration is a stimulus for muscle protein synthesis; decreases in extracellular amino acid concentration may actually shut off protein synthesis; this scheme is similar in nature to that proposed by Bohé et al (1). This may explain why

the sharp rise then fall in aminoacidemia in the soy condition resulted in a lower uptake and net synthesis than in the milk condition.

Previous studies that examined the effect of ingestion of similar quantities of crystalline amino acids on muscle protein turnover have shown that increases in net protein balance with the ingestion of 40 g crystalline indispensable amino acids (8.3 g leucine; 12) were similar in magnitude to that seen with the ingestion of only 6 g crystalline amino acids (2.2 g leucine; 9). These data suggest that, when large quantities of amino acids are ingested, amino acids are likely being directed to deamination and oxidation. In the current experiment, we observed no change in whole-body protein oxidation during the entire study protocol, which indicated that the dose of protein ( $\approx 7.5$  g indispensable amino acids) did not stimulate amino acid oxidation.

The combined stimulus of resistance exercise and protein or amino acid consumption results in a net protein balance greater than that from either stimulus alone (33, 34). Although the exercise- and feeding-induced response to a single exercise bout is small, muscle protein accumulates and fiber hypertrophy occurs over time with resistance exercise training (34). Muscle fiber hypertrophy occurs when there is a sustained positive balance between muscle protein synthesis and breakdown. Therefore, consumption of milk after resistance exercise, which sustains a more positive net protein balance acutely, should theoretically result in




**FIGURE 5.** Mean ( $\pm$  SEM) total amino acid (TAA) chemical net balance (NB) after consumption of a nonfat milk-protein beverage ( $\circ$ ) or an isonitrogenous, isoenergetic, macronutrient-matched (750 kJ, 18.2 g protein, 1.5 g fat, and 23 g carbohydrate) soy-protein beverage ( $\bullet$ ). Inset: positive area under the curve (AUC) for TAA NB after consumption of the milk or soy beverage. A 2-factor ANOVA was performed on the TAA NB data to test for main effects of time and beverage. Main effects were analyzed by using Tukey's honestly significant difference test to determine differences. The TAA NB AUC was analyzed by using a paired  $t$  test. A significant time-by-beverage interaction was found for TAA NB ( $P < 0.01$ ). \*Significantly different from the soy group,  $P < 0.05$ . Significant differences across time are represented by lowercase letters; means with different lowercase letters are significantly different,  $P < 0.05$ .  $n = 8$ .

greater muscle hypertrophy than consumption of soy protein after exercise. This ability of milk-protein consumption to enhance anabolism after resistance exercise might be particularly valuable to persons with compromised muscle function.

Our value for blood flow of  $\approx 0.21$  L/min at rest, excluding the immediate postexercise hyperemic response, compares relatively well with other resting flow values obtained by using Doppler ultrasound measurements (for a review, see reference 35). However, because our subjects were all strength-trained men, their average leg volume was  $12.7 \pm 0.7$  L; thus, our reported resting flow ( $\approx 1.56 \text{ mL} \cdot \text{min}^{-1} \cdot 100 \text{ mL leg volume}^{-1}$ ) is lower than values reported in other studies (3, 8, 28, 33). The result is that our estimates of  $R_a$  and  $R_d$  are lower than those previously reported (3, 8, 28, 33); the differences between our studies and others appear to be due, for the most part, to a lower blood flow. However, we did not observe any between-treatment effects on blood flow (Table 2), which is not surprising given that the same exercise bouts (volume and relative intensity) were performed, and similar insulin responses were observed between trials (Table 1). Hence, we acknowledge that our flow values, collected by an experienced investigator using established procedures that have been shown to be valid in a variety of situations (36–38), might be lower than what others have observed but believe it is unlikely that the between-trial differences were influenced by our measurements of flow.

In conclusion, we found that the consumption of intact dietary proteins resulted in a positive net protein balance and an increased rate of muscle protein synthesis after resistance exercise. Further analysis of area under the NB curves indicated a greater net amino acid balance across the leg, and the measures of muscle FSR indicated greater rates of muscle protein synthesis after milk

ingestion than after soy ingestion. These increases in anabolic processes were seen without any concurrent increases in whole-body protein oxidation. It appears unlikely that our results were due to differences in amino acid composition between the proteins, which were minimal. Instead, we favor the hypothesis that differences in the delivery of and patterns of change in amino acids are responsible for the observed differences in net amino acid balance and rates of muscle protein synthesis. 

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The authors' responsibilities were as follows—all authors: study conduct, data analysis, and writing and editing of the manuscript. None of the authors had a conflict of interest to declare.

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### STUDY 3

Differential effects of resistance and endurance exercise on signaling molecule phosphorylation and protein synthesis in human muscle

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In review

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**Key Words:** amino acid, stable isotope, hypertrophy, mitochondria, myofibrillar, signal transduction



## ABSTRACT

Resistance (RE) and endurance (EE) exercise stimulate human mixed-muscle protein synthesis. The different phenotypes induced by RE and EE training must result from differential stimulation of myofibrillar and mitochondrial protein synthesis specific to exercise mode. We measured the synthetic rates of myofibrillar and mitochondrial protein fractions and the activation of signaling proteins (Akt-mTOR-p70S6K) involved in translation initiation at rest and after an acute bout of unilateral leg RE or EE both in the untrained state and after 10 wk of RE or EE training. In the untrained state, both myofibrillar and mitochondrial protein synthesis increased (67% and 69%,  $P < 0.02$ , respectively) after RE. After training, only myofibrillar protein synthesis increased with RE (36%,  $P = 0.05$ ). EE stimulated mitochondrial (untrained: 154%; trained: 105%,  $P < 0.05$ ) but not myofibrillar protein synthesis, regardless of training status. An acute bout of resistance and endurance exercise increased the phosphorylation of numerous proteins in the Akt-mTOR-p70S6K pathway with comparatively minor differences between two exercise stimuli. Phosphorylation of Akt-mTOR-p70S6K proteins was increased more after 10 wk of RE than by EE training. Chronic RE or EE training modified the protein synthetic response of functional protein fractions without an obvious explanatory change in the phosphorylation of regulatory signaling pathway proteins.

## INTRODUCTION

Resistance exercise (RE) training results in increases in strength and muscle fiber cross-sectional area (CSA) (1, 2) and, particularly, of myofibrillar protein myosin and actin.

Endurance exercise (EE) training is characterized by fatigue resistance due in part to increased oxidative capacity secondary to increased mitochondrial density and thus mitochondrial protein (3-6). Acutely, the rate of mixed muscle protein synthesis (MPS), an average measure of all muscle proteins, is stimulated by resistance (7-11) and endurance (12, 13) exercise, but the nature of responses of classes or individual proteins within this mixed protein response is unknown. Resistance and endurance exercise must induce protein synthesis in differing fractions of muscle protein, most obviously myofibrillar versus mitochondrial proteins. Chronic repeated performance of resistance exercise induces a more rapid but less long lived rise in mixed muscle protein synthesis after acute resistance exercise than occurs in untrained muscle (9, 10, 14-17).

Furthermore, endurance exercise training (18, 19) as well as resistance training (11, 16, 20, 21) have been shown to increase mixed muscle protein synthesis for up to 2 and 4 days, respectively, after the last exercise session.

Phosphorylation of elements of the protein kinase B (Akt)-mammalian target of rapamycin (mTOR)-70 kDa S6 protein kinase (p70S6K) signal transduction pathway increase with exercise and feeding (22-28) and are thought to promote the development of an active translation complex. Resistance exercise both alone and in combination with feeding induces phosphorylation of Akt, mTOR, p70S6k and ribosomal protein S6 (rpS6) (29-33). Therefore, in an effort to contextualize the results of our planned measurements

of protein synthesis we also sought to measure phosphorylation of signaling proteins of the Akt-mTOR-p70S6 pathway shown by others to be responsive to resistance exercise (29-35), and endurance exercise (32, 36).

Exercise mode-specific responses have been observed in terms of AMP-activated protein kinase (AMPK) activation (32, 37, 38) and inhibition of mTOR in rodents has been speculated to be the underlying reason why resistance exercise is anabolic whereas endurance exercise is not (22). Phosphorylation and enzyme activity of AMPK, which is increased during and briefly after exercise (32, 37, 38), has been associated with inhibition of mTOR, p70S6k and 4EBP-1 phosphorylation in rat skeletal muscle (22, 39); whether a similar mechanism is at play in human muscle is unknown.

Membrane-associated integrins respond to mechanical stimuli by transducing the signal into a cellular response. Focal adhesion kinase (FAK) has been suggested as a possible integrator of load-activated stimuli and integrin signaling in hypertrophying skeletal muscle because stretch has been shown to activate FAK in muscle (40). Moreover, FAK phosphorylation is reduced in unloaded human skeletal muscle (41) suggesting that it is a reasonable candidate for sensing mechanical loads.

We hypothesized that in an untrained state an acute bout of resistance or endurance exercise would elicit a non exercise-specific rise in the synthetic rate of all protein fractions. As more and more acute bouts are carried out (i.e., comprising a training program), increasingly exercise-mode and protein-specific protein synthetic responses must occur. Thus, the primary aim of this study was to measure myofibrillar and mitochondrial protein synthesis after an acute bout of either resistance or endurance

exercise and repeat this after 10 weeks of resistance or endurance exercise training. The second aim of this study was to examine the effects of bouts of different exercise modes, carried out, in the fed state on the activation of signaling proteins regulating protein synthesis (in the Akt-mTOR-p70S6K pathway), to see how these responses were affected by endurance or resistance training. We hypothesized that the phosphorylation of all anabolic signaling molecules would be changed appropriately (increases or decreases of phosphorylation) to promote muscle protein synthesis after acute resistance but possibly suppressed after endurance exercise, at least in the untrained state for the time required for AMP/ATP ratios to normalize. After training we expected a different response in as much as training would attenuate the rise in protein synthesis (9, 10, 14-17) and that this would be accompanied by a reduced extent of change of the phosphorylation of the regulatory anabolic proteins. We also undertook to measure the responses of molecules likely to regulate activities of members of the Akt-mTOR-p70S6K pathway, namely AMPK and FAK.

## **MATERIALS AND METHODS**

### **Subjects**

Ten healthy men (mean  $\pm$  SEM: age,  $20.5 \pm 0.6$  y; mass,  $89.4 \pm 4.8$  kg; height,  $179.6 \pm 2.2$  cm,  $\text{VO}_{2\text{peak}}$ :  $43.9 \pm 2.1$  ml $\cdot$ kg $^{-1}\cdot$ min $^{-1}$ ) were recruited for the study. Subjects were not actively participating in any weightlifting activities or any programmed endurance activity ( $< 1$  day/week) for  $>8$  months prior to the study. Each participant was advised of the purposes of the study and associated risks. Participants were required to complete a health questionnaire and based on responses were deemed healthy. All subjects were non-

smokers and were not taking any medications. All subjects gave their written and verbal informed consent prior to participation. The Hamilton Health Sciences Research Ethics Board approved the project, which complies with all standards set by the declaration of Helsinki on the use of human research subjects.

### **Experimental protocol**

The subjects underwent two metabolic investigations (described later) separated by 10 wk of unilateral leg resistance or endurance exercise training. Participants served as their own controls, one leg being assigned to the resistance exercise and one leg to the endurance exercise condition. The choice of legs for training mode was randomized in a counter-balanced manner.

*Exercise Testing* At least 2 weeks before the first infusion trial, subjects reported to the Exercise Metabolism Research Laboratory for familiarization and explanation of all procedures. Afterwards, the baseline physiological characteristics of the legs were measured, in a randomized order. Single leg knee extension strength measurements were determined for both legs over several days of testing before the first and last metabolic investigation. Strength measures included isotonic single repetition maximum (1RM), Nautilus, Vancouver, WA), isometric 1RM and isokinetic (0.52 rad/s) 1RM (Biodex-System 3, Biodex Medical Systems, Shirley, NY). Participants completed three  $\text{VO}_{2\text{peak}}$  tests on separate days over the two weeks period prior to the first infusion metabolic investigation: a two-leg  $\text{VO}_{2\text{peak}}$  test and a single leg  $\text{VO}_{2\text{peak}}$  test on each leg. To estimate isotonic 1RM for the knee extension subjects were seated with their hips at 90° and their back against a backrest inclined at 30° from horizontal. The fulcrum of the

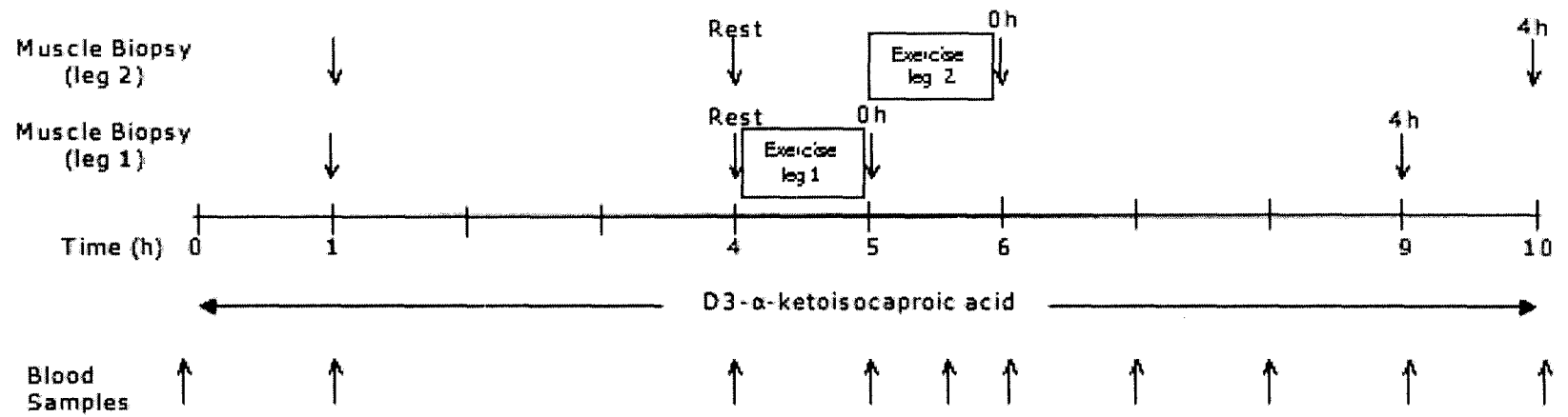
machine was aligned with the lateral aspect of the midline of the subject's knee. The leg pad of the machine was positioned ~5 cm above the subject's ankle and the rotation arm was positioned so that the subject's knee was bent to 90°. The subjects 'settings' for pin placements for seating in the machine were recorded and kept constant throughout the study. A full repetition was when the subject was able to move the weight through an arc of ~80° (from 90° to ~170°). Subjects warmed up with 8-10 repetitions using a light weight. Subjects then performed a single best effort at a weight estimated to be the subject's 1RM based on body weight and height by an experienced trainer. The weight was increased or decreased depending on whether the subject could just manage to perform the task. This estimated 1RM was checked on two subsequent occasions by simply having the subject report to the lab and asking them to once more perform a 1RM at the previously determined weight. For a 'true' 1RM to be determined the coefficient of variation (CV) between two attempts had to be less than 5%. For no subject did it require more than three attempts to determine this 1RM.

