Mathias Wernbom

Effects of an Acute Bout of Low-Load Resistance Training with Blood Flow Restriction

–with Special Reference to Muscle Damage, Hypertrophic Signaling and Satellite Cells

DISSERTATION FROM THE NORWEGIAN SCHOOL OF SPORT SCIENCES • 2011

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PREFACE AND ACKNOWLEDGEMENTS

This thesis represents research conducted at the Department of Physical Performance at the Norwegian School of Sports Sciences (NSSS / NIH), and the Lundberg Laboratory for Orthopaedic Research at the University of Gothenburg.

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Oslo and Göteborg, June 2011, Mathias Wernbom.

LIST OF PAPERS

I. Wernbom M, Augustsson J, Thomeé R. Effects of vascular occlusion on muscular endurance in dynamic knee extension exercise at different submaximal loads. J Strength Cond Res 2006: 20: 372-377.
II. Wernbom M, Järrebring R, Andreasson MA, Augustsson J. Acute effects of blood flow restriction on muscle activity and endurance during fatiguing dynamic knee extensions at low load. J Strength Cond Res 2009; 23: 2389-95.
III. Contractile function and sarcolemmal permeability after acute low-load resistance exercise with blood flow restriction. Manuscript.
IV. Resistance exercise with blood flow restriction increases protein signaling and satellite cell numbers in human skeletal muscle. Manuscript.

Since this thesis was submitted for evaluation, Paper III has been published online in the European Journal of Applied Physiology [Epub ahead of print, 27 September 2011]. Apart from this information, no changes have been made to the thesis after it was submitted.

Scientific works involving the author, but outside this thesis:

1. Wernbom M, Augustsson J, Thomee R. The influence of frequency, intensity, volume and mode of strength training on whole muscle cross-sectional area in humans. Sports Med. 2007; 37(3): 225-64.
2. Wernbom M, Augustsson J, Raastad T. Ischemic strength training: a low-load alternative to heavy resistance exercise? Scand J Med Sci Sports. 2008; 18: 401-16.
3. Nielsen JL, Aagaard P, Bech RD, Nygaard T, Wernbom M, Suetta C, Frandsen U. Rapid increases in myogenic satellite cells expressing Pax-7 with blood flow restricted low intensity resistance training. Abstract, presented at the congress of the American College of Sports Medicine, Denver Colorado, May 30 - June 2, 2011.
4. Frandsen U, Nielsen JL, Jensen L, Nygaard T, Bech RD, Wernbom M, Suetta C, Aagaard P. Short-term Bfr resistance training increase skeletal muscle myofiber size without concomitant increase in capillary density. Abstract, presented at the congress of the American College of Sports Medicine, Denver Colorado, May 30 - June 2, 2011.

Abbreviations

AMP	Adenosine monophosphate
AMPK	5'adenosine monophosphate activated protein kinase
ATP	Adenosine triphosphate
Ca^{2+}	Calcium
CaM	Calmodulin
CK	Creatine kinase
COX	Cyclooxygenase
CREB	cAMP response element-binding
DGC	Dystrophin glycoprotein complex
DGK	Diacylglycerol kinase
ECM	Extracellular matrix
eEF2	Eukaryotic translation elongation factor 2
eIF2	Eukaryotic translation initiation factor 2
eIF2B	The guanine nucleotide exchange factor for the eukaryotic initiation factor 2, which converts the inactive eIF2-GDP to the active eIF2-GTP
eIF2Bε	The epsilon subunit of eIF2B
eIF3f	The F subunit of eukaryotic translation initiation factor 3
eIF4E	Eukaryotic initiation factor 4E
4E-BPs	Eukaryotic initiation factor 4E binding proteins
EMG	Electromyography
ERK1/2	Extracellular signal regulated kinases 1 and 2
FAK	Focal adhesion kinase
FGFs	Fibroblast growth factors
FKBP12	FK506 binding protein 12, a 12-kDa protein to which the immunosuppressant drugs FK506 (tacrolimus) and rapamycin bind with high affinity
FKBP38	FK506 binding protein 38
FOXOs	Forkhead box O transcription factors
GbetaL	G-protein beta-subunit-like
Gln	Glutamine
GSK3	Glycogen synthase kinase 3
HGF	Hepatocyte growth factor
HSP	Heat shock protein
IGF-1	Insulin like growth factor 1
IKK	Inhibitor of KappaB kinase
IR	Insulin receptor
IRS-1	Insulin receptor substrate 1
JNKs	c-jun N-terminal kinases
Leu	Leucine
LIF	Leukemia inhibitory factor
LOX	Lipoxygenase
LPAAT	Lysophosphatidic acid acyltransferase
LPC	Lysophosphatidylcholine
MAFbx	Muscle atrophy F-box, also known as Atrogin-1
MAPKs	Mitogen activated protein kinases
MAP4K3	Mitogen-activated protein kinase kinase kinase kinase 3
MEK	Mitogen-activated protein kinase kinase 1, also known as MAP2K1
MGF	Mechano growth factor, a splice variant of IGF-1
MHC	Myosin heavy chain

MK2	Mitogen activated protein kinase activated protein kinase 2, also known as MAPKAPK2
MMPs	Matrix metalloproteinases
MNKs	MAP kinase-interacting serine/threonine-protein kinases
mRNA	Messenger RNA (RNA = Ribonucleic acid)
mTOR	Mammalian target of rapamycin, also known as FRAP1 (FK506 binding protein 12-rapamycin associated protein 1) and RAFT (rapamycin and FKBP target)
MuRFs	Muscle ring finger proteins
MVC	Maximum voluntary contraction (usually measured isometrically)
NCAM	Neural cell adhesion molecule, also known as CD56 and Leu-19
NFkB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NO	Nitric oxide
NOS	Nitric oxide synthase
p38MAPKs	p38 mitogen-activated protein kinases
p70S6K	70-kDa ribosomal protein S6 kinase
p90RSK	90-kDa ribosomal protein S6 kinase
PA	Phosphatidic acid
PAX7	Paired box protein 7
PDK1	3-phosphoinositide-dependent protein kinase 1
PG	Proteoglycans
PGC-1alpha	Peroxisome proliferator-activated receptor gamma coactivator 1-alpha
PGE2	Prostaglandin E2
PGF2a	Prostaglandin F2alpha
PI3K	Phosphatidylinositol 3-kinase
PKA	Protein kinase A
PKB	Protein kinase B, also known as Akt
PKC	Protein kinase C
PLA2	Phospholipase A2
PLD	Phospholipase D
PRAS40	Proline-rich Akt substrate of 40 kilodaltons
Rags	Ras GTP-binding proteins
RalA	Ras-related protein
Rapa	Rapamycin, also known as sirolimus, an immunosuppressant drug which inhibits mTOR
Ras	Ras (abbreviation of ”Rat sarcoma”), is a G protein, or a guanosine-nucleotide-binding protein
Redd	Regulated in development and DNA damage responses
Rheb	Ras homologue enriched in brain
RM	Repetition maximum, the highest load which can be lifted for a designated number of repetitions (e.g., 1RM = the heaviest weight which can be lifted only once).
ROS	Reactive oxygen species
rps6	Ribosomal protein S6, a component of the 40S ribosomal subunit
SACs	Stretch activated ion channels
SGK1	Serum/glucocorticoid regulated kinase 1
TNF-alpha	Tumor necrosis factor alpha
TSC1/2	The tuberous sclerosis complex proteins 1 (hamartin) and 2 (tuberin)
VPS34	Vacuolar protein sorting 34

PART 1. Introduction to resistance training with blood-flow restriction

The method of using tourniquet cuffs to restrict the muscle blood flow during low-load resistance exercise originated from the Japanese physiotherapist Yoshiaki Sato, who began experimenting with training in combination with pressure cuffs already in the late 1960s. He dubbed this form of training "Kaatsu training" ("ka atsu" means added pressure), and first applied it on himself, and later on clients in his training center. Shinohara et al. (1998) were the first in the English language scientific literature to demonstrate that low-load resistance training with blood flow restriction (BFR) by the use of pressure cuffs caused increases in muscle strength. Subsequently, several studies demonstrated that low load training (20-50% of 1 RM) in combination with BFR induced gains in muscle mass comparable to those seen with conventional strength training (Takarada et al., 2000b, 2002, 2004; Kubo et al., 2006).

This relatively new mode of training has been demonstrated to produce gains in muscle strength, size and protein synthesis not only in younger untrained individuals (Takarada et al., 2000, 2004; Kubo et al., 2006; Fujita et al., 2007) but also in the elderly (Yokokawa et al., 2008; Karabulut et al., 2009; Fry et al., 2010) as well as well trained athletes (Takarada et al., 2002), and in patients recovering after anterior cruciate ligament surgery (Ohta et al., 2003). Similarly, low-load BFR resistance training can also counteract unloading-induced muscle atrophy (Cook et al., 2010).

Collectively, these findings suggest that fatiguing low-load strength training with BFR can induce long-lasting elevations in muscle protein synthesis and anabolic signaling, similarly to conventional heavy resistance exercise; indicating that ischemic strength training may be a useful method in sports training, rehabilitation and other contexts.

However, as relatively little is currently known about the physiology of this type of training, multiple references will be made in this text to established models of skeletal muscle overload, including heavy resistance exercise. Part 2 will therefore provide a general physiological overview of skeletal muscle hypertrophic adaptations with increased use, before the physiology of BFR resistance exercise is discussed in further detail in Part 3. It should be noted that various synonyms appear in the literature, e.g. BFR resistance exercise, strength training with vascular occlusion, occlusion training and ischemic strength training, and these will be used interchangeably in this text.

PART 2. Skeletal muscle hypertrophy with increased use

2.1 Physiological bases of skeletal muscle hypertrophy with increased muscle use

2.1.1 Motor unit and muscle fiber recruitment

The size principle (Henneman et al., 1965; Zajac & Faden, 1985) is viewed as the governing rule for motor unit recruitment. In this scheme, motor units are recruited in order of size and contraction strength, so that small slow twitch fatigue resistant (S) units are recruited first, followed by fast twitch, fatigue resistant (FR), fast twitch intermediate fatigability (FI) and finally fast twitch, highly fatigable (FF) (Zajac & Faden, 1985). S units are composed of slow type I fibers, FR and FI units of fast type IIa fibers and FF units of fast type IIx fibers (Jones et al., 2004). The fastest fibers in humans often contain not only type IIx myosin, but a mixture of type IIx and IIa myosins and are therefore sometimes designated as type IIax.

Studying glycogen depletion after various protocols in the knee extension exercise (5 x 10 repetitions at 30%, 45% and 60% of 1RM, respectively), Tesch et al. (1998) found that overall glycogen depletion was linearly correlated with the exercise load. Interestingly, the glycogen depletion patterns indicated that not only type I but also type IIa fibers were recruited already at 30% of 1RM. Nevertheless, only the 60% protocol resulted in glycogen depletion in type IIax fibers. Beltman et al. (2004a, b) investigated alterations in phosphocreatine-to-creatine (PCr/Cr) ratios in muscle fibers. With series of brief isometric muscle actions at 39, 72 and 87% of MVC, type IIax recruitment (as judged by changes in PCr/Cr ratios) was observed only at 87% of MVC. At 39% of MVC, both type I and type IIa fibers were recruited, but type IIa less so than type I.

Collectively, multiple lines of evidence strongly support that recruitment during both concentric and isometric muscle actions follows the size principle. Regarding eccentric muscle actions and ballistic movements, there is still an active debate whether recruitment follows the size principle or if exceptions can occur. Chalmers (2008) concluded that in the vast majority of the studies (9 out of 10) which used appropriate methods to investigate the issue of selective fast fiber recruitment, the order of the recruitment followed the size principle. The research reviewed included studies on both ballistic and eccentric exercise.

While preferential type II fiber recruitment remains a possibilty, the evidence for it occurring in exercising humans is currently not convincing. The evidence is more compelling

for selective recruitment of task groups or compartments or synergists with different types of movements, and several examples of this can be found in the literature.

The importance of neural adapations (recruitment, firing patterns, etc) for strength gains is recognised by the present author, but is not discussed here. The interested reader is referred to reviews by Aagaard & Thorstensson (2003), Sale (2003) and Duchateau et al. (2006).

2.1.2 Protein content in myofibrils in skeletal muscle

The two dominant components of skeletal muscle are water and proteins, and the ratio between the two is approximately 4:1 (Houston, 1999). Total muscle protein and myofibrillar protein constitute 20% and 12%, respectively, of the muscle wet weight (Yates & Greaser, 1983). Myofibrils take up ~80-87% of the fiber volume in human skeletal muscle (Eisenberg, 1983). In turn, the myofibrils in mammalian skeletal muscle are primarily made up by the contractile proteins myosin (~43%) and actin (~22%), with the elastic filament titin contributing a further 10% (Yates & Greaser, 1983). Thus these three proteins make up ~75% of myofibrillar protein content. Nebulin, troponin, and tropomyosin contribute another ~15% and the remaining ~10% come from various other proteins (e.g., alpha-actinin, beta-actinin, desmin). Each myosin filament is composed of myosin heavy chains (MHC) and myosin light chains (MLC), with MHC contributing ~85% to the molecular weight of myosin (Yates & Greaser, 1983). Thus, MHCs form a large part of both myofibrillar and total protein content.

MHC and myosin contentrations can vary depending on factors including the type of muscle, the age and the training status of the individual. For both humans and animals, it has been reported that MHC content is slightly higher in fast muscle than in slow muscle (Carroll et al., 2004) although others have failed to find such differences in humans (D'Antona et al., 2003, Borina et al., 2010). Furthermore, aging, unloading and in particular their combination lowers myosin concentration and also actin concentration (D'Antona et al., 2003, Borina et al., 2010, Riley et al., 1998). The decrease in myosin content with aging and unloading has a direct negative impact on the specific force (tension per CSA) of the affected muscle fibers (D'Antona et al., 2003, Borina et al., 2010), as myosin content has been linearly correlated with specific force in both human and animal studies (D'Antona et al., 2003). Conversely, being physically active may help minimise negative changes in myosin content and specific force in the muscle fibers in elderly individuals (D'Antona et al., 2007).

2.1.3 Muscle fiber hypertrophy and myofibrillar protein content

Myofiber hypertrophy occurs primarily due to the accumulation of myosin and actin in the fiber. In this process, newly synthesised myosin and actin filaments are added to the periphery of each myofibril, making each myofibril larger without any apparent changes in packing density or crossbridge spacing (MacDougall, 2003). However, based on the greater increase in fiber CSA versus average myofibril area, it appears that the growth in the CSA of the muscle fiber must involve both increases in the area of the myofibrils and the number of myofibrils present in the fiber (MacDougall, 2003).