Isometric and isokinetic (concentric at an angular velocity of  $0.52 \text{ rad.s}^{-1}$ ) knee extensor peak torques were determined using the dynamometer after having a prior familiarization on the dynamometer. The order of testing for each mode was randomized. Subjects had their shoulders strapped to the chair and the chair was adjusted so that the lateral aspect of the midline of the subject's knee lined up with the dynamometer fulcrum. Chair settings were recorded and set to the same settings for subsequent dynamometer testing. The participant's knee was maintained at an angle of 70° during three isometric repetitions (5 s) with 90 s of rest between repetitions. For the isokinetic contractions, subjects carried

out 10 repetitions throughout the complete 65° range of motion. The subjects were given more than two min of recovery time between each exercise mode. All subjects were verbally encouraged to voluntarily produce their maximal force and given visual feedback of their force production. The highest peak torque value was considered as the maximal value.

VO<sub>2peak</sub> values for both legs together and separately were determined using an incremental exercise test to exhaustion (usually 7-12 min) on a Lode cycle ergometer (Groningen, Netherlands) with oxygen uptake measured continuously (AEI Technologies, Pittsburgh, PA). Exhaustion was defined at a respiratory exchange ratio >1.2, a heart rate within 5 beats.min<sup>-1</sup> of the subject's age-predicted maximal heart rate (for the two leg test), and the inability to maintain 60 rpm on the cycle ergometer at the set workload. The participant's foot was secured to the pedal to enable transmission of force when pushing and pulling the pedals. From the single leg test on the leg assigned to endurance exercise activity, a workload designed to elicit a VO<sub>2</sub> equivalent to 75% of the subject's single leg VO<sub>2peak</sub> was selected and confirmed using a 15 min test ride 1 week before the metabolic investigation.

Muscle metabolic investigation. Subjects participated in two investigations before and after training. (See Fig. 1). They were asked to refrain from any strenuous exercise for two days beforehand. Therefore, the post-training investigations were carried out at least two days after the last training session.



**Figure 1.** Infusion trial protocol



Participants were asked to record what they ate in the afternoon and evening before the first investigation and were asked to consume the same meals again before the second investigation. Subjects were instructed not to consume any food after 2000 h on the night beforehand and not to consume beverages containing caffeine for 24 h. On each study trial day, subjects consumed a defined formula beverage (2000 kJ; 82 g carbohydrate, 20 g protein and 8 g fat, Boost®, Novartis Nutrition Corporation, Mississauga, ON) in the morning (0500 h) after an overnight fast. Two and a half hours later (effectively post-absorptive), subjects reported to the exercise metabolism laboratory. A 20 g polyethylene catheter was then inserted into an antecubital vein from which a baseline blood sample was taken to determine background amino acid enrichment. After the baseline blood sample was drawn, a primed constant infusion of D<sub>3</sub>-α-ketoisocaproic acid (D<sub>3</sub>-α-KIC prime: 10 μmol · kg<sup>-1</sup>; infusion rate: 9 μmol · kg<sup>-1</sup> · h<sup>-1</sup>) was initiated. All isotopes were purchased from Cambridge Isotopes (Andover, MA), dissolved in 0.9% saline, filtered through a 0.2 μm filter and infused using a calibrated syringe pump (KD Scientific, Holliston, MA). The infusion protocol was designed so that steady state was achieved within 1 h in both the intramuscular and plasma pools (14, 42). Immediately after the beginning of the infusion the participant ingested an aliquot of Boost® (75% carbohydrate, 18% protein and 7% fat), which they ingested every 30 min (22 times) for the duration of the metabolic study. The total energy content of all the drinks provided 75% of the daily energy requirements based on the Harris-Benedict equation (43), using a physical activity level of 1.5. On the basis of the leucine content in protein and the food composition we manipulated the drink to deliver 0.1% of the labeling in d<sub>3</sub>-α-KIC mixed

into the protein drink. After baseline sampling, subjects rested for 1 h, during which time another polyethylene catheter was inserted into the contralateral arm to sample blood for the remainder of the protocol. After 1 h, a blood sample and a percutaneous quadriceps muscle biopsy was obtained using a 5 mm Bergström biopsy needle modified for manual suction under local anesthesia (1% xylocaine). The muscle was dissected free of any visible fat and connective tissue and was immediately frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$  prior to analysis. In the untrained situation a single biopsy was taken, but after training, biopsies were taken from each leg. After resting for another 3 h, another blood sample and two more muscle biopsy were taken (one from each leg) via separate incisions. Half the participants were randomized to perform the resistance exercise first and the others performed the endurance first. After 10 wk of training, the order of exercise was reversed for each participant so that participants who performed the exercised with resistance exercise first when untrained, underwent endurance exercise first in the untrained situation, and endurance first after training. The acute resistance exercise consisted of 5 sets of 8-10 repetitions at 80% 1RM of single leg knee extension and the endurance exercise consisted of single leg cycling for 45 min at 75%  $\text{VO}_{2\text{peak}}$ . The same relative intensities for the knee extension and single leg cycling were used in the second trial after training and re-testing of strength and  $\text{VO}_{2\text{peak}}$ . After the first exercise bout, in whatever mode, a blood sample was taken and then a percutaneous quadriceps muscle biopsy was taken from the leg that had exercised; the second acute bout of exercise in a different mode was then performed followed by blood sampling and

a percutaneous muscle biopsy taken from the exercised leg. The participants then rested and blood samples and biopsies were taken 4 h after each respective mode of exercise.

*Training Protocol* . After the aforementioned baseline testing was complete, subjects underwent a 10 wk training program in which one leg carried out a resistance-training program, consisting of knee extension exercise, while the contralateral leg carried out an endurance training program consisting of one-legged cycling on a cycle ergometer specially designed for this study. The participant's foot was secured to the pedal to enable transmission of force when pushing and pulling the pedals. Subjects alternated training between resistance and endurance training each day so that the first week they performed endurance training three days and resistance training twice. The following week, resistance training was carried out three times and endurance exercise twice.

The training for the resistance-trained leg began with each session consisting of three sets with 10-12 repetitions per set at 80% of their 1RM. On the third week, the volume of training was increased to four sets with 8-10 repetitions per set at 80% 1RM with the last set performed to failure (unable to lift the weight). If the participant could lift more than 10 repetitions on the last set, the training weight was increased on the subsequent session. This mode of adjusting training weight was carried on for the remainder of the protocol. On the fourth and fifth weeks, subjects performed 5 sets of 8-10 repetitions. At weeks six and seven, the training weight was increased so that participants' goal was to lift 8 repetitions for 5 sets. During weeks eight to ten, participants completed five sets of 6-8 repetitions. After the completion of the training protocol, subjects were tested again to determine their dynamic strength, isometric and isokinetic strength.

The training for the endurance-trained leg began with exercise for 30 min at 75% of pre-determined single leg  $\text{VO}_{2\text{peak}}$  for two weeks. Participants' heart rates were monitored throughout each training session. As a participant adapted to the training, workload was increased to elicit a heart rate equivalent to the heart rate observed at 75 % single leg  $\text{VO}_{2\text{peak}}$  in the untrained state. The duration of the exercise was increased in week 3 to 45 min. During the midweek session of week 4, 6, 8, and 10, participants completed interval sessions. In brief, participants alternated between two workloads designed to elicit heart rates of ~ 140-150 and ~ 160-170 for the duration of the training session. During week 5, training workload was adjusted so that participants were training at a heart rate of ~160. For participants with a below or above average maximal heart rate, workload was adjusted on an individual basis. In week 6, training time was increased to 1h. After the completion of the training protocol subjects' two leg and single leg  $\text{VO}_{2\text{peak}}$  was tested again on separate days.

### **Analytic methods**

#### *Blood sample analysis*

Blood samples were collected into heparinized evacuated containers. Whole blood (100  $\mu\text{l}$ ) was added to ice-cold perchloric acid (PCA; 0.6M, 500  $\mu\text{l}$ ), mixed and allowed to sit on ice for 10 min to precipitate all proteins. This mixture was then centrifuged at 20,000g for 2 min at 4°C. The PCA was neutralized with 250  $\mu\text{l}$  of 1.25 M  $\text{KHCO}_3$  and the reaction was allowed to proceed on ice for 10 min. Samples were then centrifuged at 15,000 rpm for 2 min at 4°C. The supernatant was stored at -20°C for further analysis of blood amino acid concentrations (44) and leucine enrichments. In the fed state, there is a

rapid equilibration between leucine and  $\alpha$ -KIC such that plasma and muscle intracellular enrichments converge (45). The PCA blood sample was derivatized by adding 50  $\mu$ l of N-methyl-N-*t*-butyl-dimethylsilyl-trifluoroacetamide + 1% *t*-butyl-dimethylchlorosilane (MTBSTFA + 1% TBDMCS, Regis Chemical).  $^2\text{H}_3$ -Leu enrichment was quantified using capillary gas chromatography-electron impact ionization-quadrupole mass spectrometry (GCMS; GC Hewlett Packard 6890, Palo Alto, CA; MSD Agilent 5973, Palo Alto, CA) by monitoring ions at  $m/z$  200 and 203. Plasma was obtained by centrifuging the evacuated tube at 4°C for 10 minutes at 4000g. Plasma was stored at -20° C for quantifying insulin and glucose concentrations. Insulin levels were determined using a commercially available radioimmunoassay kit from Diagnostic Products Corporation (Los Angeles, CA). Glucose levels were determined on neutralized blood PCA extracts using a standard enzymatic method (46).

#### *Muscle biopsy sample analysis*

Needle biopsies from the vastus lateralis were obtained under local anesthesia (1% xylocaine). A 5 mm Bergström biopsy needle modified for manual suction was used to obtain ~100-200 mg of muscle tissue from each biopsy. Biopsies were obtained from separate incisions from the same leg during each session. The muscle was dissected free of any visible fat and connective tissue and was immediately frozen in liquid nitrogen and stored at -80° C prior to analysis.

One piece of frozen wet muscle (~10–15 mg) was homogenized using the method described by Henriksson and colleagues (47) to a 50 times dilution. The homogenate was subsequently analyzed to determine the maximal activity of CS on a spectrophotometer

(Ultrospec 3000 pro UV/Vis) using a method described by Carter et al. (48) and corrected for protein content using a Bradford assay(46). A second portion of wet muscle (~10-20 mg) was saved for Western blotting; 10 mg wet muscle was added to 150  $\mu$ l of homogenizing buffer 1mM Na<sub>3</sub>VO<sub>4</sub>, 50 mM NaF, 40 mM  $\beta$ -glycerolphosphate, 20 mM Sodium Pyrophosphate, 0.5% Triton-X-100, complete mini protease inhibitor tabs (Roche, Indianapolis, IN) in Tris buffer pH 7.2). After thorough homogenization on ice, homogenates were spun in a 4°C centrifuge at 4500  $\times$  g for 10 minutes. The supernatant was removed and stored at -80 °C until Western blot analysis. A small portion was saved for Bradford assay for protein assays. (46).

Aliquots from the homogenates were boiled at 100°C for 5 min in 4  $\times$  sample buffer (300 mM Tris-HCl, pH 6.8, 50% glycerol, 8% SDS, 20%  $\beta$ -mercaptoethanol, 0.02% bromophenol blue). 50  $\mu$ g of sample were added per lane and separated by electrophoresis in running buffer (0.1% SDS, 192mM glycine, 25 mM Tris base) on 7.5-15% SDS-PAGE gels at 100 V until dye marker had passed through stacking layer and then at 200 V until the dye marker reached the gel bottom.. After electrophoresis, proteins were transferred to polyvinylidene difluoride (PVDF) membranes (Biorad, Hercules, CA) at 100 V for 2h at 4°C in transfer buffer (192mM glycine, 25 mM Tris base, 10% methanol). After transfer, the PVDF membranes were placed in blocking buffer: 5% BSA in Tris-buffered saline (50 mM Tris, 150 mM NaCl) and 0.1% Tween-20 (TBST) for 1 h. Blots were incubated in primary antibody in 5 % BSA in TBST overnight at 4°C with constant agitation. The next morning, blots were washed in TBST three times for 5 min and then incubated with secondary antibody (Amersham

Biosciences, Little Chalfont, UK) in 5% BSA in TBST for 1 h at room temperature, with continual agitation. Blots were washed in TBST three times for 5 min and then incubated for 5 min with Chemi Glow™ chemiluminescence reagent (Alpha Innotech, San Leandro, CA). Optical density measurements were obtained with a CCD camera, mounted in a Fluorchem SP imaging system (Alpha Innotech, San Leandro, CA). Once the image was captured, densitometric analysis was performed using AlphaEaseFC software (Alpha Innotech, San Leandro, CA). After detection of the phosphorylated antibody, the antibodies were stripped off the PVDF membrane by incubating the blot in stripping buffer (25 mM glycine-HCl, pH 2.0, 1% SDS) for 1h. We confirmed this stripping buffer was effective by reincubating the blot with secondary antibody then Chemi Glow™ chemiluminescence reagent and determining that no chemiluminescence was observable after incubation with stripping buffer. The blot was then washed in TBST three times for 10 minutes each, blocked and exposed to the total primary antibody overnight. All data is expressed as the ratio between the phosphorylated protein to the total protein. Primary antibodies were purchased from Cell Signaling (Beverly, MA) as follows : phospho-Akt (Ser<sup>473</sup>; 1:1000), total-Akt (1:1000), phospho-AMPK $\alpha$  (Thr<sup>172</sup>; 1:1000), total- AMPK $\alpha$  (1:1000), phospho- GSK3 $\beta$  (Ser<sup>9</sup>; 1:1000), total- GSK3 $\beta$  (1:1000) phospho-mTOR (Ser<sup>2448</sup>; 1:1000), total-mTOR (1:1000), phospho- S6 Ribosomal protein (Ser<sup>235/236</sup>; 1:1000), total-S6 Ribosomal protein (1:1000) and from Santa Cruz (Santa Cruz, CA): phospho-eIF4E (Ser<sup>209</sup>; 1:500), total-eIF4E (1:500), phospho-FAK (Tyr<sup>576/577</sup>, 1:1000), Total-FAK (1:1000), phospho-p70 S6K (Thr<sup>389</sup>; 1:1000), total- p70S6k (1:1000).

The procedure for the isolation of the mitochondria was adapted from Bezairé et al. (49) and myofibrillar protein from Bohé et al. (50). Briefly, the remaining portion of muscle was homogenized with a Dounce homogenizer in ice-cold homogenizing buffer (0.1 mM KCl, 50 mM Tris, 5 mM MgCl<sub>2</sub>, 1 mM EDTA, 10 mM  $\beta$ -glycerophosphate, 50 mM NaF, 1.5% BSA pH 7.5). The homogenate was transferred to a 2 ml Eppendorf tube and spun at 650g for 10 min at 4°C. The supernatant was transferred to another Eppendorf tube and spun at 10,000g for 10 min at 4°C to pellet the sarcoplasmic mitochondria (SM). The supernatant was removed and discarded. The pellet that remained from the original 650  $\times$  g spin was washed twice with homogenization buffer. A glass pestle was utilized to forcefully homogenize the pellet in ice cold homogenization buffer to liberate intermyofibrillar mitochondria (IM). The resulting mixture of myofibrils and IM was spun at 650  $\times$  g for 10 min at 4°C to pellet out the myofibrils. The supernatant was removed and spun at 10,000  $\times$  g for 10 min at 4°C to pellet the IM. The myofibrils, SM and IM pellets were washed three times with homogenizing buffer containing no BSA. A BSA-free homogenate was confirmed by electrophoresis. The myofibrils were separated from any collagen by dissolving them in 0.3 M NaCl, removing the supernatant and precipitating them with 1.0 M PCA. All samples were washed once with 95% ethanol and then lyophilized to dryness (Savant, Rockville, MD).

We confirmed qualitatively using electron microscopy analysis that the mitochondrial fractions were enriched for mitochondria and that the myofibrillar fraction contained exclusively myofibrils. Furthermore, we measured citrate synthase activity in the mixed muscle homogenate (homogenate prior to differential centrifugation) and each of the



mitochondrial fractions. We found that the citrate synthase activities of the mitochondrial fractions were ~3 fold higher than those of the mixed muscle homogenates.

It was determined the SM and IM protein pellets should be combined in order to quantify  $^2\text{H}_3$ -Leu enrichment. The mitochondria- and myofibrillar-enriched proteins were hydrolyzed in 6 M HCl at 100°C for 24 h. Hydrolysates were applied to a cation exchange resin (Dowex AG-50W X8, 100 - 200 mesh  $\text{H}^+$  form) and washed with 0.01 M HCl. The amino acids were eluted with 6 M  $\text{NH}_4\text{OH}$ , the heptafluorobutyric propyl esters were prepared (51), and  $^2\text{H}_3$ -Leu enrichment in the myofibrillar- and mitochondria-enriched protein fractions was determined using gas chromatography-negative chemical ionization-quadrupole mass spectrometry (GC Hewlett Packard 6890, Palo Alto, CA; MSD Agilent 5973, Palo Alto, CA) by monitoring ions at  $m/z$  349 and 352.

(Unfortunately, during processing, the mitochondria-enriched samples from 3 participants were lost, so data represent  $n=7$ ). No myofibrillar samples were lost ( $n=10$ ).

*Calculations* The fractional synthetic rate (FSR) of myofibrillar and mitochondrial proteins was calculated as the rate of tracer incorporation into mixed muscle proteins using plasma  $^2\text{H}_3$ -Leu to reflect the precursor pool enrichment, according to the previously published equation (10).

*Statistics* Sample size estimates were based on the ability to detect a 25% difference between groups in mixed muscle fractional synthetic rate using alpha at 0.05 and beta at 0.2 (2-sided), with an estimated variance in the measure based on past studies from our lab and from literature values. To protect power we added two subjects to the final calculated sample size estimate. Data were analyzed using Statistica (v 6.0, Statsoft,

Tulsa, OK) using a repeated measures analysis of variance with planned comparisons. Where a significant F-ratio was observed, post-hoc analysis using Tukey's test was utilized to identify individual differences. Significance was set at  $p < 0.05$ . Data are presented as means  $\pm$  SEM.

## RESULTS

### *Training and functional capacities*

Nine out of ten participants completed 100% of their allocated training sessions. One subject completed 80% of his training sessions. All participants maintained stable weight throughout the study (data not shown). There were no significant differences in any strength measurements between legs before training (Table 1). As expected, voluntary 1RM, isokinetic and isometric knee extension strengths increased by 75, 25 and 20%, respectively, after resistance exercise, with all increases being greater or occurring only in the resistance trained leg. We have previously shown that the same program of resistance training also elicits increases in muscle fiber and whole muscle cross-sectional area with no changes in the contralateral leg (14, 52). Measurements of functional adaptations considered to be characteristic of endurance training are presented in Table 2. There were no significant differences in single leg  $\text{VO}_{2\text{peak}}$  between legs before training. Two-leg (i.e., whole-body)  $\text{VO}_{2\text{peak}}$  did not change significantly after 10 wk of training, but there was a simultaneous 17% increase in the endurance trained single leg  $\text{VO}_{2\text{peak}}$  which was significant, with no change observed in the resistance exercise leg. Before training, 1 leg  $\text{VO}_{2\text{peak}}$  represented  $77 \pm 2\%$  of two leg  $\text{VO}_{2\text{peak}}$  capacity.

**Table 1.** Strength measurements. Values are means  $\pm$  SEM. \* significantly different from those in the untrained state (same leg); + significantly different from endurance exercise (same training state),  $P < 0.05$ .

	Endurance Exercise			Resistance Exercise		
	Untrained	Trained	% change	Untrained	Trained	% change
1RM (kg)	48 $\pm$ 3	62 $\pm$ 3*	30 $\pm$ 6	47 $\pm$ 3	79 $\pm$ 3*+	75 $\pm$ 10
Isometric peak torque (N)	299 $\pm$ 15	285 $\pm$ 16	-4 $\pm$ 4	293 $\pm$ 16	346 $\pm$ 17*+	20 $\pm$ 7
Isokinetic peak torque (N·m) 0.52 rad/s	241 $\pm$ 10	243 $\pm$ 10	1 $\pm$ 2	233 $\pm$ 11	290 $\pm$ 10*+	25 $\pm$ 4

**Table 2.** Measures of endurance capacity. Values are means  $\pm$  SEM \* significantly different from untrained (same leg), + significantly different from resistance exercise (same training state),  $P < 0.05$ .

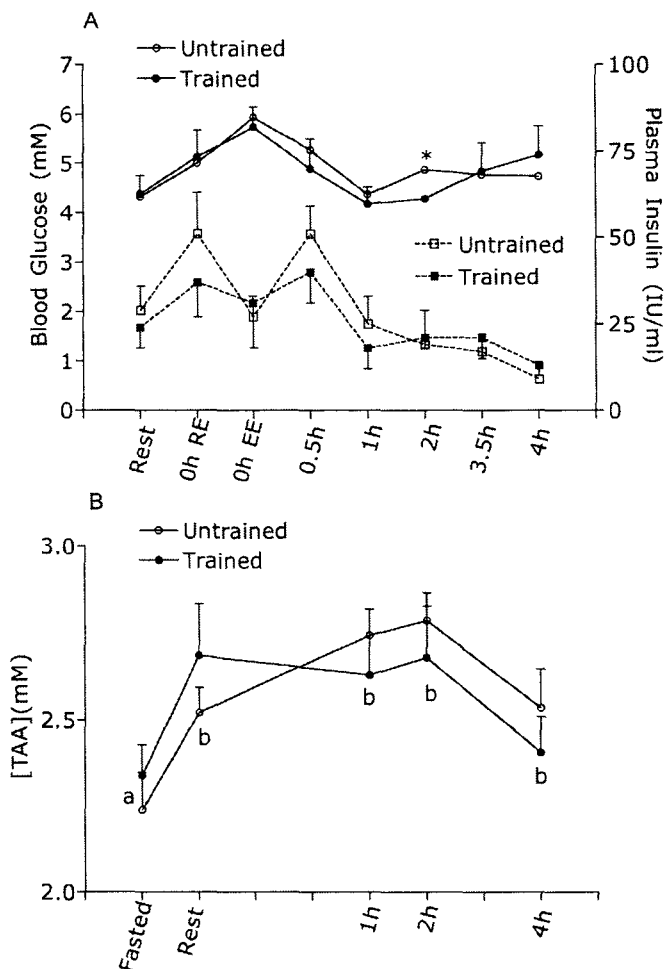
	Untrained			Trained		
Two leg $\text{VO}_{2\text{peak}}$ (L/min)	$3.9 \pm 0.2$			$3.8 \pm 0.2$		
	Endurance Exercise			Resistance Exercise		
	Untrained	Trained	% change	Untrained	Trained	% change
One leg $\text{VO}_{2\text{peak}}$ (L/min)	$2.9 \pm 0.2$	$3.5 \pm 0.2^{*+}$	$17 \pm 4$	$3.1 \pm 0.2$	$3.1 \pm 0.2$	$-2 \pm 2$
One leg $\text{VO}_{2\text{peak}}$ as a % of two leg $\text{VO}_{2\text{peak}}$	$77 \pm 2$	$90 \pm 2^{*+}$	$19 \pm 3$	$81 \pm 2$	$81 \pm 3$	$0 \pm 3$
CS activity ( $\text{mol} \cdot \text{kg}^{-1} \text{protein} \cdot \text{h}^{-1}$ )	$9.6 \pm 0.3$	$11.7 \pm .5^{*+}$	$22 \pm 8$	$9.6 \pm 0.3$	$9.0 \pm 0.2$	$-4 \pm 5$

**Table 3** Enrichment of plasma  $^2\text{H}_3$ -Leucine. Blood sample times reflect the time at which muscle biopsies were taken (except for the baseline blood sample). Units are atoms percent excess. Values are means  $\pm$  SEM (N=10).

	Blood sample time (h)					
	1	4	5	6	9	10
	1 <sup>st</sup> resting biopsy	2 <sup>nd</sup> resting biopsy	1 <sup>st</sup> recovery biopsy (leg 1)	1 <sup>st</sup> recovery biopsy (leg 2)	2nd recovery biopsy (leg 1)	2nd recovery biopsy (leg 2)
Untrained	$4.5 \pm 0.7$	$4.5 \pm 0.6$	$4.3 \pm 0.4$	$4.4 \pm 0.6$	$4.5 \pm 0.8$	$5.0 \pm 0.6$
Trained	$4.7 \pm 0.8$	$4.5 \pm 0.7$	$4.4 \pm 0.4$	$4.5 \pm 0.4$	$4.7 \pm 0.3$	$4.7 \pm 0.5$

This capacity increased to  $90 \pm 2\%$  afterwards ( $P < 0.05$ ; Table 2) only in the endurance trained leg and did not change after resistance exercise training. Citrate synthase activity, a marker of muscle mitochondrial content (53), increased  $22 \pm 8\%$  ( $p < 0.05$ ) after endurance training but no change was observed after resistance exercise training.

Feeding during the protocol resulted in the expected hyperaminoacidemia, hyperinsulinemia, and hyperglycemia (Figure 2).



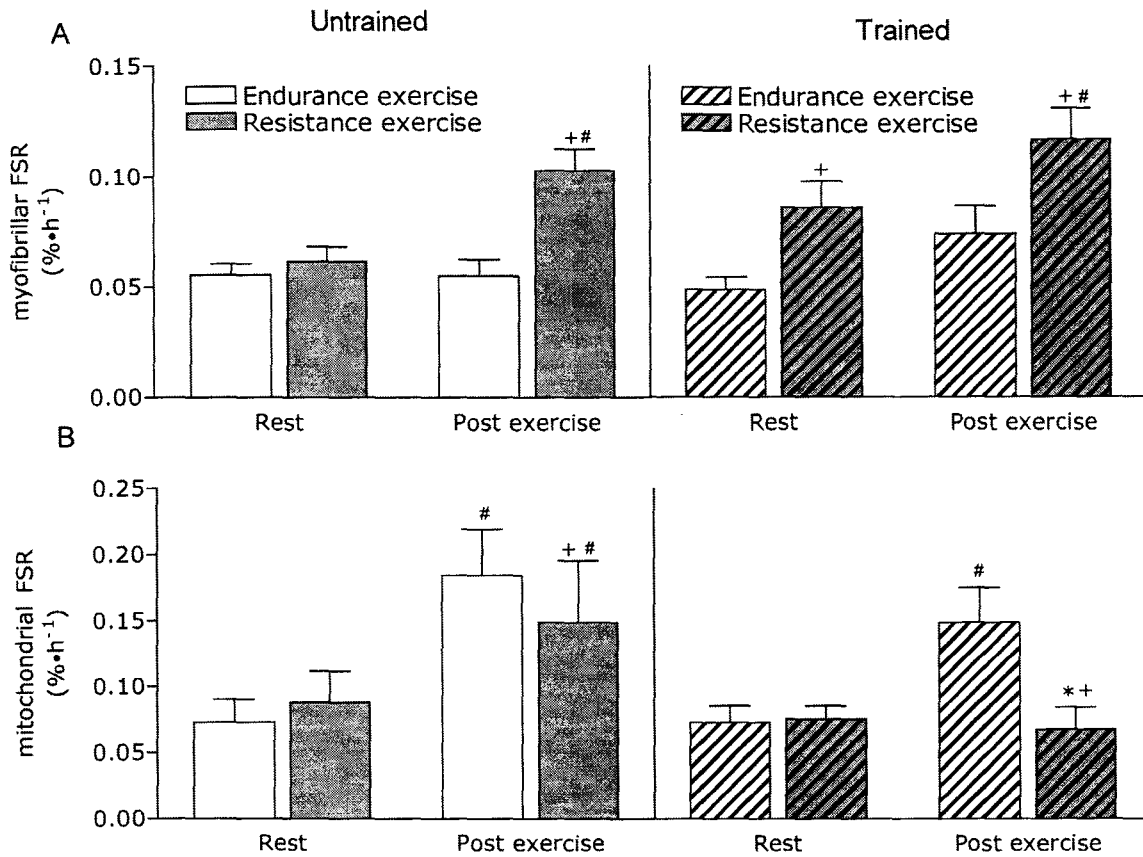
**Figure 2.** (A) Plasma glucose (mM) and insulin ( $\text{IU} \cdot \text{ml}^{-1}$ ) concentrations and (B) Whole-blood venous total amino acid concentration (mM). Values are means  $\pm$  SEM \* significantly different from values in untrained, letters depict a significant effect of time,  $P < 0.05$ .