It follows from the model proposed by MacDougall and colleagues that any resulting enhancement of the fiber force development induced by strength training would primarily be caused by the increased myofiber area. However, as already noted, specific force can vary considerably betweens different fiber types and between different individuals. Furthermore, Gilliver et al. (2009) found that specific force could vary up to threefold between fibers of the same fiber type. Regarding a possible role for myosin content in this variation, these authors argued that it seemed unlikely that there would be a threefold difference in myosin content between fibers of the same type.

Nevertheless, average values for myosin concentration can vary substantially (~2-fold) in both fiber types between young and elderly immobilised men (D'Antona et al., 2003). Moreover, the means and the standard deviations of the young subjects for myosin concentration suggest a large variability in myosin content for both fiber types even in young individuals (type I: 215 ± 84 μM, type IIa: 184 ± 82 μM). Interestingly, bodybuilders were reported to have both higher specific tension and greater fiber areas than age-matched controls (D'Antona et al., 2006). In any case, it is clear that a substantial variation in both specific tension and myosin content in muscle fibers can occur in vivo, even in healthy young individuals, thus suggesting the possibility that at least some of the variation in specific force may be due to variations in myosin concentration.

Consequently, it is tempting to speculate that strength training can increase myosin content before any gains in fiber area takes place. Similarly, Phillips (2000) proposed a sequence of events where elevated myofibrillar content occurs before fiber area increases. Indeed, studies have demonstrated early increases in myofibrillar content in both humans (Willoughby & Nelson, 2002; Willoughby & Taylor, 2004) and animals (Karagounis et al.,

2010) undergoing strength training. Myofibrillar content has also been correlated with strength in dynamic movements in humans (Cribb et al., 2007a, b).

Moreover, Balagopal et al. (1997) demonstrated significant correlations (r=0.51-0.76) between the rate of MHC synthesis and dynamic knee extension strength per kg muscle mass (measured by DEXA) in the leg. Since strength per muscle mass is arguably dependent on the specific force of fibers, these findings further support the connection between myosin concentration and specific force. In light of these findings, future studies should investigate the myosin concentration throughout the time course of a strength training period.

2.1.4 The balance between protein synthesis and degradation

Similarly to energy balance, there is a balance between protein synthesis and degradation, and any change in muscle mass is a result of an imbalance between these two processes. Thus, a greater accumulation of muscle proteins can in theory be caused by either an increased protein synthesis or a decreased degradation, or a combination of both. Repeated elevations in muscle protein synthesis (MPS) create periods of positive net protein balance that sum up to create hypertrophy (Rasmussen & Phillips, 2003).

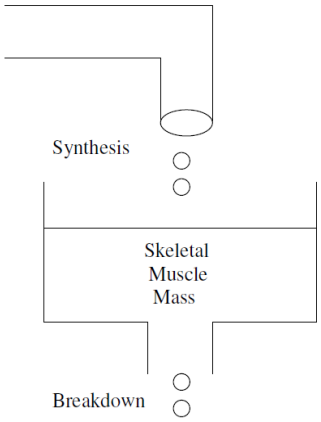


Figure 2.1. The net protein balance in skeletal muscle is determined by the rates of muscle protein synthesis and breakdown. In the long run, if MPS > MPB, hypertrophy results, whereas if MPB > MPS, atrophy occurs. Figure drawn after Houston (1999).

Both MPS and muscle protein breakdown (MPB) are stimulated by resistance exercise in the fasted state; alternatively, breakdown may be relatively unchanged especially in the trained individual (Phillips et al., 1999). In any case, MPS is relatively more elevated by the exercise than MPB (Phillips et al, 1997). Still, the net protein balance remains negative in the fasted state until proteins or amino acids are provided (Rasmussen & Phillips, 2003). In the fed state, resistance exercise typically leads to a positive net protein balance and prolonged elevations in MPS, sometimes up to 72 hours post-exercise, and feeding may also ameliorate exercise-induced elevations in MPB.

The focus on the role of MPS in protein turnover in humans has in part been due to MPS being easier to measure than MPB; but also because evidence suggests that in healthy humans, the relative changes in MPS are typically more important for net protein balance in response to feeding, exercise and disuse than changes in MPB (Rennie et al., 2010). In other words, increases and decreases in MPS are currently seen as the major underlying causes of hypertrophy and disuse atrophy, respectively, in humans.

This is not to say that MPB does not have a role in healthy muscle hypertrophy. It has been postulated that some activation of proteolytic pathways is necessary in order to facilitate remodelling (Taillandier et al., 2004). Indeed, enhanced mRNA and protein levels of the E3 ligases Atrogin-1 and MuRF1 in the ubiquitin-proteasome pathway were observed in human exercise-induced muscle hypertrophy (Léger et al., 2006). These authors suggested that the increased levels of Atrogin-1 and MuRF1 following hypertrophy in healthy muscle may be required to maintain a normal level of protein degradation and continued quality control of muscle proteins. In support, it has been demonstrated that while knockout of both MuRF1 and MuRF2 leads to striated muscle hypertrophy in mice, particularly in the heart (Witt et al., 2008), mice deficient for MuRF1 and MuRF3 develop a skeletal muscle myopathy and hypertrophic cardiomyopathy characterized by subsarcolemmal MHC accumulation, myofiber fragmentation, and diminished muscle performance (Fielitz et al., 2008).

Finally, MPB and MPS are closely correlated in the fasted state in humans 3 hours after resistance exercise (Phillips et al., 1999), which has led to the hypothesis that in the fasted state, increased protein degradation drives the increase in protein synthesis (MacKenzie et al., 2009). Specifically, increased MPB is postulated to increase the intracellular concentration of the amino acid leucine (MacKenzie et al., 2009), which provides a signal for increased MPS. The presence of leucine and other important amino acids are also necessary or permissive for the protein synthesis response to various other stimuli such as growth factors (Miyazaki &

Esser, 2009, Foster & Fingar, 2010). Thus, MPS and MPB are intertwined in the fasted state, at least in part via amino acids (particularly leucine).

Altered muscle use can influence MPS and MPB via multiple routes, where some are short term (e.g., increased translational efficiency, decreased targeting of proteins for degradation) while others are more long term (e.g., increased transcription, elevated number of myonuclei). The current view is that increased translational efficiency represents the most immediate step in upregulating protein synthesis in response to exercise (Hornberger & Esser, 2004; O´Neill et al., 2009). An overview of the different levels of regulation of the protein turnover in skeletal muscle is shown below in the picture drawn from Favier et al. (2008).

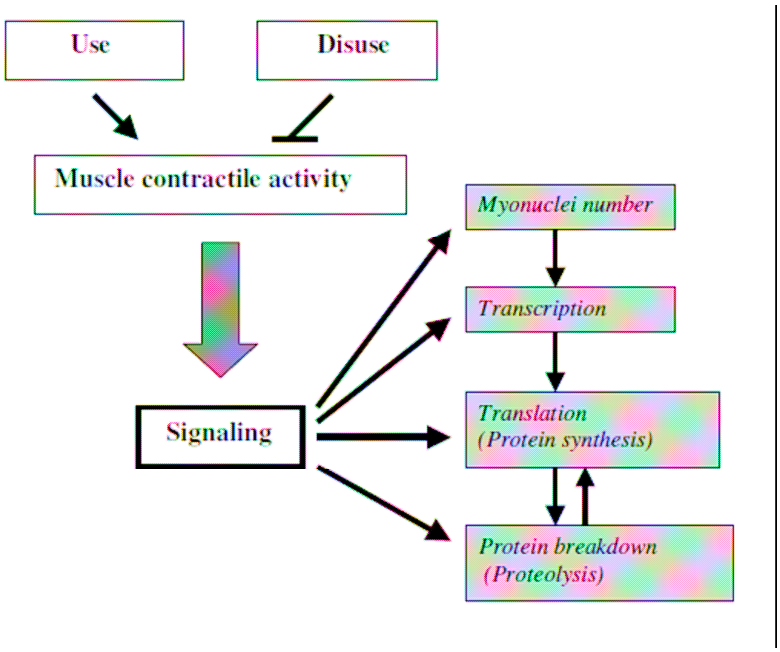


Figure 2.2. Modulation of skeletal muscle mass by altering the number of myonuclei, transcriptional and translational capacity, and the rate of protein degradation. Modified from Favier et al. (2008).

For discussion of the various steps in transcription, as well as in protein synthesis/translation and breakdown, the reader is referred to textbooks in cell biology. With regard to protein turnover, several excellent reviews can be found in the literature, for example by Proud (2007), Glass (2005, 2010), Murton & Greenhaff (2008) and Kimball & Jefferson (2010).

2.1.5 Hypertrophic adaptations - Time course and magnitude of changes

The mean muscle fiber areas (MFA) in powerlifters who had never used anabolic drugs were reported to exceed those of controls by ~50% (Eriksson, 2006). Somewhat depressingly, drug-using powerlifters had in turn muscle fibers which were ~50% largers than those of the drug-free lifters. These data indicate that muscle fibers in humans can grow with at least 50% in CSA with long-term hard systematic strength training, and at least 125% with a combination of training and anabolic drugs.

Interestingly, increases of ~40-50% in MFA and ~45-66% in type II fiber areas have been reported in relatively short-term (8-30 weeks) controlled resistance training studies (e.g., Staron et al., 1990, 1991; Pyka et al., 1994; LaStayo et al., 2000; Hulmi et al., 2009a), suggesting that considerable fiber growth may be attained in just 2-6 months, as opposed to many years of hard training. These figures are also among the highest reported in controlled training studies. However, MFA is associated with large errors and it cannot be ruled out that the extreme hypertrophy displayed by bodybuilders and powerlifters is in part due to hyperplasia. Furthermore, a single biopsy is not necessarily representative of a muscle belly, let alone a whole muscle group.

Regarding whole muscle area increases, bodybuilders and strength athletes have typically larger limb circumferences than control subjects. Interestingly, these differences were reported to be larger for the upper arm (~30%) compared to the thigh (~15-20%) and in particular the lower leg (~5%) (Sale & MacDougall, 1984; Tesch, 1993). A caveat with these data is that they were collected during 1980s, when bodybuilders for various reasons generally did not have the degree of thigh development displayed by bodybuilders today. In the study by D'Antona et al. (2006), the bodybuilders had 54% greater vastus lateralis CSA and 38% greater quadriceps CSA than control subjects. However, it should be noted that the subjects who had the greatest CSA were also users of anabolic drugs.

Data from the literature on longitudinal strength training studies supports that both the relative degree and rate of hypertrophy in the muscle groups of the upper arm are typically greater than in the thigh. In a comprehensive review, Wernbom et al. (2007) reported that the rate of gain in elbow flexor CSA with strength training was ~0.20% per calendar day (i.e., 1.4% per week), while the rate for the quadriceps was ~0.11% per day. In the study reporting the highest total increases, elbow flexor CSA was increased with 33% after 11 weeks of elbow flexor training (Sale et al., 1985), and quadriceps CSA with 34% after 20 weeks of training (Sale et al., 1990b). Finally, in the few studies directly investigating this issue, elbow

flexor CSA or thickness gains were greater than those of the quadriceps. Therefore, it seems plausible to suggest that the elbow flexors have a greater relative hypertrophic potential than the quadriceps in previously untrained subjects, presumably at least in part due to being used less in daily activities (and hence being in a less trained state).

The upper limit for exercise-induced hypertrophy in humans is not known and the individual variation in response is very large, for example ranging from –5% to +55% in elbow flexor CSA (average gain: +20%) with 12 weeks of training (Hubal et al., 2005). Similar findings were reported by Bamman et al. (2007) regarding variations in MFA gains in vastus lateralis with 16 weeks of training. Thus, there is likely to be large genetic components in the muscle mass response. However, it is interesting to note that the variation in the response to strength training is considerable also at the group level (i.e., between different studies). For example, the rate of CSA gain in the quadriceps in the literature was calculated to vary from 0.03% to 0.56% per day, and even after removing the upper value, the variation was still almost 10-fold, from 0.03% to 0.26% per day (reviewed by Wernbom et al., 2007). This strongly suggests that factors on the group level (principally the design of the training program, but also age, training status, nutrition, etc) played significant roles.

2.1.6 Hypertrophic adaptations – fiber hypertrophy vs hyperplasia

There are reports that fiber areas cannot fully explain the differences in muscle mass in the vastus lateralis and triceps brachii between bodybuilders and non-resistance trained controls (MacDougall et al., 1982, Larsson & Tesch, 1986, D'Antona et al., 2006) and between the deltoids of swimmers vs controls (Nygaard & Nielsen, 1978, cited in Larsson & Tesch 1986). In the study of D'Antona et al. (2006), the MFA of bodybuilders was only 14% greater than the controls, while the CSA of the vastus lateralis was 54% greater. Other authors have contradicted these findings and concluded that the greater muscle area in the biceps brachii in bodybuilders can be explained by increased fiber areas (MacDougall et al., 1984).

Nevertheless, in a study in which the muscle fibers were directly counted in dissected muscles from deceased humans, the tibialis anterior of the left leg had 10% more fibers than the right leg (Sjöström et al., 1991). These authors concluded that "the results imply that long-term asymmetrical low-level daily demands on muscles of the left and the right lower leg in right-handed individuals provide enough stimuli to induce an enlargement of the muscles on the left side, and that this enlargement is due to an increase in the number of muscle fibres (fibre hyperplasia). Calculations based on the data also explain why the underlying process of

hyperplasia is difficult, or even impossible, to detect in standard muscle biopsies". Sjöström et al. (1991) further noted: "The actual formation of a new fibre, either through a splitting or a regenerative process, probably occurs rapidly, in less than a week. In addition, if formation of new fibres occurs, it can probably take place anywhere in the muscle".

The comments of Sjöström et al. (1991) are also underscored by the rather high coefficient of variation of measurements typically seen with biopsy sampling, which is on the order of ~17% (Narici et al., 1996). For example, Narici et al. (1996) found no significant increase in MFA after 6 months of strength training, whereas gain in the vastus lateralis CSA at the same level was significant. This may in part have been due to the higher sensitivity of MRI to detect changes in muscle CSA. Evidence supporting exercise-induced new fiber formation in humans was presented by Appell et al. (1988), who found newly formed myotubes in the exercised leg but not the control leg after 6 weeks of one-leg cycling.

Taken together, it appears that hyperplasia may occur in humans and that the current methods of obtaining fiber areas and numbers (typically based on muscle tissue from a single biopsy at each time point), are not sensitive enough to detect an increase in fiber number. Nevertheless, even in highly trained bodybuilders, whole muscle CSA correlates better with MFA ($r = 0.75$) than with calculated fiber number ($r = 0.55$) (Alway et al., 1989), which supports the view that while hyperplasia may occur, myofiber growth is still the dominant mechanism for exercise-induced muscle hypertrophy in humans (Fleck & Kraemer, 1997).

2.1.7 Underlying hypertrophic processes – protein synthesis and myonuclear addition

A central question is if the muscle fibers can accomplish the necessary elevations in MPS for muscle mass gains with the already existing protein synthesis machinery, or whether the addition of new myonuclei from satellite cells is obligatory for significant hypertrophy. Animal studies are contradictory on this point, with some studies demonstrating that activation of satellite cells and subsequent donation of new myonuclei are necessary for fiber hypertrophy in response to anabolic stimuli, whereas others clearly demonstrate that stimulation of MPS alone can suffice, without the need for new myonuclei (O'Connor & Pavlath, 2007; McCarthy & Esser, 2007).

However, some of the studies demonstrating no role or need for satellite cell activation and myonuclear addition have relied upon manipulation of key hypertrophy-inducing molecules such as Akt (Rommel et al., 2001; Lai et al., 2004) or anabolic agents such as clenbuterol (Kline et al., 2007). As proposed by O'Connor & Pavlath (2007), the relative

importance of satellite cell activity vs. increased protein synthesis from the existing machinery may vary depending on the stimulus used to induce muscle growth. It could thus be argued that pharmacological and genetic manipulations are not representative for functional overload. Indeed, the IGF-1-PI3K-Akt axis appears to be of secondary importance in mechanically stimulated muscle growth (see later sections on the Akt and mTOR pathways).

Another issue is that the overload models employed usually differ greatly between animal experiments and human voluntary strength training. Furthermore, analogous with what seems to be the case with atrophy in rodents vs humans, the time course and the relative contributions from different mechanisms may not be the same between the species. Therefore, it seems appropriate to focus mainly on data from human studies, despite the mechanistic limitations, when discussing mechanisms of hypertropy with strength training in humans.

The first study in humans on protein synthesis and heavy resistance training was carried out by Chesley et al. (1992). Other studies soon followed and established that MPS could increase for at least 24 hours but was almost back to baseline at 36 hours (MacDougall et al., 1995). These figures have since been modified in both directions. In the untrained state and/or after a bout of strenuous exercise, MPS can be increased up to at least 48-72 hours (Phillips et al., 1997, Miller et al., 2005), whereas in the trained state, MPS may be back to baseline at 28 hours post-exercise, and possibly even sooner (Tang et al., 2008).

Given the now quite substantial number of studies investigating the issue, there is no doubt that MPS can increase in humans after a strength training session. Under healthy conditions, such elevations can be summed together if occurring over a reasonable time frame and consequently result in muscle hypertrophy (Rasmussen & Phillips, 2003). Again, a central question is whether the muscle fibers can accomplish muscle mass gains with the already existing machinery of myonuclei, via e.g. increased translation per mRNA and/or more mRNA produced by each myonuclei. It seems that the short answer is “yes, up to a point”.

Studies by Kadi, Eriksson & colleagues (Kadi et al., 1999a, b; Eriksson, 2006) have provided strong evidence for the necessity of myonuclear addition in long-term hypertrophy. Specifically, it was reported that in a population consisting of untrained controls, drug-free powerlifters and powerlifters using anabolic steroids, a very strong relationship between the CSA of fibers and the number of myonuclei ($r = 0.86$; $P < 0.0001$) was obtained with a range of areas between 2,500 and 14,000 μm^2. Similarly, Hikida et al (1998) reported a significant correlation between MFA and number of myonuclei per fiber ($r = 0.60$; $P < 0.0001$) in a group consisting of young and elderly men.

The group of Bamman has provided further strong support to the case for myonuclear addition (Petrella et al., 2006; Bamman et al., 2007; Petrella et al., 2008). In these papers, high-responders, moderate-responders and non-responders to a 16-week period of heavy resistance exercise were identified by K-means cluster analysis based on changes (or lack of thereof) in myofiber areas. Those who responded best to the strength training (i.e., the high-responders) in terms of hypertrophy (+57% in MFA) and strength gains were also those who succeeded best in upregulating the number of myonuclei (+26%) in their muscle fibers. The moderate responders managed to increase MFA with ~28% and the number of myonuclei with 9%, whereas the non-responders failed entirely to increase any of these parameters. The groups also differed in the satellite cell number per myofiber, in that the high-responders both had a greater number at baseline and a greater increase in satellite cells with training than the other two groups. Again the non-responder group appeared to fare the worst, with the lowest satellite cell number at baseline and no significant upregulation with training.

In contrast to the results of Bamman & coworkers, Andersen & colleagues reported that 14 weeks of heavy strength training induced hypertrophy of muscle fibers (Aagaard et al., 2001; Kadi et al., 2004) as well as at the whole muscle level (Aagaard et al., 2001; Andersen et al., 2005), but that the fiber hypertrophy was not accompanied by an elevated number of myonuclei (Kadi et al., 2004). The gains observed were 16% in MFA, 18% in type II fiber area, 9% in type I area and 10% in whole muscle CSA (Aagaard et al., 2001). Similarly, Hikida et al. (1998) reported no change in myonuclei per fiber, whereas fiber hypertrophy was observed in young men after 8 weeks of training (+26% in MFA) and after 16 weeks of training in older men (+37% in MFA). However, trends for enhanced myonuclear number were observed in both groups (+9% in the young men and 7% in the older men), which would likely have been significant if the groups of subjects had been larger.

Kadi et al. (2004) observed that myonuclear addition had occurred in studies where the fiber hypertrophy had exceeded 26%, but not in the range of 6.8-15.5%. They suggested that "until a certain limit of hypertrophy is reached, an increase in the area of muscle fibres can occur without the addition of new myonuclei. Existing myonuclei are able to increase their protein synthesis and support a moderate enhancement of the cytoplasmic area". Bamman & coworkers (Petrella et al., 2006) agreed with Kadi et al. (2004) and speculated on a theoretical threshold of 26-27% gain in fiber area as the upper limit for hypertrophy without new myonuclei. They also suggested an upper limit for the myonuclear domain (the area of the myofiber supported by each myonuclei) of ~2000 μm^2, beyond which myonuclear addition would be necessary for further hypertrophy. However, after inclusion of more subjects, they

observed that the myonuclear domain size could be increased to ~2250 μm^2 in extreme responders, whereas in moderate responders, the domain was 2000 μm^2 (Petrella et al., 2008).

Taken together, it appears that moderate hypertrophy can occur without myonuclear addition in humans undergoing strength training. However, it is not clear whether satellite cell activation might still be necessary for repairing damaged muscle fibers resulting from heavy loads. It is not difficult to envision a scenario in which a certain percentage of fibers may have been damaged by strenuous and quite frequent heavy resistance exercise, as employed by both Bamman et al. (9 sets in total) and Andersen, Kadi et al. (14 sets in total). Any myonuclei lost because of damage would obviously have to be replaced by new ones, which would necessitate satellite cell activation. In this context, it is interesting that increased satellite cell numbers per fiber were observed in both these series of investigations, and in Kadi et al. (2004) the increase was apparent throughout the whole training period of 90 days.

Interestingly, recent data from animal studies on mechanical overload suggest that not only may addition of new myonuclei accompany fiber hypertrophy, but the elevated numbers of myonuclei actually precede the fiber area gains (Bruusgaard et al., 2010). Moreover, preliminary data suggests that myonuclear addition can occur within 1-2 weeks in humans with very frequent BFR resistance exercise (Nielsen, Frandsen, et al., ACSM 2011 abstracts, see also Part 3 in this thesis). In any case, it is evident that more studies are needed on this topic, especially as the existing literature concerns mainly conventional heavy resistance training with coupled concentric-eccentric muscle actions.

2.2 Signaling pathways activated by increased muscle use (overload)

2.2.1 Overview of signalling pathways in muscle protein synthesis and degradation

Numerous pathways (and the kinases, phosphatases, lipases, etc, that belong to them) have been implicated in the control of protein turnover in cells, including skeletal muscle fibers. These include, but are not limited to, the family of mitogen activated protein kinases (MAPKs), the isoforms of Akt (also known as protein kinase B, PKB), and the mammalian target of rapamycin (mTOR). To date, at least six distinct groups of MAPKs have been identified: the extracellular regulated kinases (ERK) ERK1/2, the isoforms of p38MAPK (alpha, beta, delta and gamma), the jun NH2 terminal kinases (JNK) JNK1/2/3, ERK3/4, ERK5 and ERK7/8 (Krishna & Narang, 2008). In addition, for several of these, spliced variants exist.

Of the MAPKs, the ERK1/2, the p38 kinases and the JNKs are the most studied (Krishna & Harang, 2008), and this also appears to be the case in striated muscle. However, the regulation of protein turnover is yet far from completely understood. An overview of the control of protein synthesis by some signal transduction pathways is shown in below.

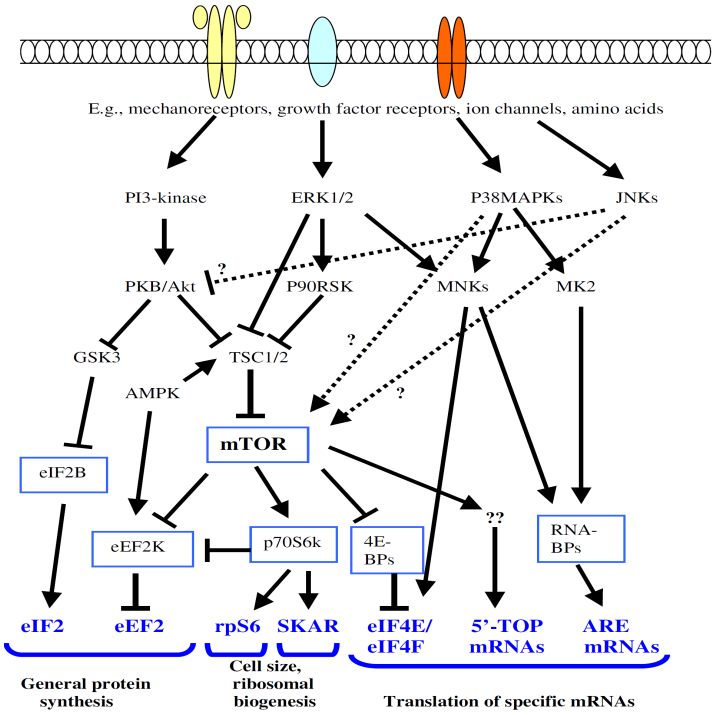


Figure 2.3. Some of the pathways regulating translation, scheme based on Proud (2007) and modified to include some recent findings. Pointed arrows = activation, blunt arrows = inhibition. Dotted lines = possible connection, question marks = uncertain significance and/or not fully known mediators.

2.2.2 The central role of mTOR in skeletal muscle hypertrophy

A central protein kinase in the regulation of muscle growth is mammalian target of rapamycin (mTOR). It exists in complexes with other proteins, and to date two mTOR complexes have been discovered, mTOR complex 1 (mTORC1) and 2 (mTORC2). The mTORC1 appears to be the most important for protein synthesis regulation via downstream effectors such as p70S6K (also called S6K), 4E-BP´s and eEF2 (Baar et al., 2006; Proud, 2007). Therefore, unless otherwise indicated, mTOR refers to mTORC1 in the present paper.

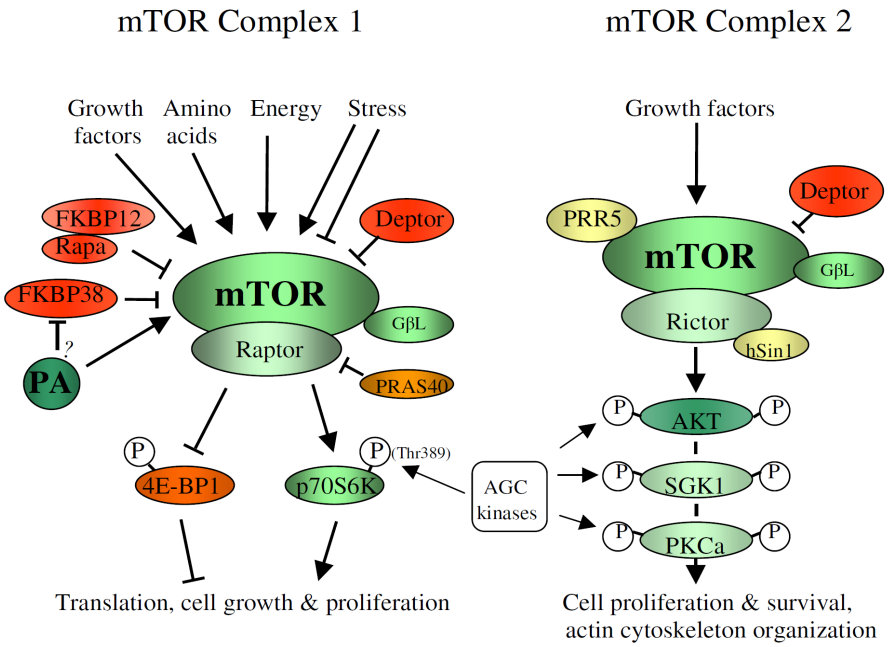


Figure 2.4. General overview of the two mTOR complexes, mTORC1 and mTORC2, and their primary roles in cells. Figure adapted from Foster & Fingar, 2010, and modified based on Foster (2009), Cully et al (2010) and Wang & Proud (2010), among others. Inhibitory proteins in red and orange.

Numerous studies to date have indicated that activation of mTOR and its downstream effectors is necessary for the induction of protein synthesis and hypertrophy in skeletal muscle (Baar et al, 2006; Hornberger et al., 2006; Drummond et al., 2009; Goodman et al., 2010). Furthermore, activation of mTOR via overexpression of the upstream positive regulator Rheb was shown to be sufficient for skeletal muscle hypertrophy (Goodman et al., 2010). Thus, there is convincing evidence for mTOR as a key player in hypertrophy.

Because mTOR is an integrator of signals from growth factors, nutrients, mechanical stimuli, cellular stresses (e.g., hypoxia) and energy status (Sandri, 2008; Foster & Fingar, 2010), there are many inputs to mTOR from several signaling cascades. In general, nutrient deprivation (in particular, essential amino acids such as leucine), hypoxia, catabolic cytokines, and excessive cellular stresses have a negative impact on mTOR signaling, and thus cell growth can be shut down by these negative inputs. Positive factors include nutrients and energy, growth factors, insulin, and mild to moderate stresses (e.g., mechanical forces).