Isotopic equilibrium was attained in plasma after 1 h of infusion and maintained throughout the tracer infusion (Table 3).

#### *Protein synthetic responses*

Resting myofibrillar fractional synthetic rate (FSR) was not different between legs prior to training (Figure 3A). After training, resting myofibrillar FSR was significantly greater in the resistance trained leg than in the endurance trained leg. Prior to training, resistance exercise resulted in a 67% increase in myofibrillar FSR. After 10 weeks of unilateral resistance exercise training, the same relative intensity of acute resistance exercise resulted in a 37% increase above resting myofibrillar FSR. Single leg cycling had no effect on myofibrillar FSR in the post-exercise period compared to rest prior to and after 10-wk of endurance training.

Resting mitochondrial FSR was not different between legs before training (Figure 3B). Both single-leg knee extension resistance exercise and cycling endurance exercise increased in mitochondrial FSR in the untrained state (Figure 3). However, this post-exercise increase in mitochondrial FSR was significantly more pronounced after endurance exercise than after resistance exercise. After training, there were no significant differences between legs or changes due to training at rest. However, the post-exercise increase in mitochondrial FSR above resting values was only observed in the endurance trained leg.



**Figure 3.** Myofibrillar (A) and mitochondrial enriched (B) FSR at rest and for the 4 h period after single- leg cycling single leg knee extension exercise. Values are means  $\pm$  SEM in  $\% \cdot h^{-1}$ . \* Training effect: significantly different from untrained values with same leg at same time; + leg effect: significantly different from endurance values, same training state and at same time; # time effect: significantly different from values at rest with same leg at same time and from those with same training state,  $P < 0.05$ .

























































































*Signalling protein phosphorylation*

Akt In the untrained state trial, Akt phosphorylation was increased by 1.5 fold above rest immediately and 4 h after acute exercise in both the endurance and resistance modes (Figure 5A). After training in both modes, resting Akt phosphorylation was 1.3 fold greater than in the untrained state. The post-exercise phosphorylation response to endurance exercise vanished after endurance training. After resistance exercise training, resistance exercise increased phosphorylation of Akt by 1.5 fold above rest immediately post-exercise, but this difference did not persist 4 h later.

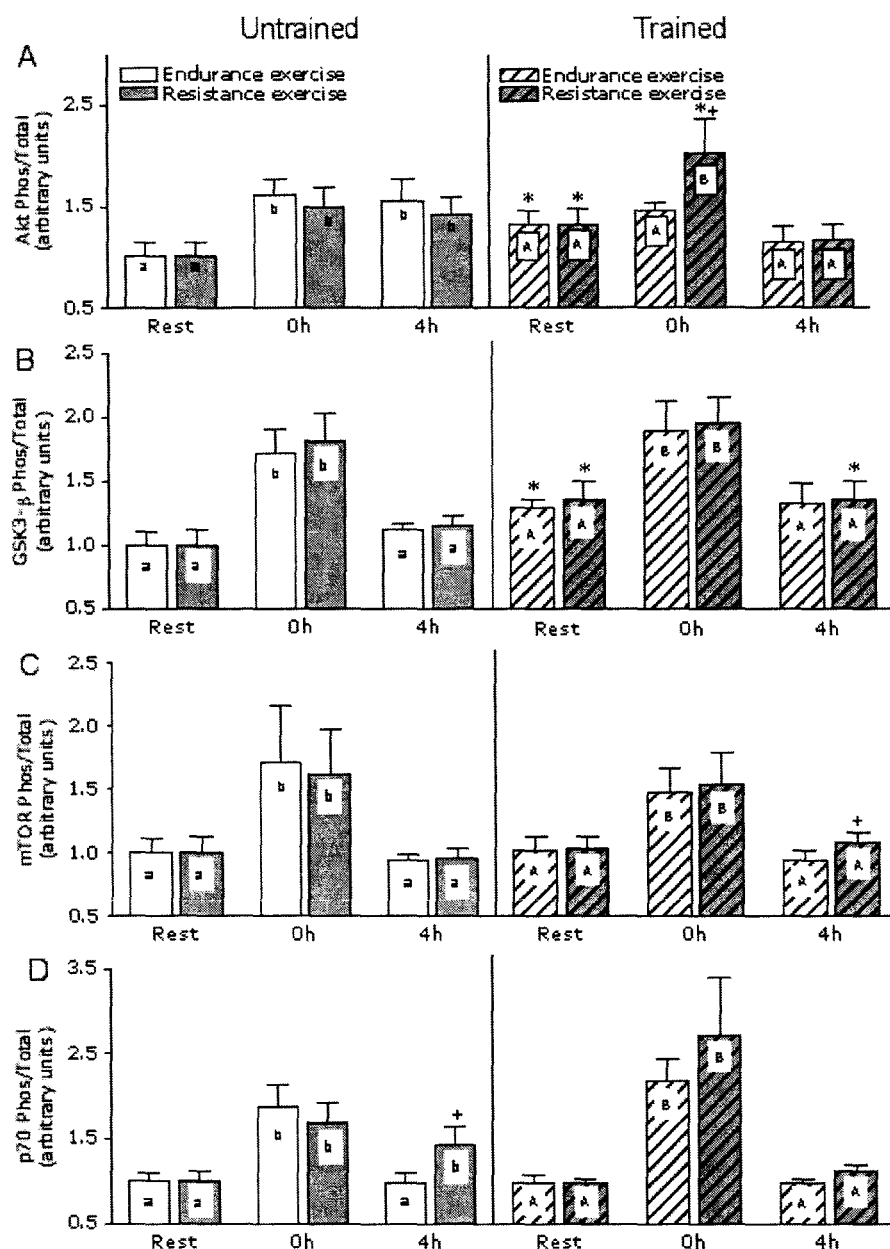
GSK-3 $\beta$  GSK-3 $\beta$  (Figure 5B) phosphorylation increased immediately after and returned to resting phosphorylation values by 4 h after acute exercise in the endurance and resistance modes before (endurance 1.7, resistance 1.8 fold increases above rest, respectively) and after training (endurance 1.5, resistance 1.4 fold increases above rest, respectively). After each type of training, resting GSK-3 $\beta$  phosphorylation was 1.3 fold greater than before training.

mTOR mTOR (Figure 5C) phosphorylation increased immediately after and returned to resting phosphorylation values by 4 h after acute endurance (untrained 1.7, trained 1.4 fold increase above rest, respectively) and resistance (untrained 1.6, trained 1.5 fold increase above rest respectively) exercise irrespective of training state. The phosphorylation of mTOR was 1.1 fold greater 4 h after acute resistance exercise after training than after endurance exercise.



Target (phosphorylation site)	Exercise mode	Untrained			Trained		
		Rest	0h	4h	Rest	0h	4h
Akt (Ser <sup>473</sup> )	Endurance Exercise						
	Resistance Exercise	NA					
mTOR (Ser <sup>2448</sup> )	Endurance Exercise						
	Resistance Exercise	NA					
p70S6k (Thr <sup>389</sup> )	Endurance Exercise						
	Resistance Exercise	NA					
rpS6 (Ser <sup>235/236</sup> )	Endurance Exercise						
	Resistance Exercise	NA					
GSK3β (Ser <sup>9</sup> )	Endurance Exercise						
	Resistance Exercise	NA					
AMPKα (Thr <sup>172</sup> )	Endurance Exercise						
	Resistance Exercise	NA					
eIF4E (Ser <sup>209</sup> )	Endurance Exercise						
	Resistance Exercise	NA					
FAK (Tyr <sup>576/577</sup> )	Endurance Exercise						
	Resistance Exercise	NA					

**Figure 4.** Examples of analysis of protein western blots. All bands for a particular antibody were obtained from a single Western blot.



**Figure 5.** Ratio of phosphorylated to total Akt (Ser<sup>473</sup>) (A), GSK3β (Ser<sup>9</sup>) (B), mTOR (Ser<sup>2448</sup>) (C), p70S6 kinase (Thr<sup>389</sup>) (D) at rest, immediately (0 h) and 4 hours (4 h) after single-leg knee extension (resistance) or cycling (endurance) prior to and after 10 wk of unilateral resistance or endurance training. Values are means ± SEM in arbitrary units. \* Training effect: significantly different from untrained values with same leg at same time; + leg effect: significantly different from endurance values, same training state and at same time; time effect: letters depict a significant effect of time (lower case represent untrained and upper case represent trained),  $P < 0.05$ .

p70S6k Acute exercise before training in both modes of exercise increased p70S6k phosphorylation above resting values immediately after exercise (Figure 5D, endurance 1.9 fold, resistance 1.7 fold). p70S6k phosphorylation remained 1.4 fold elevated above rest 4h after resistance exercise, whereas after endurance exercise it had returned to baseline. After training acute endurance and resistance exercise caused a similar pattern of immediate post exercise stimulation of p70S6k phosphorylation (endurance 2.2 fold, resistance 2.7 fold), but by 4h, p70S6k phosphorylation was no different from that at baseline.

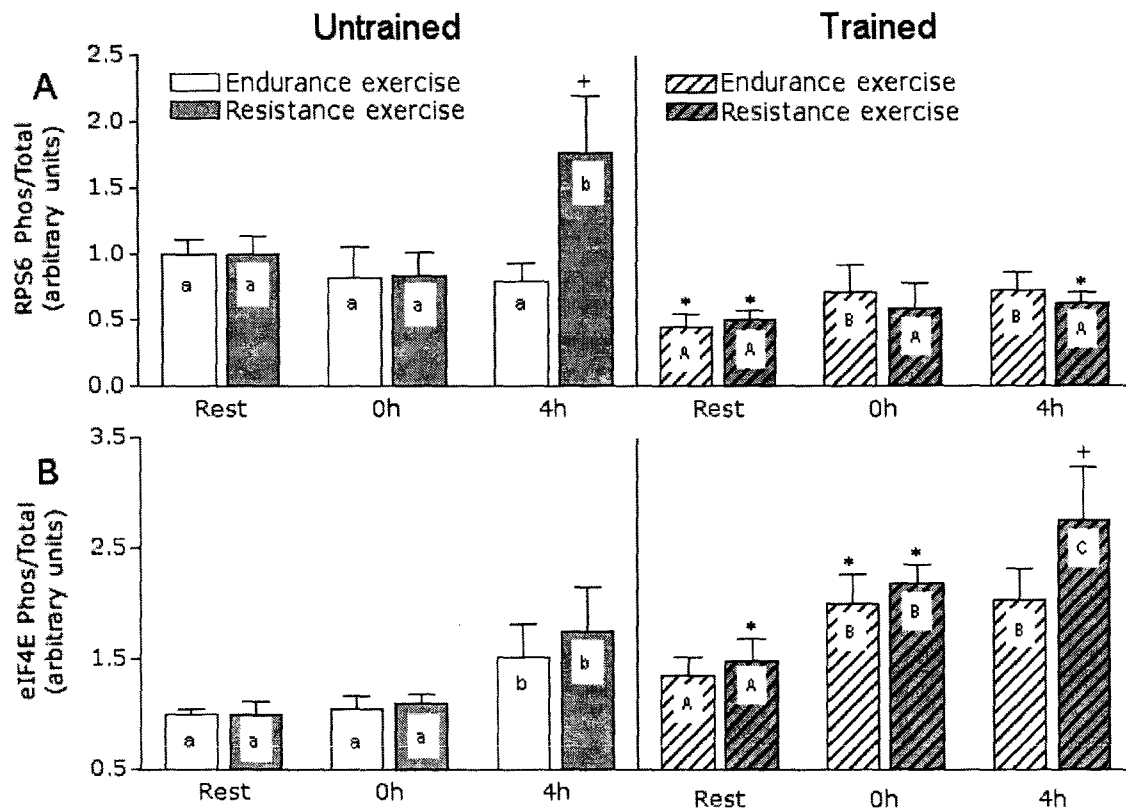
rpS6 rpS6 phosphorylation was significantly increased by 1.8 fold 4 h after acute resistance exercise before training (Figure 6A). Endurance exercise had no effect on rpS6 phosphorylation. After training rpS6 phosphorylation was 50% lower at rest than in the untrained state. Endurance exercise increased rpS6 phosphorylation by 1.6 fold immediately and 4 h after exercise but resistance exercise had no significant effect after training.

eIF4E In the untrained state, eIF4E phosphorylation did not change immediately after acute exercise in either mode. (Figure 6B). However, 4 h after each type of exercise, eIF4E phosphorylation was significantly increased above resting values (endurance 1.5 fold, resistance 1.8 fold). At rest, after resistance training, eIF4E phosphorylation was 1.5 fold greater than in the resting condition in the untrained trial. Increased eIF4E phosphorylation was seen immediately (1.5 fold above trained rest) and persisted until 4h after both modes of exercise in the TR trial. At 4 h post exercise, the resistance trained

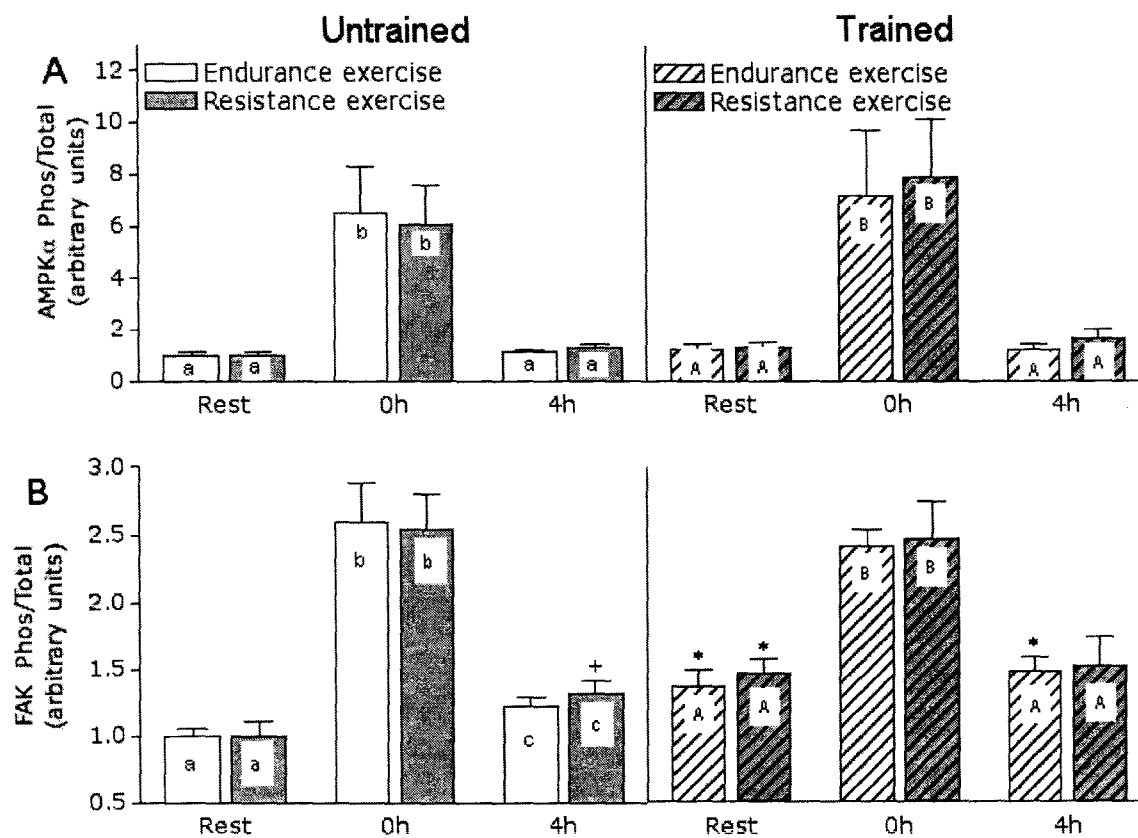
leg had significantly greater (1.8 fold greater than rest) eIF4E phosphorylation than its endurance trained (1.5 fold greater than rest) counterpart.