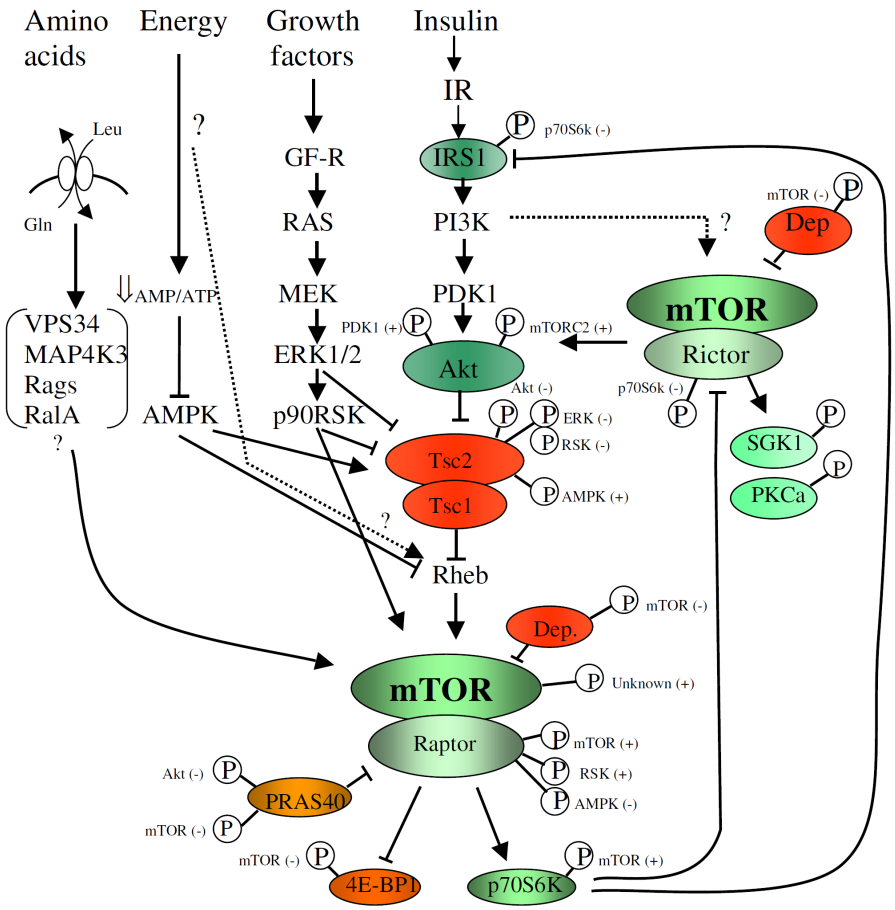


Figure 2.5. More detailed overview of some of the inputs to mTORC1 and mTORC2, with emphasis on mTORC1 and downstream effectors S6K1 and 4E-BP1. Figure adapted from Foster & Fingar (2010) and modified based on Cully et al (2010) and Wang & Proud (2010), among others. Pointed arrows = activating actions, blunt arrows = inhibitory effects. Phosphorylations (P) can either activate (+) or inhibit (-) the protein in question.

A number of investigations have now shown that the mTOR pathway, and especially the downstream effector p70S6K1, can be activated in resistance exercise in humans (e.g., Eliasson et al., 2006; Tannerstedt et al., 2009; Terzis et al., 2007, 2010; Hulmi et al., 2010). In some studies, correlations between phosphorylation of p70S6K at the site Thr389 and increases in muscle mass (Terzis et al., 2007) and MPS (Kumar et al., 2009, Burd et al., 2010a) were reported, thus supporting data from animal studies (Baar & Esser, 1999).

Similarly, Burd et al. (2010b) reported that phosphorylation of 4E-BP1 (and thereby relieving its inhibitory effect on the initiation factor eIF4E) was correlated with increased MPS. The abundance of the initiation factor subunit eIF2Bepsilon, a positive regulator of global protein synthesis, was recently also shown to be dependent on mTOR, and the levels of eIF2Be increased with resistance training (Mayhew et al., 2011). Conversely, it was demonstrated that rapamycin blocked the early heavy resistance exercise-induced elevations in mTOR signaling and protein synthesis (Drummond et al., 2009). Taken together, there is compelling evidence for the importance of mTOR in hypertrophy resulting from overload.

What is not clear is how mTOR and the downstream effectors are activated with mechanical loading. Early studies suggested that this was via the Akt pathway, but as discussed below this is probably not the major route. A more promising candidate is phosphatidic acid (PA). PA-mediated mTOR activation has now been demonstrated in several studies. It was suggested by Hornberger & colleagues that mechanical stimulation of mTOR signaling in skeletal muscle is caused by activation of phospholipase D (PLD), which then produces PA which in turn activated mTOR-p70S6K. This idea is very attractive, because the two isoforms of PLD, PLD1 and PLD2, are found at strategic places for force transmission in the muscle fiber, near the Z-disks and the sarcolemma (Hornberger et al., 2006).

However, it was also pointed out by these authors that other routes for PA production probably were active, because the intracellular concentration of PA continued to increase even after PLD activity had reverted back to baseline. Indeed, preliminary results indicate that PLD is not primarily responsible for mechanically induced elevations in PA (Dr Troy Hornberger, personal communication, see also Hornberger 2011). These findings, if confirmed, will shift the focus to other candidates for PA production such as isoforms of lysophosphatidic acid acyl transferase (LPAAT) and diacylglycerol kinase (DGK). The isoforms LPAAT alpha, theta and zeta are expressed in human skeletal muscle (West et al., 1997; Li et al., 2003; Tang et al., 2006) and overexpression of LPAAT theta leads to activation of mTOR-p70S6K (Tang et al., 2006). Similarly, DGK zeta activates p70S6K via mTOR (Avila-Flores et al., 2005). The possible relevance of these enzymes, and indeed also of PLD1 & PLD2, for mTOR signaling in response to strength training in humans is currently unkown.

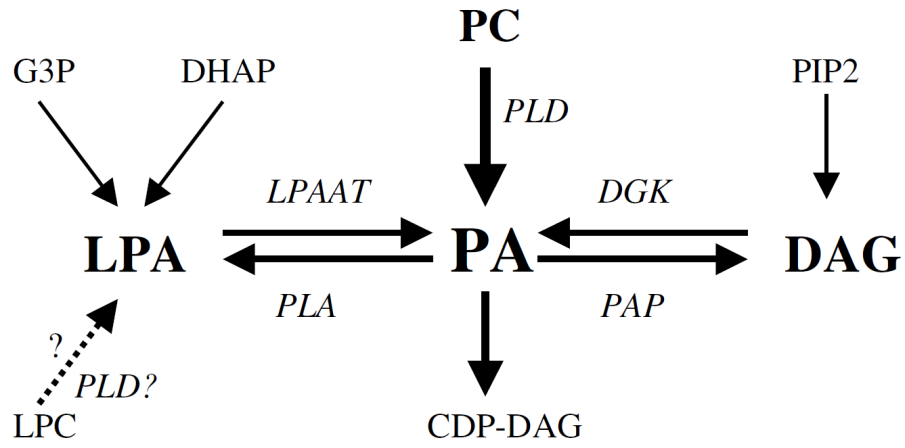


Figure 2.6. Possible routes for production of phosphatidic acid (PA). G3P = glycerol-3-phosphate, DHAP = dihydroxyacetone phosphate, LPC = lysophosphatidylcholine, PLD = phospholipase D, LPA = lysophosphatidic acid, PLA = phospholipase A, PC = phosphatidylcholine, DAG = diacylglycerol, CDP-DAG = cytidine diphosphate DAG, DGK = diacylglycerol kinase, PAP = PA phosphohydrolase, PIP2 = phosphatidyinositol-4,5-biphosphate. Redrawn and modified from Hornberger et al. (2006b).

Another candidate for increased mTOR signaling is VPS34 (vacuol protein sorting 34). VPS34 was shown to be activated by eccentric muscle actions in rats, and this activation correlated with activity of p70S6K at 3 hours post-exercise (MacKenzie et al., 2009). It was also noted that the intracellular concentration of leucine increased after resistance exercise, and that exogenous leucine stimulated VPS34 activity and p70S6K. Based on these findings, it was suggested that increased muscle protein breakdown from resistance exercise caused the observed increased intracellular concentration of the amino acid leucine, which in turn triggered activation of VPS34 and mTOR.

These authors also proposed a model in which resistance exercise initially triggered mTOR activation through PA production, which was then followed with VPS34 activation, and that some growth factor could have been responsible for mTOR activation at late time points (between ~6-18 hours post). Interestingly though, it was recently shown that amino acid and glucose-induced mTOR signaling are dependent on PLD and that VPS34 is upstream of PLD (Xu et al., 2011). This finding suggests that PLD may be activated later rather than sooner with eccentric exercise, or that PLD activation is biphasic, but in either case at least in part mediated via VPS34. A few possible routes to mTOR-p70S6K and protein synthesis, as well as some of the mediators of protein breakdown, are shown in the figure below.

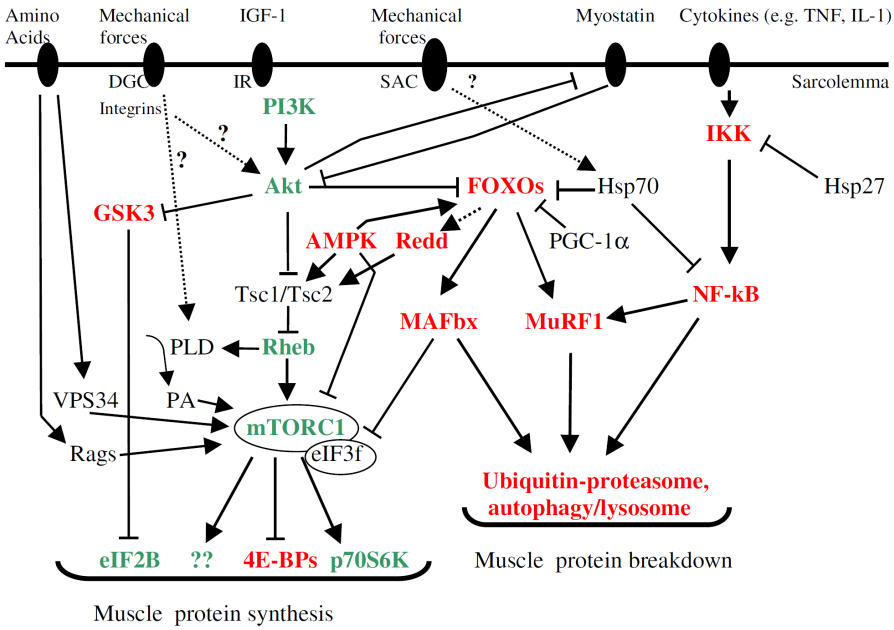


Figure 2.7. Some mediators of hypertrophic and atrophic signaling. Note that Akt, HSP27, HSP70 and PGC-1alpha can have positive effects on mTOR-p70S6K via inhibition of transcription factors and ligases involved in proteolysis, e.g., NF-KB, FOXOs, MAFbx and MURFs. Note also that cytokines activate proteolytic pathways and molecules (in red), which is not always the case (see text). Adapted from McCarthy & Esser, (2010), Glass (2005, 2010), Sandri et al. (2006) and Sandri (2008).

2.2.3 Akt/PKB in skeletal muscle hypertrophy and in prevention of atrophy

In the late 1990s and early 2000s, several papers were published which implicated a crucial role for mTOR and its downstream effectors in skeletal muscle hypertrophy in response to various stimuli, and Akt as the upstream kinase primarily responsible for the mTOR activation. Bodine et al. (2001) investigated several models of hypertrophy in rodents, including synergist ablation, reloading after hindlimb suspension, and constitutively activated Akt (cAkt). In this study, it was demonstrated that rapamycin almost completely blocked the whole muscle and fiber hypertrophy induced by 7 and 14 days of functional overload or reloading, as well as the hypertrophy resulting from cAkt. The loading-induced hypertrophy (~25 and ~44% after 7 and 14 days) was accompanied by marked increases in both total and phosphorylated Akt, as well as phosphorylated p70S6K and 4E-BP1. Furthermore, the

binding of the eukaryotic initiation factor (eIF) eIF4E to eIF4G increased (thus forming the initiation factor eIF4F, which is an import event of translation initiation). This increase was abolished by rapamycin, to values that were even below control.

A previous investigation by the same research group (Rommel et al., 2001) similarly demonstrated that IGF-1 induced hypertrophy in myotubes was Akt-dependent (as judged by the effects of the PI3K inhibitor LY294002), and that cAkt induced dramatic myotube hypertrophy. In this study, while the effects of rapamycin were marked, the attenuation of hypertrophy was less than with the LY294002, suggesting the involvement of other targets of Akt, in addition to mTOR. This was proposed to be GSK3, which was phosphorylated and inhibited by Akt, which in turn resulted in the activation of the initiation factor eIF2B and enhanced protein synthesis.

Subsequent studies by the same group (Lai et al., 2004) and other research groups (Pallafacchina et al., 2002) supported Akt as a central kinase upstream of mTOR in the regulation of MPS. It was also reported that IGF-1 inhibited up-regulation of the atrophy-inducing ubiquitin ligases MuRF1 and MAFbx/Atrogin-1 by blocking nuclear translocation of FOXO transcription factors (Sandri et al., 2004; Stitt et al., 2004) and it was suggested that this was an important mechanism for IGF-1 induced hypertrophy (Sachek et al., 2004).

However, in some of the studies (e.g., Bodine et al., 2001; Pallafacchina et al., 2002) rapamycin abolished all of the effects of cAkt and functional overload on intact muscle, suggesting that mTOR was the most important route downstream of PI3K-Akt. Subsequently, Hornberger and colleagues demonstrated that mechanical stimulation of mTOR signaling in various models systems of skeletal muscle was independent of Akt (Hornberger et al., 2004, 2005, 2006, 2007; Hornberger & Chien 2006; O'Neil et al., 2009), which has been confirmed by other groups (Hamilton et al., 2010). Furthermore, inactivation of the IGF-1 receptor did not prevent overload-induced hypertrophy and activation of the mTOR pathway in mice (Spangenburg et al., 2008; Witkowski et al., 2010), although Akt was still activated. Finally, activation of mTOR via overexpression of the upstream positive regulator Rheb was shown to be sufficient for skeletal muscle hypertrophy (Goodman et al., 2010).

Several investigations have shown that Akt/PKB can be activated by resistance exercise in humans (e.g., Cuthbertson et al., 2005; Dreyer et al., 2006, Mayhew et al., 2009). However, similarly to findings from animal studies, Akt does not appear to play a role in the early activation of mTOR to strength training. For example, Eliasson et al. (2006) reported that while phosphorylation of p70S6K (p-p70S6K) at the site Thr389 increased, phosphorylation of Akt was decreased after eccentric exercise. A similar observation was made by Deldicque

et al. (2008), who used predominantly eccentric training. Furthermore, Terzis et al. (2010) reported that increasing strength training volume from 1 to 5 sets of 6 repetitions at 85% of 1RM resulted in greater p-p70S6K, while p-Akt decreased with training volume! Collectively, it appears that Akt is not the main route to mechanically induced early mTOR signaling.

However, it can be argued that IGF-1 and Akt are still necessary for optimal hypertrophy. For example, the IGF-1 receptor deficient mice in Spangenburgs studies were smaller in body size and muscle mass than their control counterparts. Moreover, Akt may be involved in later mTOR signaling, stimulated by some growth factors(s) and/or some other factor. Support for such a notion can be found in the study of Mayhew et al. (2009), who reported that protein synthesis was elevated at 24 hours post-exercise, and that p-Akt was also elevated at 24h. Furthermore, Léger et al. (2006) observed that hypertrophy of human muscle after strength training was accompanied by an increase in phospho-Akt, phospho-GSK-3beta and phospho-mTOR protein content. Finally, Akt could indirectly affect mTOR-p70S6K via dampening the negative effects of Atrogin-1 and MuRFs on eIF3f, although this inhibition could also be caused by HSPs and PGC1-alpha (see figure 2.7) and the SAPKs (see below). In summary, the roles of Akt in human resistance training models are currently unclear.

2.2.4 ERK1/2 in the regulation of muscle mass

ERK1/2 are typically activated by growth factors, including IGF-1 (Haddad & Adams, 2004), and are also sensitive to mechanical forces and other exercise-related stimuli such as acidosis and ROS in skeletal muscle (Wretman et al, 2001). Acidosis potentiated the effects of mild stretches, but the strongest ERK1/2 responses were seen during severe stretches and eccentric contractions (Wretman et al., 2001). Martineau & Gardiner (2001) also showed that the ERK pathway is mechanosensitive, and in their study, phospho-ERK2 tracked linearly with muscle tension. Both human exercise studies (e.g., Tannerstedt et al., 2009) and stretch models of cultured myocytes (Atherton et al., 2009) indicate that ERK1/2 is quickly activated by mechanical activity and that phosphorylation then decreases rapidly (after ~15 minutes).

A role for ERK1/2 in muscle hypertrophy is suggested by the observation that inhibition of ERK1/2 by PD098059 abolishes IGF-1 induced hypertrophy in vivo (Haddad & Adams, 2004). Furthermore, MAPKs, and specifically ERK1/2, were reported to be necessary for maintainence of muscle mass (Shi et al., 2009). ERK1/2 was also implicated in testosterone-induced proliferation in L6 myoblasts (Fu et al., 2011). In rats, a combination of resistance exercise and insulin produced greater elevations of MPS than resistance exercise alone, and

PD098059 not only blocked this but lowered MPS to below control values, suggesting that ERK1/2 contributes to exercise-induced hypertrophy (Fluckey et al., 2006). In some cell types, ERK1/2 has been shown to activate mTOR, via inhibition of TSC1/TSC2 by ERK1/2 or p90RSK, a downstream effector of ERK1/2 (Proud, 2007; Foster & Fingar, 2010).

On the other hand, inhibition of ERK1/2 did not prevent and even slightly enhanced stretch-induced hypertrophy in myotubes, which however was dependent on mTOR (Sasai et al., 2010). Activation of ERK1/2 has also been associated with atrophy and muscle wasting conditions (Penna et al., 2010). Furthermore, ERK1/2 is activated already at relatively low concentric forces both in rat and human skeletal muscle (Wretman et al., 2001; Richter et al., 2004). Thus, the roles of ERK1/2 in muscle hypertrophy and atrophy are currently unclear.

Studies from the Blomstrand group in Stockholm have shown that conventional heavy resistance exercise (Karlsson et al., 2004) and eccentric exercise (Tannerstedt et al., 2009) cause early increases in p-ERK1/2, which are transient and return to baseline within ~1 hour. In contrast, Hulmi et al. (2010) reported that a protocol of 1 x 15 at 1RM did not increase p-ERK1/2 at 30 minutes post-exercise, but that a 5 x 10RM regime caused an increase at this time point. Seemingly in contrast to earlier studies, Burd et al. (2010b) reported that p-ERK1/2 was increased at 4h after resistance exercise. Deldicque et al. (2008) also showed a late response in p-ERK1/2, in this case at 24 hours post-exercise. These observations suggest the possibility for a biphasic response in ERK1/2. Alternatively, the response of p-ERK1/2 may be affected other factors as well, such as whether the exercise was performed in a fasted or fed state, which has differed between studies in the literature.

ERK1/2 could be involved in mTOR signaling via inhibition of TSC1/2 (either by ERK1/2 itself or via p90RSK). ERK1/2 has been suggested to be involved in early mTOR signaling in response to overload (Miyazaki et al., 2011). However, the disparities in the general time courses of p-ERK1/2 and p-p70S6K after exercise, and the rather ubiquitous response of ERK1/2 to exercise in general (including endurance exercise), as well as the observation that ERK1/2 inhibition did not inhibit stretch-induced hypertrophy in myotubes, raises questions regarding the importance of ERK1/2 for mTOR signaling and MPS.

In addition to impacting mTOR signaling, ERK1/2 is also known to phosphorylate MNK1 at the site Ser209, although it should be noted that this phosphorylation site is also a target of p38 (Proud 2007, see Figure 2.3). Increased phosphorylation of eIF4E at Ser209 has been observed in conjunction with resistance exercise (Drummond et al., 2008b, 2009) and feeding (Smith et al., 2008) and in the same studies, this phosphorylation differed between subjects who responded to feeding and/or resistance exercise compared to those who had little

or delayed response in protein synthesis. Accordingly, phospho-eIF4E at Ser209 has been suggested to increase protein synthesis (Smith et al., 2008; Drummond et al., 2008b, 2009). However, increased p-eIF4E at Ser209 may actually decrease the binding of eIF4E to the 5´-cap structure of the mRNA, which in turn could decrease translation (Proud, 2007).

Yet another possibility for ERK1/2 to increase MPS is via eIF2B activity, which regulates general protein synthesis (Proud, 2007). Increasing evidence supports the importance of eIF2B, in particular the subunit eIF2Bepsilon (eIF2Bε), for exercise-induced elevations in protein synthesis (e.g., Kubica et al., 2005; Mayhew et al., 2011). Interestingly, elevated abundance of eIF2Bε was one of two signaling hallmarks of high-responders to resistance exercise in the study of Mayhew et al. (2011), the other being elevated phosphorylation of p70S6K at Thr421/Ser424. Elevated activity of eIF2Bε can also be accomplished by decreased phosphorylation at Ser539, an event which de-inhibits and thus increases the activity of eIF2Bε. Dephosphorylation of eIF2Bε at Ser539 has been observed in human studies (Burd et al. 2010a).

One well-known route to decreased p-eIF2Bε is Akt /PKB-mediated inhibition of GSK3-beta (figure 2.3). Notably though, several other pathways, including ERK1/2 and p38, were reported to positively influence both eIF2B activity and protein synthesis in some cell types (Kleijn & Proud, 2002) and this effect was not mediated via decreased phospho-eIF2Bε at Ser539 or increased abundance of eIF2Bε. Later studies from the same group has revealed that repression of the phosphorylation of another site, Ser525, is crucially involved in amino acid control of eIF2Bε activity and protein synthesis in human cells (Wang & Proud, 2008). Interestingly, this was independent of mTOR. Given the previous finding of an influence of ERK1/2 and p38 pathways on eIF2B activity, and that ERK1/2 and p38 are both involved in amino acid signaling (Casas-Terradellas et al., 2008), it is tempting to speculate that activation of ERK1/2 and/or p38 may in part activate MPS via enhanced eIF2B activity, in addition to their possible inputs to mTOR.

2.2.5 The enigmatic family of p38MAPK – inducers of both hypertrophy and atrophy?

Two other members of the MAPK family, p38 and JNK, are often termed the stress activated protein kinases (SAPKs). The SAPKs are activated in cells in response to various types of cellular stresses, such as UV radiation, ROS and TNF-alpha (Clavel et al., 2010). The p38 family consists of four different isoforms (alpha, beta, delta and gamma), which sometimes

have opposite functions (Lovett et al., 2010). Adding to the complexity is the finding that splice variants exist for p38alpha and beta (Jiang et al., 1997). The p38alpha and p38gamma are the most abundant isoforms in skeletal muscle (Ruiz-Bonilla et al., 2008, Lovett et al., 2010). The p38alpha was the first isoform to be discovered and it is still quite often referred to in the literature as simply p38. In many papers in the literature, the identity of the isoform(s) studied was not stated which makes it difficult to interpret the results.

P38 has been directly implicated in atrophy and muscle wasting induced by cytokines such TNF-alpha, and by ROS, via the induction of MuRF1 and Atrogin-1/MAFbx (Li et al., 2005; Adams et al., 2008; McClung et al., 2010). In complete contrast to these results, another study reported that p38 counteracted atrophy via nuclear exclusion of FOXO3a and decreased induction of Atrogin-1 (Clavel et al., 2010). Moreover, p38 is also associated with hypertrophy in myotubes undergoing passive stretch (Hanke et al., 2010). In C2C12 myocytes, the p38alpha/beta inhibitor SB-202190 decreased protein synthesis with 40% (Hong-Brown et al., 2004), which is consistent with a role for p38 in global protein synthesis in some cell types (Kleijn & Proud, 2002). Furthermore, Norrby & Tågerud (2010) found a strong correlation ($r2 = 0.7737$) between the phosphorylation of the p38 downstream target MK2 (at T317) and the increase in muscle weight by chronic stretch. In line with this observation, an association between cell size and MK2 was reported by Cully et al. (2010). Finally, p38 is necessary for testosterone-induced fiber hypertrophy in mice (Brown et al., 2009) and for creatine enhancement of differentiation in C2C12 cells (Deldicque et al., 2007).

As noted by Norrby & Tågerud (2010), p38 thus appears to respond to both catabolic stimuli (such as TNF-alpha) and anabolic stimuli (such as stretch and functional overload). Norrby & Tågerud (2010) theorised that phosphorylation of MK2 at T317 would lead to export of the p38-MK2 complex from the nucleus to the cytoplasm, decreasing transcription of atrophy-inducing factors and increasing anabolic activities in the cytoplasm instead.

Interestingly, the same authors that originally made the connection between p38 downstream of TNF-alpha and muscle atrophy (Li et al., 2005) also demonstrated that TNF-alpha is a mitogen and stimulates satellite cell activation in skeletal muscle (Li, 2003) and that TNF-alpha is very important for muscle repair and regeneration, via p38 (Chen et al., 2007). Further studies have shown that p38, in particular p38alpha, is critical for myogenesis. TNF-alpha is also involved in mechanotransduction activation of p38 (Zhan et al., 2007).

In many studies, the doses of TNF-alpha have been supraphysiological, i.e., clearly higher than values encountered in vivo even in many pathological states (Zhan et al., 2007). Importantly, only pathological levels of TNF-alpha inhibit instead of promote myogenesis

(Chen et al., 2007; Zhan et al., 2007). Accordingly, many of the investigations in the literature may have studied pathological roles and not physiological roles of TNF-alpha and p38.

These bimodal effects are paralleled by the bimodal effects of ROS. At low levels, ROS promote cell growth, whereas at high levels, senescence and apoptosis occur (Sauer et al., 2001). In L6 myoblasts, hydrogen peroxide (H_2O_2) at low levels was shown to stimulate cell growth (Caporossi et al., 2003) and protein synthesis (Orzechowski et al., 2002). Orzechowski et al. (2002) found the maximal effect on MPS (+90%) at 10µM H_2O_2, but already 1µM H_2O_2 increased MPS by 52%. In contrast, McClung et al (2009, 2010) found that 24 hours of exposure to 25µM H_2O_2 in C2C12 myotubes reduced myofiber diameter and that inhibition of p38 rescued this decrease. As noted above, p38 leads to elevated ROS production, but ROS can also in turn activate p38 (McClung et al., 2010; Cully et al., 2010).

Notably, p38 has been demonstrated to be both necessary and sufficient for activation of the mTOR pathway, and p38 is also necessary for amino acid sensing by mTOR (Cully et al., 2010). Moreover, activation of p38 increased the size of human cells, whereas knockdown of p38 isoforms resulted in decreased size in a number of cell types. Importantly, Cully et al. (2010) found that only low doses of H_2O_2 induced mTOR-p70S6K activation. For p38, the relation was such that the higher the concentration of H_2O_2, the higher the phospho-p38 (Cully et al., 2010). A recent study (Zheng et al., 2011) indicates that p38alpha is the isoform responsible for activation of the mTOR-p70S6K pathway. Interestingly, these authors also reported that in response to energy stress, p38beta inhibited mTOR via inhibition of Rheb.

The isoforms of p38 have distinct functions, including in skeletal muscle. In satellite cells, p38alpha stimulates differentiation and inhibits proliferation, while the p38gamma does the reverse and thus prolongs the proliferation (Lassar, 2009). This latter effect appears to be important for optimal increases in myogenic precursors, fiber numbers and fiber sizes after injury (Gillespie et al., 2009). Furthermore, p38gamma is necessary for angiogenesis and mitochondrial biogenesis with endurance exercise via PGC-1alpha (Pogozelski et al., 2009).

Interestingly, PGC-1alpha mRNA is downregulated in various conditions of muscle atrophy and upregulated by exercise, which counteracts FOXO3 action (Sandri et al., 2006). Furthermore, p38gamma has also been implicated in the prevention of corticoid-induced muscle atrophy by testosterone, via nuclear localisation of PGC-1alpha and subsequent exclusion of FOXO1 and FOXO3a (Qin et al., 2010). Nuclear localisation of PGC-1alpha may thus in part explain the earlier observations of Hickson et al. (1990) that both exercise and anabolic steroids can counteract or attenuate corticoid-induced muscle atrophy.