AMPK AMPK phosphorylation increased by 6 fold immediately after acute exercise of both modes in both the untrained and trained states, but returned to baseline values by 4 h after exercise (Figure 7A). There was no effect of mode of exercise or training.

FAK In the untrained state, immediately after acute exercise of each mode, FAK phosphorylation increased (endurance 2.6, resistance 2.5 fold) and remained significantly elevated above baseline for 4h (Figure 6B, endurance 1.2 and resistance 1.3 fold), but phosphorylation was significantly greater at this time after resistance exercise. After both modes of training resting FAK phosphorylation was significantly greater than before training (endurance 1.4, resistance 1.5 fold). After both modes of training, FAK phosphorylation was elevated above rest immediately after (endurance 1.8, resistance 1.7 fold) but returned to baseline at 4h after exercise.



**Figure 6.** Ratio of phosphorylated to total rpS6 (Ser<sup>235/236</sup>) (A), eIF4E (Ser<sup>209</sup>) (B) at rest, immediately (0 h) and 4 h after single-leg knee extension (resistance) or cycling (endurance) before and after 10 wk of unilateral resistance or endurance training. Values are means  $\pm$  SEM in arbitrary units. \* Training effect: significantly different from untrained values with same leg at same time; + leg effect: significantly different from endurance values, same training state and at same time; time effect: letters depict a significant effect of time (lower case represent untrained and upper case represent trained),  $P < 0.05$ .



**Figure 7.** Ratio of phosphorylated to total AMPKα (Thr<sup>172</sup>) (A), FAK (Tyr<sup>576/577</sup>) (B) at rest, immediately (0h) and 4 h after single-leg knee extension (resistance) or cycling (endurance) prior to and after 10 wk of of unilateral resistance or endurance training. Values are means ± SEM in arbitrary units. \* Training effect: significantly different from untrained with same leg at same time; + leg effect: significantly different from endurance same training state and at same time; time effect: letters depict a significant effect of time (lower case represent untrained and upper case represent trained),  $P < 0.05$ .

## DISCUSSION

We have characterized the human muscle protein synthetic response and intracellular phosphoprotein signaling alterations (Akt-mTOR-p70S6) that occur after both endurance and resistance exercise and examined how it is altered with exercise training. We observed that in untrained muscle, resistance exercise stimulated both myofibrillar and muscle mitochondrial protein synthesis. We contend that this is the first study to report an increase of human muscle mitochondrial protein synthesis after acute exercise.

After 10 wk of unilateral resistance training the protein synthetic response was more specific, in that only myofibrillar, and not mitochondrial, protein synthesis increased after an acute bout of resistance exercise. Furthermore, resistance training resulted in a greater resting rate of myofibrillar protein synthesis. Before and after training single-leg endurance exercise, performed at the same relative intensity, stimulated only mitochondrial protein synthesis. Single leg endurance exercise did not acutely stimulate myofibrillar protein synthesis regardless of training state.

In the untrained trial, both forms of exercise increased Akt and mTOR phosphorylation. Only resistance exercise increased rpS6 phosphorylation. Furthermore, increased p70S6 phosphorylation seen immediately after both forms of exercise, remained elevated at 4h after resistance exercise only. Our observation mirror those of previous workers who showed increased Akt and mTOR phosphorylation with no concurrent change in p70S6k or rpS6 phosphorylation after endurance exercise (32, 36). A different response was observed after 10 wk of training; we observed higher resting Akt, GSK3- $\beta$ , eIF4E and

FAK phosphorylation. Similarly, Léger et al. (54) observed increased Akt, GSK3- $\beta$  and mTOR phosphorylation at rest after 8 weeks of resistance exercise training. This increased resting phosphorylation status could explain the observed higher resting protein synthesis after resistance training or it may allow for translation initiation to be activated more rapidly after exercise in the trained state. Phosphorylation of several of the key proteins (Akt, p70S6k, GSK3- $\beta$ ) which had remained elevated 4 h after exercise in the untrained state returned to baseline at this stage in the trained state. We propose that exercise training shifts the activation state of key anabolic signaling molecules to a heightened state of “responsiveness” so that they are activated and deactivated more rapidly than in the untrained state. This training enhanced increase in ‘signaling efficiency’ is congruent with a more rapid but much shorter response in the trained versus the untrained state, which we have recently reported (14).

Resistance exercise training stimulates mixed MPS at rest and after an acute bout of resistance exercise (15, 16, 20). Those studies have also shown that resistance training attenuates the acute post exercise increase of mixed MPS to the same relative exercise intensity (15, 16, 20). We did not find an attenuation of myofibrillar protein synthesis after 10 wk of resistance exercise training. This observation is similar to that reported by Kim et al. (20). The differing response of mixed and myofibrillar protein synthesis could be due to dampening of the synthetic response of non-myofibrillar proteins while maintaining the synthesis of myofibrillar proteins after training. In support of this we observed that the increase in mitochondrial protein synthesis seen after resistance exercise in the untrained state was absent after 10 wk of resistance training. This response



does not appear to be mediated by changes in the phosphorylation status of known regulatory signaling proteins; however it is possible that increased specific gene transcripts for myofibrillar proteins remain elevated after resistance training, promoting production of myofibrillar proteins, whereas those for mitochondrial/sarcoplasmic proteins are not.

Several workers have reported an increase in mixed MPS after endurance or dynamic exercise of various kinds (12, 13). However, to our knowledge, no other study has examined the relative rates of myofibrillar or mitochondrial protein synthesis after endurance exercise. Miller et al. (55) used dynamic leg extensor exercise and reported an elevation of in myofibrillar and sarcoplasmic (which would include the sub-sarcolemmal mitochondria that are not adherent to the myofibrils) MPS for 48 h after a 1h bout. Based on this work (55) and the results of others (12, 13) we proposed that we would observe increases, albeit one that may have been delayed due to AMPK activation (22, 39), in both mitochondrial and myofibrillar protein synthesis following endurance exercise, although we did not see such a phenomenon. The single leg cycling model we used and the single leg kicking model (55) both involve endurance work, since both elicit increases in mitochondrial protein. However, the intensity of muscular work (W per kg active muscle) of the vastus lateralis in the single leg kicking exercise would be greater. This difference in work intensity could explain why our response was confined to mitochondrial proteins, but Miller et al. (55) reported increases in myofibrillar protein synthesis. Those authors cited the cellular tensegrity hypothesis (56) as a reason for the robust stimulation of protein synthesis. As a test of this hypothesis we quantified FAK

phosphorylation as a reasonable loading responsive protein complex (41, 57) but found it to be phosphorylated equally in all conditions, a result which did not illuminate our protein synthetic data. We did however observe an elevation of mitochondrial protein synthesis after an acute bout of one-leg cycling before and after training consistent with the observation of an increased mitochondrial content with endurance training (3-6).

Scheper et al. (58) speculate that eIF4E phosphorylation at Ser<sup>209</sup> subsequent to formation of the initiation complex may allow for the eIF4F complex to detach from the 5'-cap during elongation, allowing for the rapid recruitment of the next initiation complex or rendering the cap binding factors available for translation of different proteins. Before training, both forms of exercise resulted in an increased eIF4E phosphorylation at Ser<sup>209</sup> 4h after exercise. After resistance exercise training, resting eIF4E phosphorylation was elevated. Furthermore, both modes of exercise increased eIF4E phosphorylation immediately and it remained elevated for 4 h. In the resistance trained leg, eIF4E phosphorylation was significantly greater than that observed in the endurance trained leg at 4 h post exercise. Limited data exist on the activation of eIF4E in skeletal muscle. Williamson et al. (59) found that in untrained participants, resistance exercise acutely stimulated Mnk1, the upstream kinase of eIF4E, but this was not associated with an increase in eIF4E phosphorylation immediately after exercise. In consonance with the observations made by Williamson et al. (59), we did not observe an increase in eIF4E phosphorylation immediately after exercise in our untrained participants.

Atherton et al. (22) used electrical stimulation in isolated rat muscle to mimic endurance exercise or resistance exercise. AMPK was activated immediately and 3 h after low

frequency stimulation mimicking endurance exercise, but was not altered after high-frequency stimulation mimicking resistance exercise. Coffey et al. (32) found that when trained cyclists and weightlifters performed exercise in their familiar disciplines, no activation of muscle AMPK was observed. However, when they undertook a novel form of exercise, (cycling for weightlifters and weightlifting for cyclists) an increase in muscle AMPK phosphorylation was observed immediately after exercise and this returned to baseline within 3 h after exercise. Our results are similar in that AMPK phosphorylation was elevated immediately after exercise, but returned to baseline within 4 h post exercise. In contrast to previous reports (32), we found that 10 wk of training was insufficient to blunt the exercise stimulation of AMPK activity seen in athletes who had trained in their respective disciplines for years. In rat skeletal muscle, evidence exists that an increase in AMPK phosphorylation is associated with a reduced activation of mTOR, p70S6k and 4EBP-1 (22, 39). Nevertheless, we observed a simultaneous increase in mTOR and p70S6k phosphorylation above resting values coincident with the increased AMPK phosphorylation. Although unclear, in the current study it is possible that AMPK, mTOR, and p70s6k were concomitantly increased by the feeding-induced hyperaminoacidemia and hyperinsulinemia during exercise, and that these factors may override the inhibitory effect that active AMPK has on mTOR (29).

These findings provide evidence that the human skeletal muscle protein synthetic response to different modes of exercise is, as hypothesized, specific for proteins needed for structural and metabolic adaptations to the particular exercise stimulus, and that this specificity of response is altered with training to be more specific. Furthermore, our

findings indicate that several factors involved in translation initiation explained little of this differential protein synthetic response. The only notable changes were that resistance exercise resulted in a more prolonged (p70S6k) or later (rpS6 and eIF4E) activation of signaling components than with endurance exercise. It is possible that due to the timing of muscle samples, we missed changes in phosphorylation status of the target phosphoproteins measured or perhaps there are other phosphorylation sites (that were not quantified) or phosphoproteins that are involved in exercise-induced adaptations to muscle protein synthesis. In contrast, we propose that, as opposed to marked changes in signaling protein activation, at least of those proteins that would appear to be most obviously affected, that exercise and training-specific changes gene transcription may be involved in determining the specific nature of the protein synthetic response that we observed and the changes in phenotype seen with training. What controls these upstream transcriptional changes remains to be answered.

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### **Summary of major findings and suggestions for future directions**

Skeletal muscle is the largest single reservoir of protein in the human body and aside from its obvious role in performing mechanical work, it can serve as a source of amino acids for glucose production and as a nitrogen source during times of reduced nutrient availability such as during disease or after injury. Skeletal muscle, mostly by virtue of its mass, utilizes large quantities of glucose and lipids as fuel and is also a significant storage site for these substrates. Post-prandially, skeletal muscle is the largest single site of glucose disposal and as such is of primary importance in the development and etiology of type 2 diabetes (56). In addition, second only to the liver, the single biggest contributor to resting metabolic rate (RMR) is skeletal muscle (8, 27). Thus, declines in muscle mass due to disease, aging, and disuse can result in detrimental consequences, including reductions in the capacity for glucose uptake, lipid clearance, reduced resting metabolic rate, and ambulatory ability. Both exercise and feeding promote a net retention/reclamation of muscle mass and we are beginning to understand the importance of effectively manipulating these variables in offsetting, or even reversing, declines in muscle associated with the conditions of muscle loss described above. The underlying mechanisms of how exercise and feeding affect muscle protein turnover are incompletely understood. The studies contained within this thesis have added to our understanding of the control of muscle protein turnover by examining divergent effects of protein type, mode of exercise and training status.

In Study 1 a model for the study of resistance exercise training was established that allowed study within a given individual of both acute and repeated short-term

exposure to resistance exercise. The results from this work showed that skeletal muscle hypertrophy occurs with resistance training without any significant acute changes in circulating anabolic hormones following exercise. Importantly, unilateral resistance training resulted in muscle hypertrophy only in the exercised limb. Thus, phenotypic changes, at least measured outwardly as changes in muscle fibre and muscle cross-sectional area and fibre type, were not occurring in the non-exercised contralateral limb. Thus, these data indicated that the contralateral limb could act as a good control comparator for studying the effects of acute or repeated resistance exercise, or endurance exercise as it was used in Study 3, or the impact of feeding manipulations on these variables. In fact, this model has been utilized in a number of investigations arising from our lab (29, 37, 42, 49). As a corollary, in Study 3, we also observed no substantial between-leg cross-over effects of specific exercise training to the contralateral leg. That is, we saw no non-specific strength changes in the endurance trained leg and no hallmark endurance-type changes were observed in the resistance trained leg. Therefore, the unilateral model of exercise is an effective study design because it allows for within-person comparisons to be made and therefore, has stronger statistical power since between-person variability is eliminated. This model also allows for comparable systemic changes in hormones, metabolites, and substrates (most importantly amino acids), to be studied in a single experiment. This represents a cost-effective method since the infusion of stable isotope probes on two occasions in the same person, untrained (pre-training) and trained, would necessitate twice as much isotope. In addition, variability due to temporal



changes mediated by a number of potential mechanisms including diet, seasonal, and diurnal, are reduced when a single time point is used to study changes in protein turnover.

Experiments in which crystalline amino acids have been manipulated to investigate protein turnover represent an excellent experimental model. The problem with use of crystalline amino acids, however, is that they are far less likely to be consumed as a food source on a habitual basis by anyone other than a clinically monitored population. It is far more likely that whole foods or functional food products would be consumed, and likely tolerated, as versus crystalline amino acids. In addition, it is clear that consumption of intact proteins promotes different patterns of aminoacidemia and, as a result, different changes in metabolism and protein turnover. In Study 2, it was shown that fluid skim milk consumed after resistance exercise resulted in a more positive net amino acid balance and a greater stimulation of muscle protein synthesis versus an isonitrogenous, isoenergetic, and macronutrient-ratio matched soy drink. This is one of the first studies to report on the effect of intact dietary proteins on muscle protein metabolism following exercise (18, 52, 53) and the first to report a differential effect of different protein sources on changes in protein turnover in skeletal muscle following resistance exercise.

Following a resistance exercise bout new protein is added to skeletal muscle because of the synergistic effect of feeding and exercise results in a net positive muscle protein balance. Over a number of exercise sessions, the addition of new protein to the muscle after each session produces muscle hypertrophy. Therefore, based on our findings from Study 2, it would be expected that individuals engaging in resistance training who consume milk after each resistance exercise session would gain more muscle mass than

people consuming soy. This hypothesis was tested in a follow-up study to our initial acute work. Hartman et al. (24) found that during a 12 week resistance training program, participants who consumed fluid skim milk beverages immediately and 1h following each resistance training session showed greater hypertrophy than participants consuming soy or carbohydrate energy control beverages. These studies, when considered together, provide a proof-of-principle that acute findings (24, 55) are predictive of changes in muscle mass with longer-term adaptations (24, 55). Moreover, these data (24, 55) provide some validation of a model for protein accretion resulting from resistance exercise and feeding and thus adds credence to the idea that a pulsatile incremental addition of new proteins with each round of resistance exercise (38, 43) is what gradually sums to result in hypertrophy.

There are a number of unresolved research questions that arise on consideration of the work detailed in Study 2. Future work in this area could obviously take on a number of different directions, but some of the more salient studies that could be undertaken are outlined below:

1. An examination of the effect of resistance training and different types of dietary protein consumption in individuals with compromised muscle function, such as the elderly, by conducting a longitudinal training study similar to Hartman et al. (24). Recently, Elliot et al. (18) found that whole-milk may be more effective in promoting net amino acid uptake after resistance exercise than skim milk.

Therefore, if feasible, it would be ideal to include 4 groups ingesting: skim milk, whole milk, soy, or an energy (carbohydrate) control beverage. In addition to

taking measures to assess muscle hypertrophy and strength in a similar manner to Hartman et al. (24), measures of functional abilities should also be made as an important outcome for the elderly is an improvement in tasks of daily living. Interestingly, a recent review (25) details how whey protein or skim milk powder should be evaluated in terms of fortification of blended foods for so-called vulnerable groups. While the focus of this review was malnourished infants and children and people living with HIV-AIDS, a similar argument could be set out for the elderly and particularly those in institutionalized care.

2. Milk is comprised of ~80% casein and ~20% whey protein. In studies of whole-body protein metabolism at rest, it was shown that ingestion of whey protein promotes protein synthesis and yet stimulates oxidation (15), and that casein suppresses protein breakdown (7, 15). Comparing muscle protein turnover after exercise in response to different proportions of whey and casein would be of interest in order to determine an optimal combination of whey and casein to promote anabolism. A number of infant formulas have recognized the potential for maximizing anabolism that is critical in growth and as such have ratios of whey to casein closer to human breast milk protein that is composed of 65% whey and 35% casein.

From the data in Study 3, a number of novel insights were obtained. A comprehensive review of the literature in this area indicates that our report is the first study documenting measurement of mitochondrial protein synthesis following exercise and to show the effect of mode of exercise and exercise training on key proteins involved

in translation initiation. Where skeletal muscle is concerned, particularly in humans, the most often reported variable with respect to protein synthesis is mixed muscle protein synthesis, which represents an average synthesis rate of all skeletal muscle proteins. There are reports that both resistance (5, 10, 32, 40) and endurance (9, 47) exercise both stimulate mixed muscle protein synthesis and yet we know that only resistance exercise leads to hypertrophy (4, 40, 41, 51, 58) and endurance exercise promotes increased mitochondrial content (19, 23, 26, 34). Hence, it would seem likely that the exercise mode would likely specifically stimulate the synthesis of proteins known to increase in content, or change in isoform, as a result of that type of exercise. Moreover, we hypothesized that such a response may become more protein-specific with an increased number of bouts of exercise. Data from Study 3 sheds insight into the nature of the increase in protein synthesis seen following different modes of exercise. Furthermore, our data helps to reconcile the observations that both resistance (5, 10, 32, 40) and endurance exercise (9, 47) acutely stimulate mixed muscle protein synthesis, while resistance and endurance exercise training lead to very different phenotypic adaptations (4, 48, 51). It was found that resistance exercise stimulates myofibrillar protein synthesis while endurance exercise does not. Our observation provides direct data showing why resistance exercise training increases contractile protein content within skeletal muscle, as exemplified through greater muscle fiber area, while endurance exercise training does not (4, 40, 41, 51, 58). Conversely, it was found that endurance exercise stimulated mitochondrial protein synthesis in both the untrained and trained states, most likely accounting for the observation that endurance exercise increases mixed muscle protein

synthesis (9, 47). Mixed muscle protein synthesis has been reported to increase between 0.03 (47) and 0.04 (9) %/h following endurance exercise at 40-45% of  $\text{VO}_{2\text{peak}}$ . If we assume that mitochondria make up ~ 10% of muscle proteins, calculations based on the change in mitochondrial FSR in Study 3 would indicate that the increase in mitochondrial protein synthesis seen in our study would account for one third of the mixed muscle protein synthesis increase seen by others. Therefore, the increased synthetic rate of other proteins, such as sarcoplasmic proteins and/or collagen (although not measured) are likely contributing to the responses seen in mixed MPS in response to endurance exercise (9, 47), to account for the difference between mixed muscle protein synthesis and mitochondrial protein synthesis. However, we cannot discount that the higher intensity exercise protocol (75% of  $\text{VO}_{2\text{peak}}$ ) used in Study 3 might have resulted in a different mixed protein synthetic response.

Time-course data shows that the rise in mixed muscle protein synthesis may be short lived; following 45 min of walking at 40%  $\text{VO}_{2\text{peak}}$  the rise in muscle protein synthesis seen at 1h following exercise returned to levels not different from baseline by 3h following exercise(47). We measured protein synthesis of mitochondrial and myofibrillar proteins over 4h following exercise. Therefore, the rates represent the synthesis of proteins over the entire 4h period. It is possible that had we taken more frequent biopsies, we would have seen a larger increase in mitochondrial protein synthesis in the initial period following exercise that returned to baseline later in the recovery period.

Previous studies have observed that resistance exercise training attenuates the increase in mixed muscle protein synthesis seen following acute exercise (10, 29, 32, 39-41, 49). This attenuation of mixed muscle protein synthesis was speculated to be a result of a reduction in the synthesis of non-contractile proteins, and not myofibrillar proteins (29). Our observations that prior to training both myofibrillar and mitochondrial protein synthesis were elevated during recovery from resistance exercise, and that following training only myofibrillar protein synthesis increased lends evidence to this speculation. The effect of endurance exercise training on the post-exercise stimulation of muscle protein synthesis has not been studied before. Our data showed that a period of training did not result in an attenuation of the acute post-exercise increase in mitochondrial protein synthesis observed. Our study was only 10 weeks in duration, however, and therefore, it cannot be assumed that if a longer training had been used that an attenuated mitochondrial protein synthetic rate might have been observed. Assuming that there is only a finite capacity for mitochondria to expand then one would predict that at some point the increases in mitochondrial protein synthesis would be attenuated and that a rise in mitochondrial protein synthesis would represent is a renewal of potentially damaged mitochondria.

In Study 3 we also reported on the change in signaling protein phosphorylation in response to exercise. This study is the first to show the effect of endurance exercise in the fed state and resistance and endurance exercise training on the post-exercise response of the Akt-mTOR pathway. In agreement with data obtained from others (3, 11, 22, 33, 35, 45, 46), our study showed that endurance exercise increased the phosphorylation of Akt

and GSK-3, but not downstream targets of mTOR, such as p70S6k and rpS6. Similar to previous studies (11, 14, 16, 17, 20, 28, 30, 31, 50), we found that the mTOR pathway was activated in response to resistance exercise. The activation of p70S6k and rpS6, downstream targets of mTOR, did not occur until 4h after resistance exercise in the untrained trial; however, increases were seen immediately after resistance exercise following training. In support of the observation that resistance training results increased resting mixed muscle protein synthesis (29, 39, 57, 58) and a more rapid but much shorter rise in mixed muscle protein synthesis (29, 49), we found a higher resting phosphorylation of Akt, GSK3- $\beta$ , eIF4E and FAK and an immediate increase in phosphorylation of all proteins immediately following exercise in the resistance trained leg. In addition, this is the first report to show changes in the phosphorylation of eIF4E in response to exercise. We found differences in activation of eIF4E due to mode of exercise and after training; specifically, activation of eIF4E occurred sooner following both endurance and resistance exercise in the trained state and to a greater extent after resistance training.

While the work in study 3 lent some insight into the regulation protein in response to exercise in the fed state, we acknowledge to get a true measure of the effect of exercise alone on the control of muscle protein synthesis, this study would have needed to be performed in the fasted state. Indeed, it has been found that feeding alone has a substantial effect on the phosphorylation status of some of the signaling proteins measured, and that effects may have been masked by not taking a rested, fasted biopsy in this study.

There are a number of questions that arise from the work detailed in Study 3. Due to the complexity of regulation within skeletal muscle and a variety of influencing factors, the research possibilities are many. For example, the phenotypic adaptation to exercise training we observed likely involved the transcription of a number of genes. In fact, our data provide strong support for the concept that the specific nature of the protein synthetic response seen with differing exercise modes is likely strongly influenced by the transcriptional response. Furthermore, the complex integration of the signaling pathways involved in the translation of protein is far from being understood. Development of new technologies including gene microarrays to explore changes in transcription, with confirmation by RT-PCR, identification of proteins involved in the signaling and their measurement using Western blots combined with measurement of the synthesis of individual proteins (myosin heavy chain, mitochondria) will go a long way to defining how protein turnover is regulated in response to exercise and feeding. Some examples of potential future research studies are outlined here:

1. I established, based on previous work, and refined for the requirements specific to our work methods to isolate IMF and SS mitochondria for our laboratory as part of this project (For greater details see Appendix 1). Unfortunately, because there was a limited amount of mitochondrial protein from each fraction, the IMF and SS mitochondria needed to be combined to obtain a reliable measurement of overall mitochondrial protein synthesis. It is possible that these two mitochondrial pools respond differently to exercise and training, therefore, it would be very interesting to measure the protein synthesis of each of these mitochondrial protein



pools. With larger biopsies, this is likely possible, as both Bohé et al. (6) and Rooyackers et al. (44) managed to obtain sufficient protein from the SS mitochondria pool alone to measure protein synthesis.

2. We have an idea of the time course of changes in mixed muscle protein synthesis following resistance exercise in untrained and trained participants (49). It would be valuable to study the time-course of myofibrillar and mitochondrial protein synthesis following resistance and endurance exercise and how training affects this response. There are a number of study designs that could be employed to answer this research question; however, a major limitation to research in this area is the number of biopsies that can be taken from one person. In Study 3, a total of 15 biopsies were taken from each participant, likely an upper limit. A design similar to that used by Tang et al. (49) could be employed, although with two groups of participants, one group participating in resistance and the other in endurance exercise and training.
3. To date studies in humans have looked at signaling protein in young, healthy individuals. It would be of interest to examine the cell signaling in response to exercise and feeding in the elderly, or other populations with compromised muscle mass or function, particularly in response to exercise and feeding.
4. In Study 3, we did not measure changes in gene transcripts. However, exercise training would likely change the transcription of relevant genes involved in the phenotypic adaptation associated with the mode of exercise (36, 54). Therefore, it

would be of interest to explore changes in gene transcription acutely and with training in future work.

5. We know that activation of the Akt-mTOR signaling cascade is associated with increases in protein synthesis, particularly with resistance exercise (11, 14, 16, 17, 20, 28, 30, 31, 50) and feeding (1, 2, 12).(13, 21). What we do not know is which particular mRNAs are transcribed with exercise and which of those are subsequently translated.
6. Another key question to be answered is how the physical stimulus of exercise is transduced into a cellular signaling response. Discovery of new proteins involved in the control of protein turnover is likely to occur. Determination of the functional role of these proteins will lend new insight into the control of protein turnover. For example, how mTOR is regulated by GβL, Rheb, tuberlin and hamartin is incompletely defined, particularly in humans in exercise situations. The answer to these questions will likely come from researchers employing molecular biology techniques in cells and animal models. It is critically important to link the changes in signaling protein activation with the phenotypic response since changes in signaling protein phosphorylation show tremendous redundancy between exercise modes and yet the phenotypic response is much different. In addition, all attempts should be to describe that phenotypic response in as much detail as possible.

## **Conclusion**

The studies within this thesis have used the unilateral model of exercise to examine the effects of different protein and exercise modes on muscle protein turnover. By measuring protein turnover using infusion of stable amino acid tracers, amino acid concentrations in blood and muscle by HPLC, and intracellular signaling proteins abundance and phosphorylation status by Western blot techniques, some new insight has been gained in the characterization of the responses of human skeletal muscle to both exercise and feeding. In addition, some of the factors that are responsible for changes in human muscle protein turnover in response to feeding, exercise and training (both endurance- and resistance-based) are now somewhat clearer. Added to the current scientific literature, these studies will help improve our understanding of the underlying mechanisms that control skeletal muscle protein turnover in humans.