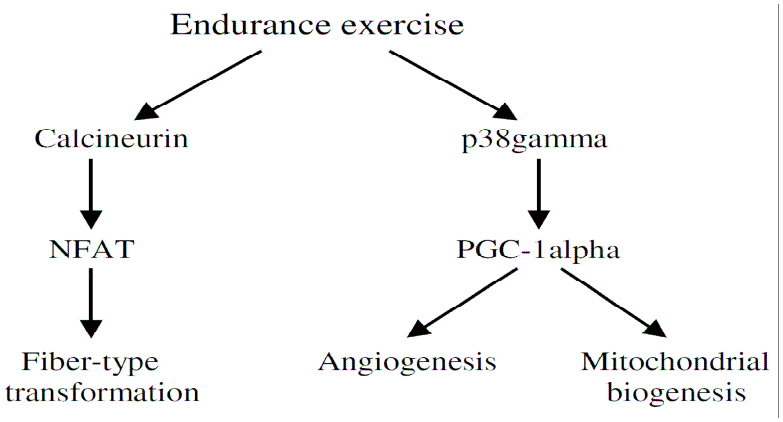


Figure 2.8. p38gamma is involved in endurance exercise adaptations. After Pogozelski et al (2009).

P38 was reported to be phosphorylated in response to a regime of sprint-interval cycling training (Gibala et al., 2009), an exercise mode which induces adaptations similar to traditional endurance training, and not resistance training. Unfortunately, it was not reported which isoforms that were activated. However, Boppart et al. (2000) found that the higher band in western blotting was p38gamma, whereas the lower band was p38alpha, as verified with isoform-specific antibodies. Notably, in response to marathon running, only the phosphorylation of p38gamma was markedly increased (Boppart et al., 2000). Thus, it may have been p38gamma which increased in the study of Gibala et al. (2009).

A number of studies (e.g., Deldicque et al, 2008; Hulmi et al, 2010) have demonstrated marked early increases in the phosphorylation of p38 in response to high-resistance exercise, which although transient appear to last up to ~1-2 hour after exercise (Karlsson et al., 2004, Tannerstedt et al., 2009). Again, in many studies it is not apparent which isoform was studied. Hulmi et al. (2010) found that both the lower band and the higher band of phospho-p38 (probably corresponding to p38alpha and p38gamma, respectively) were increased at 30 min post-exercise. Furthermore, a 5 x 10RM protocol induced a stronger response in the p38gamma band than a 1 x 15 at 1RM protocol, while p-p38alpha increased to a similar relative degree in both regimes. Thus, it could be that the 5 x 10RM protocol induced mitochondrial and angiogenic adaptations via the p38gamma - PGC-1alpha route, in part explaining the effects of moderate to high repetition protocols on strength-endurance.

It is intriguing that p38 and the other SAPK member, JNK, respond to the same mechanical stimuli as those which induce a strong p-p70S6K response, in particular stretch

and eccentric exercise (e.g. Wretman et al., 2001; Boppart et al., 2000, 2006; Hornberger et al., 2005; O'Neil et al., 2009; Tannerstedt et al., 2009). O'Neil et al. (2009) suggested that this might indicate a common activator. They also determined that JNK2 and p38 were not downstream of PA. However, although it was argued that p38 and JNK2 probably were not involved in the stimulation of the PA-mTOR pathway, they could not rule out this possibility. Finally, as already noted, p38 may affect MPS independently of mTOR via influencing eIF2B. Taken together, roles for p38 isoforms in load-induced hypertrophy seem very likely.

2.2.6 JNK

The isoforms of JNK1 and JNK2 respond to mechanical stimulation such as high-force muscle actions and stretch in both rat and human skeletal muscle (Martineau & Gardiner, 2001, 2002, Boppart et al., 1999). JNK also responds to marathon running and eccentric exercise in humans (Boppart et al, 2000), and with a stronger response to isokinetic eccentric exercise than to concentric exercise (Boppart et al., 1999). This latter finding agrees with Martineau & Gardiner (2001), who found that phosphorylation of JNK increased linearly with the level of tension imposed on the muscle. O'Neil et al. (2009) reported that JNK was activated after an acute bout of eccentric exercise in rats. In this study, both p38 and JNK were activated for many hours post-exercise, and this was the case also for p70S6K.

Collectively, these findings suggest that JNK has some role(s) in the adaptation of skeletal muscle, particularly to high-force exercise. Interestingly, in the same study that reported that p38 could prevent atrophy by nuclear exclusion of FOXO3a, it was also observed that JNK could achieve this (Clavel et al., 2010). Furthermore, JNK was recently reported to be both necessary and sufficient for mTOR signaling in some cell types (Fujushita et al., 2011), which is yet another interesting parallell with p38.

However, JNK has also been shown to have negative effects on muscle fiber hypertrophy (Brown et al., 2009), and on IGF-1 signalling (Grounds et al., 2008), and to be involved in the negative effects of myostatin on muscle mass (Huang et al., 2007). Of special interest, a JNK inhibitor nearly abolished the growth-inhibitory effects of myostatin on C2C12 myoblasts (Tan et al., 2009). JNKs also appear to be involved in muscle dystrophy, and in damage and cell death resulting from excessive stretch (Kolodziejczyk et al., 2001; Tan et al., 2009). It therefore seems that high and chronic JNK activation of certain isoforms can cause a range of negative effects such as insulin resistance, muscle growth inhibition, severe damage and muscle wasting.

As previously noted, however, the same protocols or stimuli which activate p70S6K also often activate isoforms of JNK. One possible solution to this paradox is that similarly to ROS, the duration and/or amplitude of JNK activation may have a decisive influence on whether the effects will be beneficial or detrimental. Alternatively, the JNK response with eccentric exercise may simply be related to muscle damage, which in healthy muscle usually is at its greatest with the first bout and then decreases with subsequent training. The possibly opposite roles of JNK1 and JNK2, as well as the multiple splice variants of each, make the physiological significance(s) of JNKs even more elusive.

2.2.7 AMPK

In most cell types, including skeletal muscle, AMPK (5' adenosine monophosphate-activated protein kinase) is one of the key regulators of metabolism (Winder & Thomson, 2007), and a master regulator of the homeostatic balance between cell growth and cell atrophy (Gordon et al., 2008). AMPK is activated during metabolic stresses (energy depletion), when AMP/ATP and inorganic phosphate (Pi)/phosphocreatine (PCr) ratios are high. Conditions that may activate AMPK thus include (but are not limited to) muscle contractions, ischemia, glucose deprivation and heat shock (Winder & Thomson, 2007, Gordon et al., 2008).

When activated, it stimulates glucose uptake and fatty acid oxidation and inhibits energy consuming processes such as protein synthesis, lipogenesis, and cholesterogenesis (Winder & Thomson 2007). If the energy state is low, activated AMPK can thus direct the cell to focus on ATP production to support cellular metabolic needs. When energy levels are high, inactivation of AMPK permits maintenance and growth of cell size (Gordon et al., 2008).

A series of studies from the Gordon lab (reviewed by Gordon et al., 2008) on rat models of functional overload and eccentric contractions have provided evidence for an inhibiting effect of activated AMPK (as judged by p-AMPK Thr172) on mTOR signaling and hypertrophy in skeletal muscle. Notably, AMPK (Thr172) was shown to be strongly inversely correlated with gains in muscle mass resulting from functional overload, and blocking AMPK rescued aging-associated impairment of overload-induced muscle hypertrophy.

Similarly, Aguilar et al. (2007) showed that the small cell size resulting from deletion of p70S6K in myotubes was largely rescued by inhibition of AMPK. Intriguingly, inhibition of AMPK by transfection with kinase-dead AMPK into muscle fibers resulted in hypertrophy not only in wild type mice but even more so in p70S6K-deficient mice, and sensitivity of growth to rapamycin was restored with AMPK inhition (Aguilar et al., 2007). These findings

suggest that downregulation of AMPK activity in itself can result in hypertrophy, even when activated p70S6K is not present in the muscle fiber.

AMPK inhibits mTOR by activating TSC1/2 and by an inhibiting phosphorylation of raptor, a binding partner of mTOR in the mTOR complex 1. Moreover, AMPK can not only decrease protein synthesis but also increase myofibrillar degradation in myotubes, and elevate the mRNA of atrophy-related genes such as Atrogin-1 and MuRF1 (Krawiec et al., 2007, Nakashima & Yakabe, 2007). As previously discussed, Atrogin-1 and possibly also MuRF1 & MuRF2 target the translation regulatory subunit eIF3f for degradation, and eIF3f is required for mTOR signaling leading to muscle hypertrophy (Csibi et al., 2010). Collectively, these observations may at least in part explain the dual effects of AMPK on protein synthesis and degradation. For these reasons, a chronic increase in AMPK activity, such as may occur in elderly individuals, is detrimental to muscle mass. On the other hand, a shorter activation of AMPK is beneficial for glucose uptake in the muscle.

AMPK has been found to be acutely activated in some human studies on strength training and other modes of exercise, but reports are contradictory. Phosphorylation of AMPK at Thr172 has been reported after high-volume strength training (Dreyer et al., 2006, Holm et al., 2010) and high-intensity interval cycling (Gibala et al., 2009), modes that are associated with metabolic stress. Performing resistance exercise in a fed state may inhibit AMPK activation that otherwise could result from the training (Witard et al., 2009). Also, as with ERK1/2 , one cannot rule out the possibilty that a transient activation occurred, which was not detected some studies. In support of this scenario, Wilkinson et al. (2008) reported elevated AMPK (Thr172) immediately after strength training in the fed state, which had reverted back to baseline by 4 hours. Nevertheless, resistance exercise does not always lead to increased p-AMPK even in the fasted state (e.g., Tannerstedt et al., 2009, Apro & Blomstrand, 2010).

The findings of Dreyer et al. (2006) of concurrent activation of MPS and AMPK have led some to question the view of Gordon et al that AMPK is inhibitory for protein synthesis. At least three aspects deserve discussion in this context: timing, duration and amplitude of the AMPK response. Gordons group (reviewed in Gordon et al., 2008) determined that activation of AMPK before an eccentric exercise bout abolished MPS and p-p70S6K, even up to 24 hours post-exercise. Although these findings need confirmation, they suggest that the timing of the AMPK response may be essential. Thus, it could be speculated that if it instead occurs well into the exercise bout, vital signals for MPS may already have taken place and AMPK might not in this case be inhibitory once recovery starts.

Duration of the p-AMPK response is also a possible factor, and it appears that AMPK activation is relatively short-lived with resistance exercise. A short-lasting duration may not be sufficient for proteolysis and/or inhibition of protein synthesis to have significant negative effects. Finally, even though Dreyer et al. (2006) observed concurrent activation of MPS and AMPK, AMPK could still have had an inhibitory effect on the protein synthesis at the time points investigated by these researchers. It is noteworthy that the rise in MPS, both in relative and absolute terms, was relatively modest compared to peak values reported in the literature.

2.2.8 Brief perspective - MAPKs

The roles of MAPKs in skeletal muscle are obviously manyfold and seemingly at times contrary even within specific isoforms of each family. As discussed above, ERK1/2, p38MAPK and JNK have all been linked with both atrophy and hypertrophy in skeletal muscle. Interestingly, chronically elevated phosphorylations of all three MAPKs in the resting state have been observed in elderly men compared to younger men (Williamson et al., 2003). Even more intriguingly, in response to an acute resistance exercise bout, increased phosphorylations of the MAPKs occurred in the young men, while decreases in phosphorylations were seen in the older men.

Commenting on the positive and negative effects of MAPKs and another pathway, NFkappaB, Kramer & Goodyear (2007) suggested: “this apparent paradox may be resolved by at least two explanations: timing and specificity. First, exercise causes brief but robust perturbations in cellular stress, including increases in ROS and metabolism that quickly stabilize postexercise. Diabetes and cachexia are chronic conditions of continual ROS production, inflammation, and hypermetabolism. Alternatively, distinct NF-KB complexes may be selectively activated by exercise compared with pathological conditions”.

It should come as no surprise to the reader that the present author largely shares this view. Depending on the combinations, strengths and durations, the downstream responses to activation could be very different. Other explanations for the contrasting findings include cross-talks and redundancy between the pathways. For example, both p38 and ERK1/2 may phosphorylate MNKs, p70S6K at (Thr 421/Ser 424) and activate mTOR, and possibly also eIF2B. Indeed, inhibition of all three major MAPKs results in profound fiber atrophy (Shi et al., 2009). Thus, inhibiting or observing just one MAPK in isolation may not be enough to clarify its physiological significance.

2.3 The resistance exercise stimulus

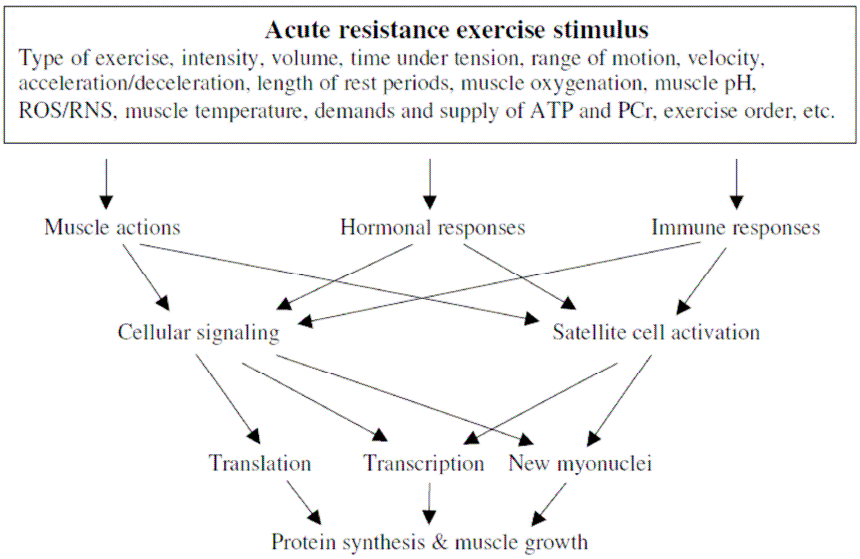


Figure 2.9. Some events stimulated by resistance exercise, with reference to muscle growth. Adapted from Spiering et al. (2008), modified based on Toigo & Boutellier (2006) and Wernbom et al. (2007).

As is obvious from the figure above, strength training with high loads can directly and indirectly activate numerous processes, ultimately leading to muscle growth. An important observation is that significant hypertrophy is seen mainly in the exercised muscle(s) and not in the other muscles (Adams, 2002b; Goldspink et al., 2006). In cases where there is an increase in for example the contralateral untrained limb muscle size after unilateral strength training of the other limb, this is generally negligible (1-2%) (e.g., Hubal et al., 2005).

However, dismissing the possibility of contributions from a systemic response is likely to be an oversimplification, and several examples of interesting interactions between local mechanisms and systemic responses have been indicated with strength training in clinically relevant situations. It could also be argued that the distinction between local and systemic responses is artificial, as it is sometimes difficult to classify certain candidate mechanisms as purely belonging to either of the two. Still, for the clarity of discussion, local and systemic responses will initially be treated in separate paragraphs in this text.