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## APPENDIX 1

Development of a new method for measurement of myofibrillar and mitochondrial protein synthesis in human skeletal muscle using GCMS

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**Title:** Development of a new method for measurement of myofibrillar and mitochondrial protein synthesis in human skeletal muscle using GCMS

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**Key Words:** amino acid isotope, mitochondria, myofibrillar

## **Introduction**

All proteins within the body are remodeled by continual and ongoing breakdown and synthesis (8). This turnover of proteins ensures the repair and maintenance of the proteins as a system to ensure the functional quality of the protein itself should it become damaged through, for example, oxidation or mechanical stress (11). Over time, when the rate of synthesis exceeds that of breakdown there is an accretion of protein within a tissue; such as occurs during growth phases in young children or when overload is imposed in skeletal muscle (i.e., hypertrophy). In contrast, when the rate of protein breakdown exceeds synthesis there is a loss of protein. For example, in situations of overt and marked muscle wasting – HIV AIDS, sepsis, renal failure – protein breakdown is thought to be the predominant process (8, 11).

Much knowledge has been gained over the past 25 years on the effect of exercise and feeding on skeletal muscle protein synthesis by use of primed constant infusions of stable amino acid isotope tracers. Over a period of hours, isotopically labeled amino acids are infused into an individual with blood samples and muscle biopsies taken at given timepoints to determine incorporation of the isotope into proteins against some measure of the precursor concentration of amino acids to get some idea of the pool from which amino acids are drawn before incorporation (9). In order to quantify incorporation of the isotope measurement of the ratio of isotopically labeled amino acid to its naturally abundant counterpart is required. For this measurement to be made on all muscle proteins the entire muscle sample is hydrolyzed in acid, the resultant free amino acids are cleaned, derivatized and run on a mass spectrometer (MS) of some form. The fractional rate of

synthesis of the muscle protein is calculated by measuring the rate of change in enrichment of the labeled amino acid into the protein over time in relation to the labeling of the pool from which the amino acid is incorporated into protein.

The most commonly reported measure of protein synthesis is to use all proteins contained within a muscle biopsy, which would yield a rate of synthesis that comprises a variety of proteins including mostly contractile, enzymatic, signaling, mitochondrial and cytoskeletal/structural proteins. Therefore, the measure of mixed muscle protein synthesis represents an average measure of synthesis rates of many proteins, and is often referred to as mixed muscle protein synthesis. Because of the different functional significance of each protein within the muscle, various researchers have developed methods to separate out individual proteins of interest including: myofibrillar, actin, myosin, sarcoplasmic and subsarcolemmal mitochondria using tissue homogenization and differential centrifugation (1, 4, 10). Studies measuring the much more abundant protein fractions, myofibrillar (which comprises 60-65% of all muscle proteins) and sarcoplasmic (i.e., non-myofibrillar), have been relatively forthcoming since these methods are simpler and have been available for some time. However, studies reporting the effects of interventions on mitochondrial protein synthesis have been far fewer owing to the fact that mitochondrial protein comprises only a small fraction, likely no more than 10%, of the total muscle protein pool. Indeed, the amount of mitochondrial protein from rat skeletal muscle yields ~ 1 and 2-4 mg/g muscle wet weight of subsarcolemmal (SS) and intermyofibrillar (IMF) mitochondria, respectively (7). Most human muscle biopsies taken from the vastus lateralis muscle weigh ~70-100 and likely no more than 200 mg



wet weight. Therefore, the expected yield of mitochondrial protein from muscle biopsy tissues would be less than 1 mg. This amount of protein is challenge to measuring using current GCMS methods. Therefore, it is not surprising that limited research is available on the regulation of mitochondrial protein synthesis with exercise or feeding.

There are two subpopulations of mitochondria in the muscle, IMF mitochondria are located between myofibrils and SS mitochondria are located beneath the sarcolemma. In humans, both populations of mitochondria have been shown to adapt to endurance exercise training by increasing in number (5, 6). To date, studies in humans, have only measured the rates of protein synthesis of SS mitochondria at rest (3, 10). Rooyackers et al. (10) were unable to obtain the IMF mitochondria because the proteolytic enzymes required to do so interfered with the measurements of the rate of synthesis of myofibrillar proteins. Recently, Bezaire et al. (2) have successfully demonstrated that strong mechanical disruption of myofibrils is effective in releasing IMF mitochondria. Therefore, the aim of this study was to adapt previous methods (1, 10) and combine them with other more recent methods of isolating IMF mitochondria (2) in order to isolate both IMF and SS mitochondrial proteins for measurement of isotopically labeled amino acids using the GCMS.

## **Methods**

**Subjects** Ten healthy men (mean  $\pm$  SEM: age,  $20.5 \pm 0.6$  y; mass,  $89.4 \pm 4.8$  kg; height,  $179.6 \pm 2.2$  cm,  $\text{VO}_{2\text{peak}}$ :  $43.9 \pm 2.1$  ml·kg<sup>-1</sup>·min<sup>-1</sup>) were recruited for the study. Subjects were not actively participating in any weightlifting activities or any programmed endurance activity ( $< 1$  day/week) for  $>8$  months prior to the study. Workup of the

technique was performed on muscle biopsies of individuals with similar characteristics to the study participants. Each participant was advised of the purposes of the study and associated risks. Participants were required to complete a health questionnaire and based on responses were deemed healthy. All subjects were non-smokers and were not taking any medications. All subjects gave their written and verbal informed consent prior to participation. The Hamilton Health Sciences Research Ethics Board approved the project, which complies with all standards set by the declaration of Helsinki on the use of humans as research subjects.

**Biopsies** Needle biopsies from the vastus lateralis of young healthy males were obtained under local anesthesia (1% xylocaine). A 5 mm Bergström biopsy needle modified for manual suction was used to obtain muscle tissue from each biopsy. The muscle was dissected free of any visible fat and connective tissue and was immediately frozen in liquid nitrogen and stored at -80°C prior to analysis.

**Tissue processing:** Tissue homogenization followed by differential centrifugation was used to separate cellular protein components of different densities. Once the tissue homogenate is obtained, a slow speed spin (~650g for 10 minutes) is used to pellet the myofibrillar protein. The supernatant is then removed and transferred to another tube and centrifuged at a higher speed (12,000g) to pellet the lighter mitochondria. We needed to establish the optimum means by which to homogenize human muscle tissue in order to obtain both SS and IMF mitochondria and myofibrillar protein. Furthermore, we needed to confirm the speed and duration of differential centrifugation to obtain myofibrillar and mitochondrial separation.

First, a search of the current literature was conducted in order to identify potential homogenizing buffers (Table 1) and homogenizing hardware (Table 2) to separate myofibrillar protein from mitochondrial protein. Each homogenizing buffer and homogenizing apparatus was systematically tested using ~100-150mg human muscle tissue to test for maximal mitochondria yield with minimum contamination of each fraction. All assays were performed on ice. The citrate synthase activity (CS) assay was used as a measure of the mitochondrial content of each fraction. In short, the different fractions obtained from the systematic testing of each homogenizing attempt were resuspended in the buffer being tested containing 0.1% Triton-X to solubilize the mitochondrial inner and outer membranes. A Bradford assay was performed in order to determine protein yield. Then an equal amount of protein from each fraction was tested for CS activity. Essentially, we were looking for a homogenizing protocol that would yield the lowest possible CS activity in the myofibrillar fraction and a high CS activity in the isolated mitochondrial fractions.

Citrate Synthase (CS) activity: The protocol for the CS activity assay was as follows; one milliliter of 0.1 M Tris buffer, pH 8.0, heated to 37°C, was added to a cuvette containing 10 µl of 10mM DTNB in 0.1M Tris buffer and 2 µl of 30 mM acetyl-CoA in water. To this, a volume of the muscle homogenate was added to obtain 12 µg protein and mixed. The spectrophotometer (Ultrospec 3000 pro UV/Vis) was zeroed, and 10 µl of 50 mM oxaloacetic acid in 0.1M Tris buffer were added to start the reaction. Absorbance were recorded at 412 nm every 30 s for 5 min. Rates of change in absorbance represent conversion of substrate and so are expressed relative to protein content.

Gel electrophoresis: Once we believed we had an effective protocol established, we tested for the presence of myofibrillar proteins in each fraction by using gel electrophoresis. In short, an equal amount of protein from each homogenate fraction was mixed in a 1:1 ratio with 2 x SDS-sample buffer (30% glycerol, 10% 2-mercaptoethanol, 2.3% SDS, 62.5mM Tris-HCl buffer pH 6.8, 0.01% Bromphenol blue) and boiled at 100°C for 2 min. It was then loaded onto a 8-cm x 7.3-cm x 1.5-mm SDS-polyacrylamide gel consisting of a 4% stacking layer and an 8% separating layer, both containing 30% glycerol. The gel was run overnight (17–18 h) at 10 mA in a cooled room (0–4°C) room. The gels were stained with Coomassie blue after electrophoresis visually inspected for the presence of MHC isoforms. A positive test would reveal the presence of MHC isoforms in the myofibrillar fraction but not in the mitochondrial fractions.

Electron microscopy: Finally a transmission electron microscope was used to visualize each fraction to confirm the presence of mitochondria in the IMF and SS fractions and contractile proteins in the myofibrillar fraction. Briefly, representative samples from each fraction were initially fixed in 2% glutaraldehyde in a 0.1M sodium cacodylate buffer and then post fixed in 1% osmium tetroxide in 0.1M sodium cacodylate buffer. Samples were dehydrated with ethanol and embedded in Spurr's resin. Thin sections (70 nm) were cut using an ultramicrotome (Ultracut E; Reichert, Vienna, Austria), placed on Cu/Pd grids (200 mesh size) and stained for 5 min in uranyl acetate followed by 2 min in lead acetate. Sections were viewed using a range of magnifications (5000–30,000x) with a transmission electron microscope (TEM; Japanese Electron Optics Laboratory 1200 Ex, JEOL, Tokyo, Japan). Micrographs from each of the fractions (myofibrillar, IMF and SS)

were examined for the presence of mitochondria, myofibrils and other cellular organelles. Representative images were photographed using a 1-s exposure time and digitized using a white-light illuminator (C-80 Epi-Illumination UV Darkroom, Diamed, Mississauga, ON, Canada).

## **Results and Discussion**

It was found that a slight modification of the buffer used by Bezaire et al (2) (100 mM KCl, 5 mM  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 5mM EDTA, 50 mM Tris HCl, 1.5% BSA, pH 7.4) using an all-glass Dounce homogenizer with the loose-pestle for initial homogenization was most effective for obtaining SS mitochondria (Figure 1 and 2B). Furthermore, using a glass pestle in a large round bottom eppendorf to apply a large amount of manual force was effective at liberating IMF mitochondria from the myofibrillar pellet (Figure 1 and 2C). From the EM micrographs (Figure 2A) and CS activity assays (Figure 1), it is apparent that not all mitochondria were liberated from the contractile proteins. Furthermore, examination of the IMF micrograph revealed that there could be a very small amount of contamination of contractile protein found in the IMF enriched portion (Figure 2D).

In order to further confirm this method was effective in obtaining a mitochondrially-enriched fraction, the protocol was repeated with murine muscle. A small amount of the homogenate obtained following the first Dounce homogenization was set aside before the protocol was completed. The CS activity of equal amounts of protein from both the SS fraction was 192% greater than the whole muscle homogenate (Figure 3). This is further evidence that the SS fraction is enriched in mitochondrial protein.

There appears to be a threshold for the mass of muscle that will yield sufficient mitochondria. Pieces of muscle weighing less than 100 mg yielded little to no measurable mitochondria. Furthermore, after all samples from the study for which this procedure was developed were processed (range 75-175 mg), an initial run of one participant's IMF and SS samples showed that there was not enough protein to measure the GCMS signal. Therefore, it was determined that in order to obtain sufficient protein to obtain data for this experiment, the IMF and SS fractions needed to be combined. Furthermore, after the protein fractions were hydrolyzed, cleaned up and derivatized, the entire derivative needed to be concentrated prior to manual injection into the GCMS to get a large enough signal to analyze. In confirmation of observations that small amounts of human muscle tissue yield very low amounts of mitochondrial protein, it has also been observed by others that a minimum of ~150mg of muscle is needed to obtain IMF and SS mitochondria (Graham Holloway, University of Guelph, personal communication). Therefore, based on our data we can make several recommendations. First, that a minimum biopsy weight for obtaining isolated muscle mitochondria be at least 150mg. In addition, we would recommend that any homogenizing buffer not contain bovine serum albumin (BSA), which interferes with the estimation of protein content for Western blot analysis. It is likely that not including BSA in the homogenizing buffer will be as effective as including it. Indeed, SS mitochondria were successfully obtained from murine skeletal muscle using a homogenizing buffer without BSA (Figure 3). However, more work will need to be done to verify that the homogenizing buffer without BSA is effective at preserving mitochondria during processing in human skeletal muscle. Further

confirmation that the sarcoplasmic fraction is viable and concentrated enough to perform Western blots will need to be made. However, if this is achieved the entire muscle biopsy will be able to be used with the following homogenizing procedure to obtain myofibrillar, collagen, sarcoplasmic (for Western blotting), IMF and SS mitochondria proteins (Figure 4).

**Table 1.** Homogenizing buffers

Buffer	KCl (mM)	Tris HCl (mM)	Tris Base (mM)	EDTA (mM)	EGTA (mM)	Hepes (mM)	MgSO <sub>4</sub> •7H <sub>2</sub> O (mM)	MgCl <sub>2</sub> (mM)	Sucrose (mM)	BSA (%)	pH
1	100	50	NI	5	NI	NI	5	NI	NI	1.5	7.4
2	135	NI	NI	NI	2	10	NI	NI	NI	0.25	7.4
3	NI	NI	NI	1	1	10	NI	NI	NI	0.25	7.4
4	10	NI	25	NI	NIN	NI	NI	1.5	250	NI	7.6
5	NI	NI	NI	NI	1	10	NI	NI	70	NI	7.4

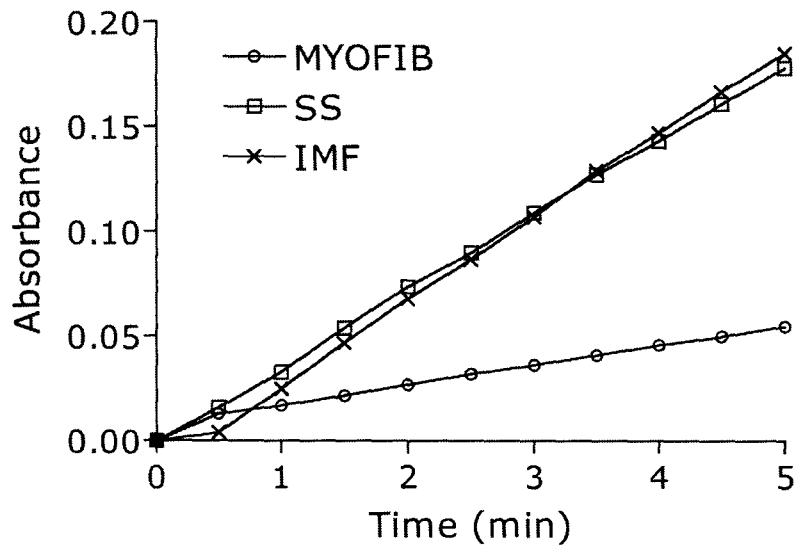
NI – Not included in buffer



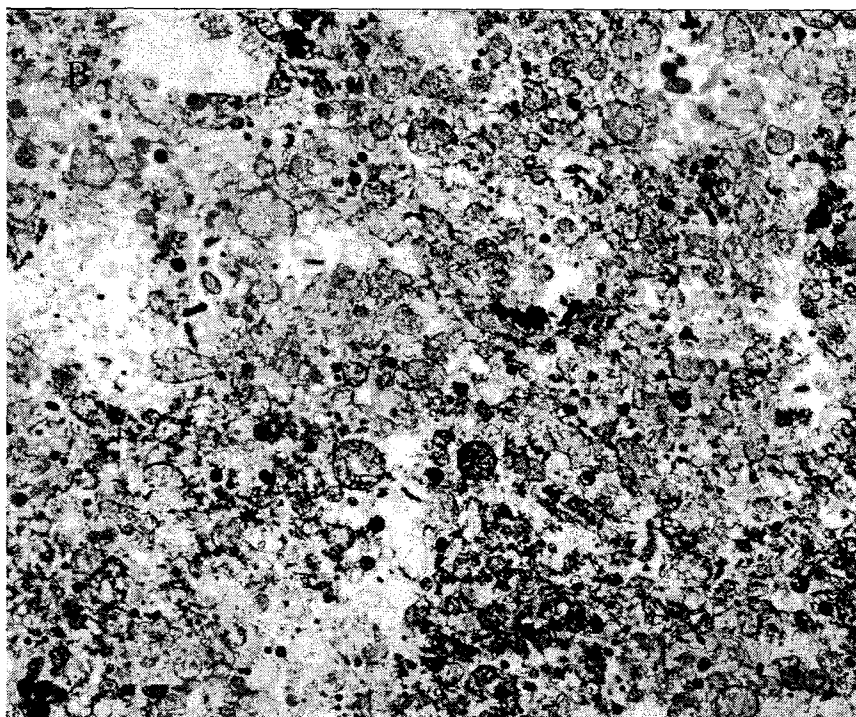
**Table 2.** Homogenizing apparatus examined with variables to manipulate

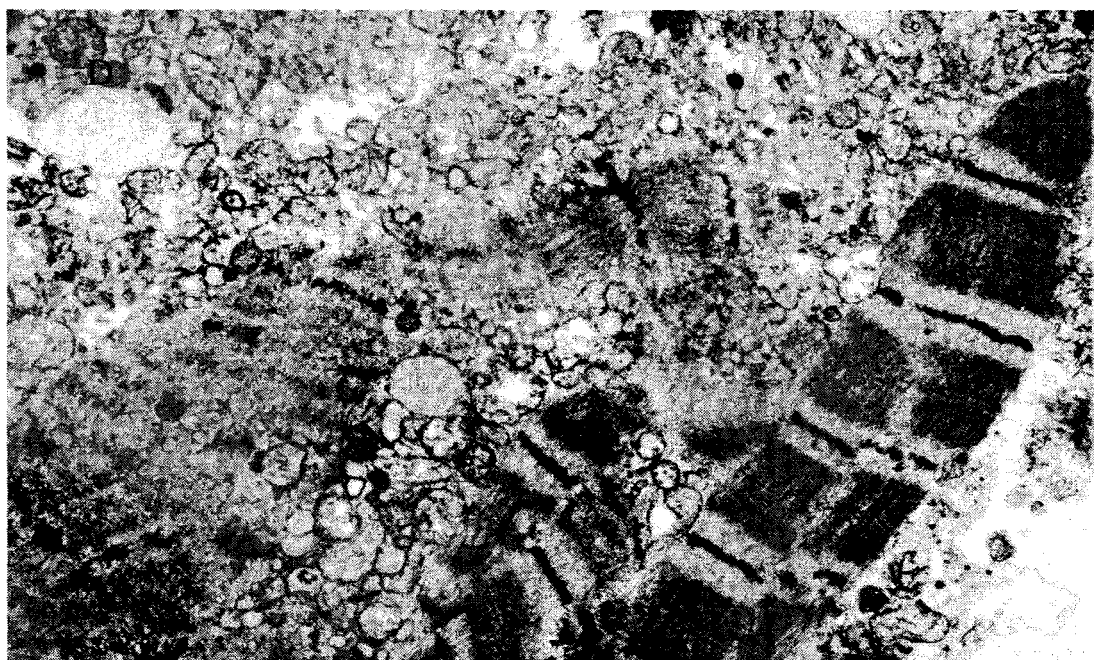
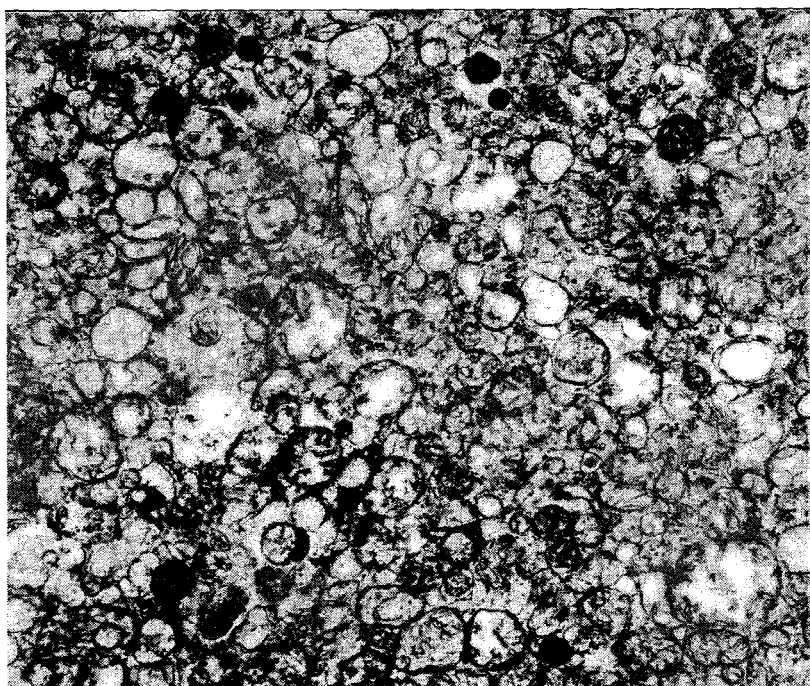
<b>Homogenizer</b>	<b>Manipulations</b>
Dounce glass homogenizer with loose-fitting pestle	<ul style="list-style-type: none"> <li>- Volume of homogenizing buffer</li> <li>- Number of pestle passes</li> <li>- Force applied to pestle</li> </ul>
Dounce glass homogenizer with tight-fitting pestle	<ul style="list-style-type: none"> <li>- Volume of homogenizing buffer</li> <li>- Number of pestle passes</li> <li>- Force applied to pestle</li> </ul>
Glass pestle in round bottom epindorf	<ul style="list-style-type: none"> <li>- Force applied to pestle</li> </ul>
Polytron (draws tissue into a long shaft containing rotating blades)	<ul style="list-style-type: none"> <li>- Speed of blades</li> <li>- Pulse versus continuous operation</li> <li>- Homogenization time</li> </ul>
Scissors	<ul style="list-style-type: none"> <li>- Volume of homogenizing buffer</li> <li>- Number of snips</li> </ul>

**Figure 1.** Change in absorbance of 12  $\mu\text{g}$  myofibrillar (MYOFIB), subsarcolemmal (SS) and intermyofibrillar (IMF) mitochondria protein in the citrate synthase activity. CS values are MYOFIB 10.3, SS 38.2 and IMF 43  $\text{mol}\cdot\text{kg}^{-1}\cdot\text{protein}\cdot\text{h}^{-1}$

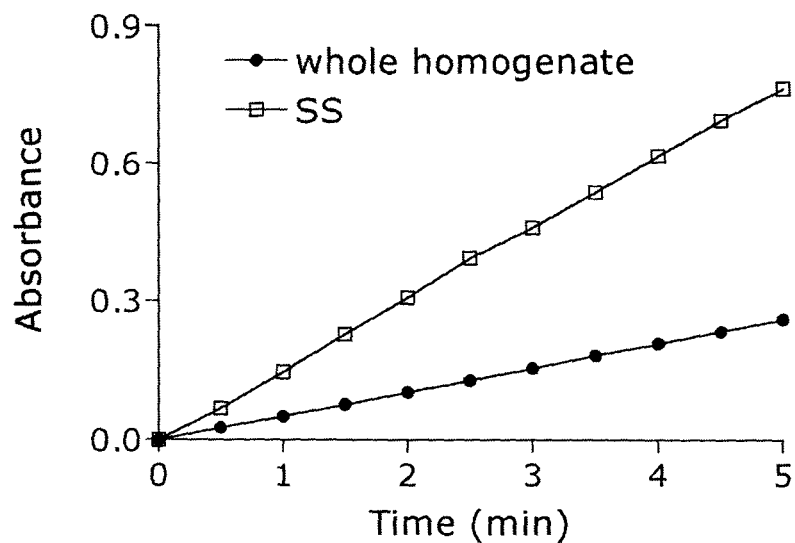


**Figure 2.** Transmission electron micrograph of fractions A: Representative image of myofibrillar fraction (magnification is x7500) B: Representative image of SS mitochondria fraction (magnification is x5000) C: Representative image of IMF mitochondria fraction (magnification is x15,000) D: Some myofibrillar protein contamination in the IMF mitochondria fraction (rarely observed; magnification is x6000)

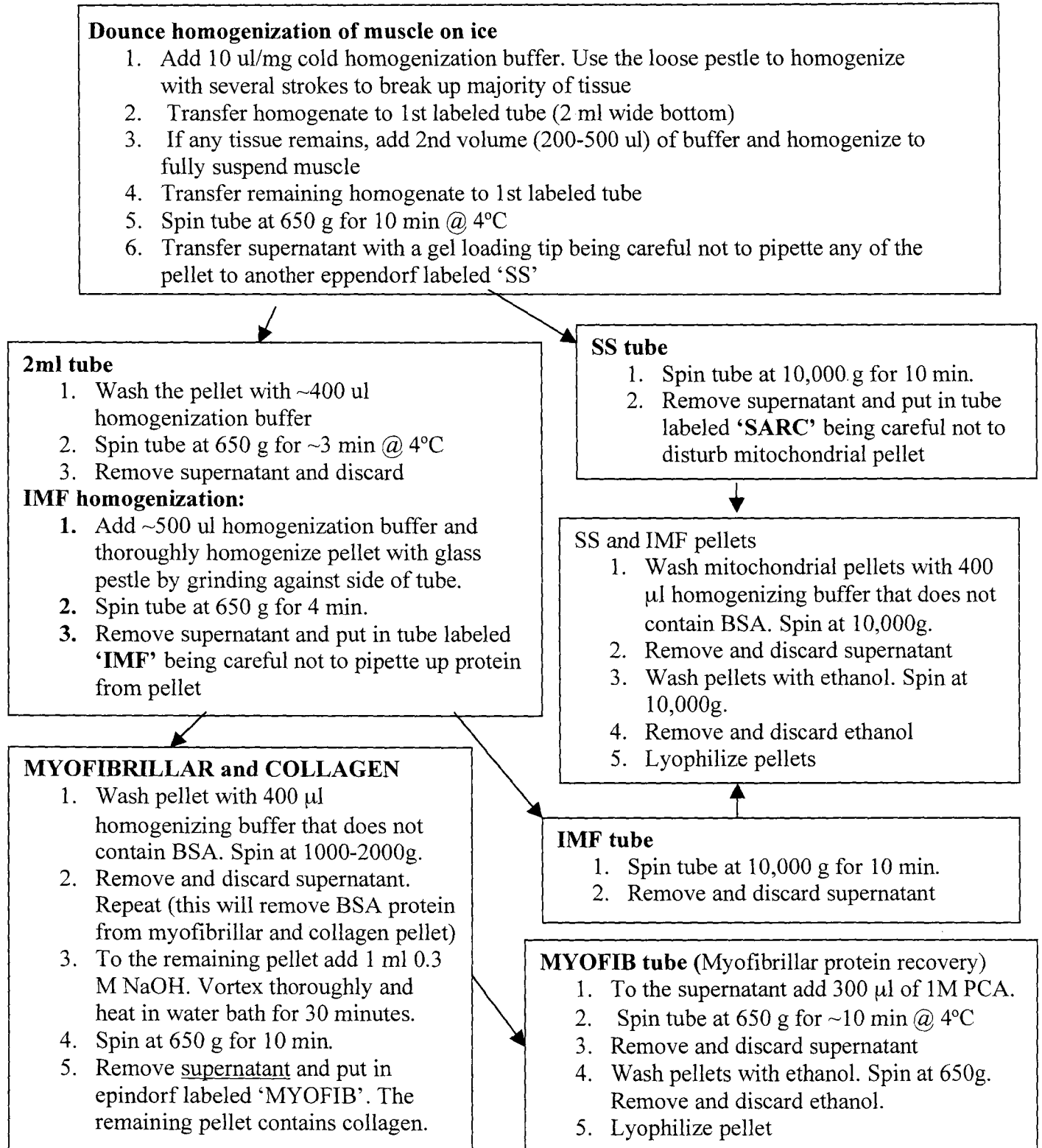




**Figure 3.** Change in absorbance of 7.4  $\mu\text{g}$  of mouse whole mixed homogenate and subsarcolemmal (SS) mitochondria protein in the citrate synthase activity. CS values are whole homogenate 156 and SS 455  $\text{mol} \cdot \text{kg}^{-1} \text{protein} \cdot \text{h}^{-1}$



**Figure 4.** Protocol for separating collagen, myofibrillar, sarcoplasmic, IMF and SS mitochondria fractions.



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