2.3.1 Mechanotransduction and local growth factors

Several mechanisms intrinsic to the muscle are possibly involved in the localised hypertrophic response to resistance training, including but not limited to: autocrine/paracrine growth factors, transduction of mechanical stimuli to intracellular signals (mechanotransduction), intracellular signaling induced by changes in the local environment, and changes in the content of receptors of hormones, growth factors, cytokines, and other ligands. The interested reader is referred to excellent reviews by Vandenburgh (1992), Hornberger & Esser (2004); Huijing & Jaspers, (2005), Burkholder (2007) and Hornberger (2011).

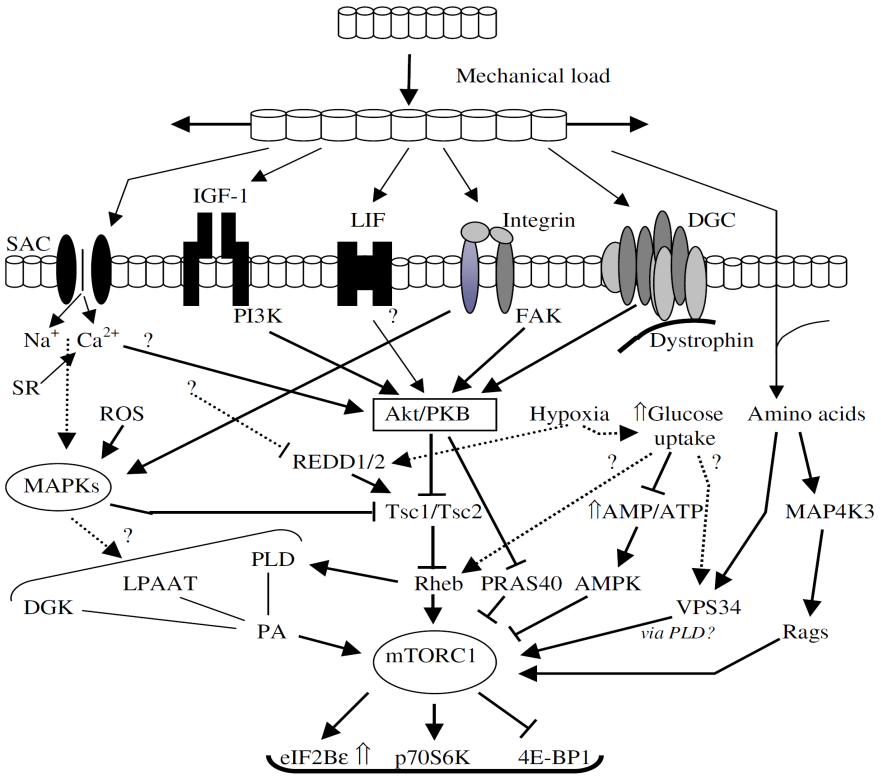


Figure 2.10. Simplified scheme of how mechanical loading of the membrane and cytoskeleton may lead to activation of the mTOR pathway via multiple mechanisms in the skeletal muscle fiber. For clarity, cytoskeletal structures have been omitted, as well as some of the inputs and targets of mTOR. Figure adapted from Spangenburg (2009) and modified based on Lee et al (2009), Hornberger (2011), Xu et al (2011), Mayhew et al. (2011) and Buller et al (2011), among others.

Multiple putative mechanosensors exist in the muscle fibers, of which integrins have already been mentioned. Other candidates include, but are not limited to, the dystrophin-glycoprotein-complex (DGC), caveolins, stretch-activated ion channels (SAC) and the lipid bilayer in the sarcolemma (Hornberger & Esser, 2004, Burkholder, 2007). These have all been implicated in mechanically induced signaling.

Integrin signaling is essential for mechanically induced upregulation of MHC II and myosin content (Hanke et al., 2010). Paradoxically however, Boppart & colleagues (2006) found that forced upregulation of alpha7-beta1-integrins in mice resulted in both less damage and less phosphorylation of multiple kinases including Akt, mTOR, p70S6K, ERK1/2, p38 and JNK after eccentric exercise. This seems to indicate that not only was the overall damage less, but the sensitivity to mechanical stimuli was also decreased. Perhaps this could be due to less stimulation of other mechanotransducers than integrins, such as SACs, or less transmembrane passage of molecules (e.g., growth factors).

SACs appear to be necessary for both muscle hypertrophy adaptations and optimal mTOR-p70S6K signaling (Spangenburg & McBride, 2006; Butterfield & Best, 2009). SACs have also been implicated in the satellite cell activation in response to mechanical stretch (Tatsumi, 2010). Based on a series of their own experiments and the results of other leading researchers, Tatsumi and colleagues proposed an elegant model for stretch activation of satellite cells.

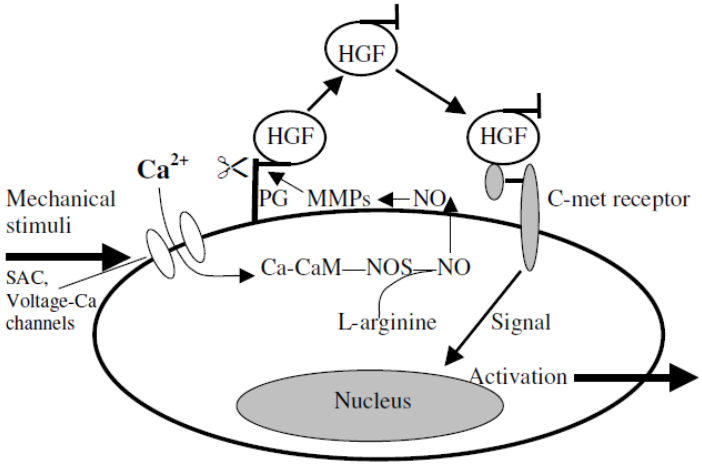


Figure 2.11. Scheme of how mechanical stretch may lead to activation and ultimately to increased number of satellite cells, myonuclei and new fibers in skeletal muscle. See text for details. Figure adapted from Tatsumi (2010).

In this model, stretch leads to opening of SACs in the satellite cells, in a manner dependent on the Calcium-CaM-NOS-NO pathway. Nitric oxide (NO) produced by nitric oxide synthase (NOS) diffuses out of the cell and activates matrix-metalloproteinases (MMPs), which in turn cleave proteoglycans (PG) in the extracellular matrix (ECM). Hepatocyte growth factor (HGF) is stored in the ECM bound to PG, and is released by the proteolytic actions of the MMPs. HGF can then bind to the c-met receptor on the satellite cell to activate it.

In addition to being upregulated at the mRNA level and/or increased in the circulation, several local growth factors such as IGF-1, fibroblast growth factors 1 and 2 (FGF1 & FGF2) and HGF (as discussed above) can be released from their storage sites within or outside the muscle fibers and therefore also be a part of a local almost immediate mechanotransduction process. Similarly to HGF, IGF-1 is stored in the ECM and can be freed upon the activation of MMPs and/or other proteases such as calpains.

FGF1 (also called acidic FGF) and FGF2 (basic FGF) are both stored inside the muscle fibers and lack a conventional secretory signal sequence, but upon temporary ruptures in the sarcolemma, these can leak out into the extracellular space (McNeil & Khakee, 1992). Simultaneously with leakage of FGFs, influx of calcium occurs, triggering PLA2 and the subsequent production of arachidonic acid, lysophospholipids, and their metabolites such as prostaglandins. This scenario has been termed the “damage sensor hypothesis” of mechanotransduction (Grembowicz et al., 1999; Miyake & McNeil, 2003).

FGFs have been shown to increase proliferation of satellite cells, and in the case of FGF2 also to activate quiescent satellite cells (Yablonka-Reuveni et al., 1999). Moreover, blocking FGF-receptors with antibodies abolished mechanically stimulated hypertrophy of skeletal muscle cells in culture (Clarke & Feeback, 1996). Similarly, neutralizing antibodies against either bFGF or IGF-I into the muscle at the time of lesion reduced the number and diameter of regenerating myofibres, suggesting a delay in proliferation and/or fusion of activated satellite cells (Lefaucheur & Sébille, 1995). Therefore, FGFs seem important for the normal response to mechanical stimuli, as well as to muscle damage, in skeletal muscle.

In humans, it was shown that bedrest reduced the plasma levels of FGFs and creatine kinase (CK), whereas resistance exercise increased the concentrations and at the same time counteracted bed-rest induce muscle atrophy (Clarke et al., 1998). Furthermore, a moderate correlation was found between changes in muscle fiber CSA and CK values, indicating a connection between wounding of the sarcolemma and the trophic state of the muscle fibers.

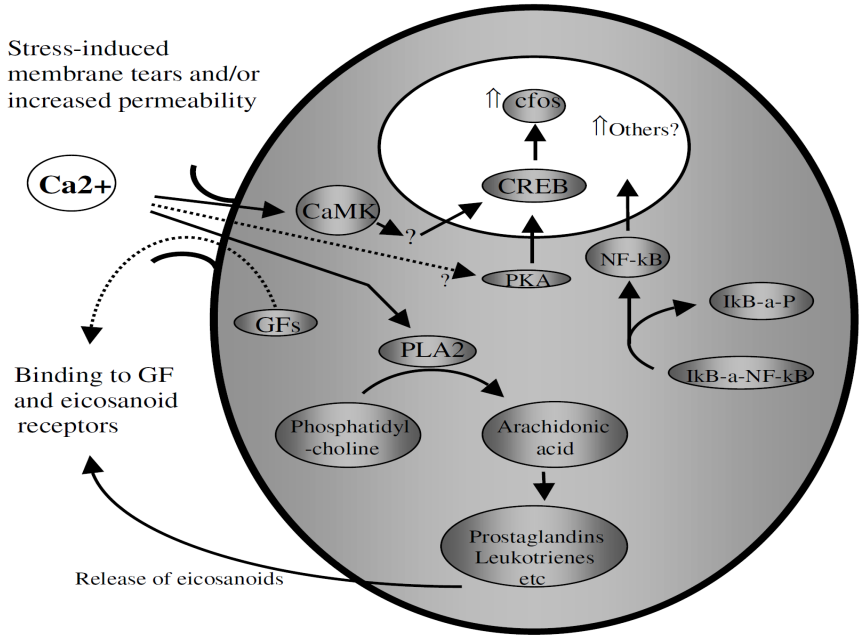


Figure 2.14. The "damage sensor hypothesis". Mechanical stress may lead to tears or increased leakiness in the sarcolemma, leading to efflux of growth factors (GFs) and influx of ions such as Ca2+ and Na+, in turn leading to various cell responses. Figure adapted from Miyake & McNeil (2003).

However, the hypothesis of mechanically induced membrane ruptures has been challenged (Allen & Whitehead, 2010). According to these authors, there is rather an increase in the general permeability of the sarcolemma, presumably caused by activation of SACs, which is responsible for the leakage in and out of the muscle fiber. Also of note is that in some cell types, FGF2 has been shown to use non-conventional routes of exit (Taverna et al., 2003; Nickel, 2010). Finally, not only mechanical stress but also metabolic and hypoxic stress may lead to greater permeability of the sarcolemma (Fredsted, 2007). In any case, increased leakage of molecules and ions into and out of the muscle fibers could be important mechanisms for various hypertrophic stimuli, including strength training.

2.3.2 Systemic responses

Heavy-resistance exercise can result in acute changes in the plasma levels of several hormones and growth factors. Hormones and systemic growth factors that have been shown to

increase acutely in plasma as a result of a strength training bout include growth hormone (GH), testosterone, cortisol, insulin-like growth factor 1 (IGF-1), insulin, and catecholamines (Kraemer & Ratamess, 2005). To date, most of the research has been focused on the anabolic hormones GH and testosterone, and the catabolic hormone cortisol. The sometimes marked acute increses in concentrations in response to strength training, in particular to so called "hypertrophic" or bodybuilding type protocols (e.g., 10RM loads, short interset rest periods), led several groups to suggest that the hormonal response, especially GH, is of importance for gains in muscle size and strength.

However, the evidence for a role for GH in exercise-induced muscle hypertrophy is limited. McCall et al. (1999) reported significant correlations between the acute increases in GH and the increases in type I and type II fiber area in the biceps brachii in response to a period of strength training, but there was no relationship between increases in GH and increases in whole muscle area for the biceps brachii. Goto et al. (2005) reported significant correlations between acute exercise-induced elevations in GH and increases in quadriceps CSA after a training period. While these correlations appear to support a role for GH, they do not prove causality. It is possible that the increased levels of GH simply reflected a greater level of effort. Additionally, elevated levels of GH may be caused by and/or occur in parallel to local processes, which could themselves be important for the hypertrophic response.

Furthermore, studies examining exogenous GH in combination with resistance exercise have generally not shown any significant additive effects on muscle anabolism (reviewed by Rennie, 2003). It was perhaps not surprising then that West et al. (2009, 2010), using a within subject design, did not find any differences in elevations in MPS, gains in muscle size and strength between unilateral arm training only and unilateral arm training followed by an intense lower body protocol, even though the hormonal responses were drastically in favor of the combined protocol.

However, Rønnestad and colleagues (2011) found a slight advantage in both strength and elbow flexor CSA gains for combined unilateral arm training followed by an intense lower body protocol vs a pure unilateral arm protocol. Also, there are several variants of GH as well as other peptides that are released from the pituitary gland in response to resistance training (Hymer et al., 2006). Other circulating factors that may be changed by exercise include myostatin (Walker et al., 2004), follistatin (Hansen et al., 2011), follistatin related gene (FLRG) (Willoughby, 2004), opioids (Kraemer et al., 1993) and myokines (Pedersen, 2009). Thus, it may be premature to rule out the systemic responses.

PART 3. The physiology of blood flow-restricted resistance training

3.1 Motor unit and muscle fiber recruitment

Given the low loads typically used in BFR resistance exercise, it would seem that the degree of recruitment of motor units and muscle fibers would be correspondingly low. However, restricting the muscle blood flow with a tourniquet has been shown to decrease the endurance and increase the electrical activity (as measured by EMG) of the working muscle during low-intensity exercise. For example, Takarada et al. (2000b) showed that the EMG of the biceps brachii during low-intensity resistance exercise (40% of 1RM) combined with partial occlusion of the muscle blood flow was almost equal to that observed during conventional heavy resistance exercise (80% of 1RM).

In an early study on low-intensity dynamic knee extensions performed to exhaustion during cuff occlusion, Ingemann-Hansen et al. (1981) observed glycogen depletion in both fiber types as well as low creatine phosphate levels in muscle biopsies taken immediately after exercise. Recruitment of all fiber types, including type IIx, during BFR knee extensor training already at low work loads was confirmed by Krustrup et al. (2008). Yasuda et al. (2005) reported type II fiber hypertrophy as well as increased whole muscle CSA after a period of low-intensity strength training (20% of 1RM) with partial BFR.

Collectively, these findings show that type II fibers are recruited also during very low-intensity ischemic strength training. Indeed, Meyer (2006) argued that the enhanced hypertrophic response to exercise with BFR may simply be due to the recruitment of large motor units and the resulting mechanical load on the muscle fibers in these units. The underlying basis for the involvement of the type II fibers in BFR resistance exercise even at low load is probably related to fatigue in type I fibers, thus necessating the recruitment of type II fibers in order to continue the exercise (reviewed by Wernbom et al., 2008). For example, Greenhaff et al. (1993) demonstrated that BFR during electrical stimulation in human quadriceps markedly increases the rate of glycogenolysis and the degree of PCr depletion in type I fibers compared to free-flow conditions.

3.2 Hypertrophic adaptations to BFR resistance training

3.2.1 Hypertrophic adaptations - Time course and magnitude of changes

In some studies, BFR training regimes with a total of 12 sessions per week have been tried successfully over short periods (1-2 weeks), resulting in significant increases in muscle CSA and strength despite the very short training periods (Abe et al., 2005a,b, 2006; Fujita et al., 2008). The CSA in the quadriceps was reported to increase with 3.5% after one week of training (Fujita et al., 2008) and with 7.7% after two weeks (Abe et al., 2005a), corresponding to a rate of increase of 0.5-0.55% in CSA per calendar day. These figures are among the highest reported in the literature, and 4-5 times higher than the average rate of CSA gain with conventional heavy strength training (reviewed by Wernbom et al., 2007).

However, marked hypertrophy has been noted even with only 2 sessions per week of BFR resistance training with multiple sets to failure (Takarada et al., 2002, 2004). Furthermore, the rates of quadriceps CSA gains noted with twice weekly BFR strength training in these studies were impressive (0.18-0.22% per day) and appeared to be in the upper range of the rates typically resulting from multiple-set heavy resistance training at the same frequency of training (average rate ~0.11% per day, range 0.03-0.26%), as reviewed by Wernbom et al. (2007).

The rapid muscle area gains reported by Abe et al. (2005a) were also manifest at the fiber area level, with gains of 6% in type I area and 28% in type II area (Yasuda et al., 2005). One may rightly be skeptic to increases of this magnitude in such a short time, and ask if these were real increases and not due to residual swelling. However, muscle strength increased in proportion to the CSA in these studies, and muscle swelling resulting from any damage should have subsided by 2 weeks. Moreover, new data from other research groups appears to confirm the possibility of very rapid gains in muscle size and strength.

The present author is fortunate to be part of a low-load BFR training project led by Professor Per Aagaard and colleagues at the University of Southern Denmark in Odense. Preliminary results show increases in mean areas of type I and type II fibers of 20% and 21% respectively, and a corresponding 20% increase in MVC after a mere two weeks of training at 20% of 1RM to failure (Nielsen et al, and Frandsen et al, abstracts presented at the 2011 American College of Sports Medicine congress). In this study, the number of training sessions during the two weeks were 21 in total, thus the training frequency was on average 1.5 times per day (in reality, once a day on some days, twice on others, with a few rest days). Importantly, the ~20% gains in MFA and MVC were measured 8 days after the training period ended, thus any residual swelling was most likely negligible, and the gains in MVC indirectly supports this by showing that the CSA increase were functional.

These rates of increases are likewise among the highest reported in the literature. To put them into perspective, the mean fiber area elevations are at least equivalent to those reported in a previous study from the same group of researchers after 14 weeks of heavy resistance training, 3 times per week (Aagaard et al., 2001). Interestingly, after just one week (7 sessions) of BFR training, the gains in type I and II fiber areas were even larger (35% and 47%), but at this early time point it is likely that some swelling was still present due to microdamage to the muscle fibers imposed by the training bouts.

In summary, it seems clear that muscle size can adapt at remarkably fast rates with very frequent BFR training, and that even with a normal amount of training sessions per week, the muscle size gains still are comparable with many conventional routines.

3.2.2 Underlying hypertrophic processes – protein synthesis and myonuclear addition

Fujita et al. (2007) reported that low-load BFR resistance training increased MPS at 3 hours post-exercise, thus giving a mechanistic explanation behind the hypertrophy with low-load ischemic strength training. A newer study from the same group (Fry et al., 2010) confirmed this finding, this time in a group of older men.

In the preliminary results of Nielsen et al., satellite cell (SC) number per muscle fiber increased 4-fold (0.101 ± 0.027 to 0.383 ± 0.065) ($p<0.01$). Likewise, Pax7-positive SC per myonuclei increased 3-fold (0.040 ± 0.010 to 0.119 ± 0.027) ($p<0.01$). Myonuclei number per muscle fiber increased 35% (2.51 ± 0.22 to 3.30 ± 0.65) ($p<0.05$) and nuclear domain did not change with training (1917 ± 248 vs. 2053 ± 514 μm^2 /nuclei). Finally, mean muscle fiber CSA was related to Pax7-positive SC per muscle fiber pre and post training ($r=0.6$-0.7) ($p<0.05$).

Perhaps the most important observations of these new results are that myonuclear addition seems essential for myofiber growth, and that the availability of satellite cells is in turn crucial for optimal increases in myonuclei. This is very much in agreement with the results of Petrella et al. (2008). Furthermore, the increase in SC seems remarkably large. Given that SC numbers per fiber are much lower than the numbers of myonuclei per fiber, it may be that large increases in SCs were needed for myonuclear addition. Also, is possible that some damage was present that necessitated muscle repair.

3.3 Signaling pathways activated by low load BFR resistance exercise

Relatively little is currently known with regard to the pathways activated by low-to-moderate load BFR (ischemic) strength training. This section will therefore be necessarily (and perhaps also mercifully) quite short.

mTOR

A few investigations have now determined that the mTOR pathway and the downstream effector p70S6K (as judged by phosphorylation of the site Thr389) can be activated in BFR resistance exercise in humans (Fujita et al., 2007; Fry et al., 2010). Also, the eukaryotic elongation factor 2 (eEF2) was dephosphorylated (Fujita et al., 2007). This means that the inhibition of eEF2 was less, and thus that translation elongation was probably facilitated, which is also positive for MPS. However, this enhanced activation by dephophorylation of eEF2 may or may not be mTOR-p70S6K dependent. In contrast to p-p70S6K, p-4E-BP1 did not change in the paper of Fry et al. (2010).

Interestingly, in a follow up on the same subjects as in Fujita et al. (2007), Drummond et al. (2008) reported that REDD1, a negative regulator of mTOR, was decreased at 3 hours post-exercise. Notably, myostatin was also decreased. However, these responses did not differ between BFR and control training (at the same load and volume as BFR), while both p-p70S6K and protein synthesis clearly differed at 3 hours post between the groups. Therefore, downregulation of REDD1 and myostatin may have been permissive but not decisive for the positive anabolism in the BFR condition.

Akt/PKB

Both Fujita et al. (2007) and Fry et al. (2010) showed that Akt/PKB can be activated by BFR resistance exercise at 3 hours post-exercise in humans. However, as previously noted, Akt/PKB may be of secondary importance in early exercise-induced mTOR signaling. In support, p-Akt increased similarly between BFR and control training conditions in the study by Fujita et al. (2007), whereas MPS clearly differed. Therefore, the role of Akt may be questioned also in BFR strength training.

Increased p-FOXO3a and decreased MuRF1 and Atrogin-1 mRNA at 8 hours post-BFR resistance exercise was reported by Manini et al. (2010). However, as discussed previously, many other candidates for this response exist in addition to Akt. Interestingly, Drummond et al. (2008) reported an increase in MuRF1 mRNA at 3 hours post, suggesting that MuRF1 may

have a biphasic response, much like with heavy resistance exercise (e.g., Mascher et al., 2007). Also, MAFbx/Atrogin-1 did not change in either Drummond et al. (2008) or Mascher et al. (2008). Notably, Mascher et al. (2008) demonstrated a blunted MuRF1 mRNA upregulation after the second bout of exercise. One could speculate that MuRF1 is involved in the breakdown of myosin in myofibrillar remodelling, which may be greater with the first bout.

ERK1/2

Fry et al. (2010) reported that at 1 hour post-exercise, p-ERK1/2 was increased ~6-fold with the BFR protocol but not with the control training. At 3 hours, p-ERK1/2 still tended to be elevated (~5-fold), but this was not significant. Fry et al. (2010) also observed increased MNK1 phosphorylation, which is a target of ERK1/2. Theoretically, this could increase MPS. As noted before, however, MNK1 is also a target for p38 and this kinase was not investigated by Fry et al. (2010).

p38MAPK

To the best of the knowledge of the present author, no study has investigated p38 in response to BFR resistance exercise.

AMPK

Fry et al. (2010) reported no changes in AMPK phosphorylation at Thr172 in older men in response to BFR resistance training immediately after exercise and at 1 and 3 hours post-exercise. To the knowledge of the present author, the response of AMPK to BFR exercise has not been investigated at later time points.

PART 4: Initial observations on signs of muscle damage with BFR resistance training

The present author started pilot experiments on BFR resistance training in late 2002-early 2003. The first subject was the author himself, who by then had been performing heavy strength training on a more or less regular basis for about 18 years (for the upper body at least, for the lower body, about 8 years). The other subjects were the authors main supervisor at the time, Dr Jesper Augustsson, and some of the authors fellow gym training friends and physiotherapists, who were more or less talked into trying out this new mode of exercise.

One of the first observations arising from these early pilot studies was that not only was BFR training "all-out" to failure quite painful during the bout because of the enhanced accumulation of metabolic by-products, but in many cases this mode of training produced remarkable delayed onset muscle soreness (DOMS) in the days afterwards, and some reported other symptoms of muscle damage such as muscle cramping and markedly reduced range of motion. The author himself had difficulties walking down stairs and a noticeably altered walking pattern even on level surfaces because of the severe soreness caused by these first experiments. The DOMS lasted almost a week and was on par with that caused by some of the more severe heavy leg workouts that the author had undertaken.

Interestingly, the literature on BFR training that had been published up until then did not mention the occurence of DOMS with this new mode of exercise. At this point, the author started hypothesising on possible similarities between BFR resistance exercise and conventional heavy strength training, despite the vast differences in the loads used during training. These hypotheses are still developing to the present day, and provided the impetus for the studies presented in this thesis.

PART 5: Research aims

The aims of this thesis were:

1) To characterise the effects of blood flow restriction (BFR) on muscle endurance at different exercise loads in the knee extension exercise (paper I)
2) To document the occurrence and time course of DOMS with low-load BFR resistance training (papers I-III)
3) To investigate the muscle activation patterns in the quadriceps (vastus lateralis and medialis) with low load BFR resistance training (paper II)
4) To document the fatigue and recovery of muscle force capacity and contractility after an acute bout of low-load BFR resistance training (paper III)
5) To investigate whether signs of muscle damage were present at the muscle fiber level after an acute bout of low-load BFR resistance training (paper III)
6) To investigate the occurrence and the time course of intracellular anabolic signaling with low-load BFR resistance training (papers IV)
7) To investigate the effects of an acute bout of low-load BFR resistance training on activation and proliferation of satellite cells (paper IV)

Specifically, it was hypothesised that:

1. BFR would decrease the endurance in dynamic exercise
2. Fatiguing low-load BFR resistance exercise would be associated with high levels of muscle activity
3. Fatiguing low-load BFR resistance exercise would induce both acute (minutes, hours) and more long-lasting (days) decrements in voluntary force capacity, and some signs of damage would present at the muscle fiber level
4. Low-load BFR resistance exercise performed all-out to concentric torque failure would induce both acute (minutes, hours) and more long-lasting (days) increases in mTOR-p70S6K signaling
5. Low-load BFR resistance exercise performed all-out to concentric torque failure would cause increases in satellite cell numbers

PART 6: Methods

This thesis presents data from three separate studies. The first study (paper I) was carried out in 2003 and early 2004. The second study, the "EMG experiment" (paper II) was performed in 2007. The third study (paper III and IV), the "muscle damage and signaling experiment", was carried out between 2008 and 2010. Analyses of muscle tissue samples from the third study are still running. All three studies involved the knee extensors (quadriceps) and the acute effects of blood flow restriction during low-load resistance exercise on the performance and the responses of this muscle group.

6.1 Subjects

A total of 39 healthy subjects (20-45 years of age, males n=30 and females n=9), who were students and employees at the University of Gothenburg and the Norwegian School of Sport Sciences, completed the experiments. All of the subjects were physically active, and many of them performed strength training on a regular basis, or had at least previous strength training experience. The participants were instructed not to train their quadriceps during the last 3 days before the main exercise bouts, and not to change their diets during the experimental periods. The subjects were informed of the experimental risks and signed an informed consent document before the investigations. The studies were approved by the Human Ethics Committee at the Faculty of Medicine, University of Gothenburg, Sweden, and the Regional Ethics Committee of Southern Norway, and complied with the standards set by the Declaration of Helsinki.

6.2 Study design

All three experiments were carried out with pre-tests, the exercise protocol(s), and repeated post-tests, i.e., a pre-post-design. A within-subject design was used, in which one of the legs (randomised) performed unilateral dynamic knee extension exercise (in a conventional machine with weight stack for resistance) with blood flow restriction, and the other leg performed resistance exercise without restriction.

In the first two studies (paper I and II), both legs performed exercise to concentric torque failure, and the order was randomised (i.e., some subjects started with the BFR leg and

others started with the free-flow leg). In the third study, the BFR leg was always trained first and the free-flow leg repeated the exact same number of repetitions and sets as the BFR leg (i.e., the amount of work was matched between the two conditions).

Outcome measures included number of repetitions completed (papers I-III), EMG (paper II) delayed onset muscle soreness (DOMS) (papers I-III), ratings of perceived exertion (RPE) and ratings of acute pain (papers I-II), maximum voluntary isometric torque capacity (MVC) (paper III), passive tension (paper III), tetranectin antibody staining (immunohistochemistry) on frozen muscle sections (paper III), satellite cell antibody staining (immunohistochemistry) on frozen muscle sections (paper IV) and intracellular signaling determined by western blotting (paper IV).

6.3 Exercise protocols

The exercise protocols in all studies involved dynamic knee extensions with coupled concentric-eccentric muscle actions (i.e., the concentric "lifting" phase was followed by the eccentric "lowering" phase). In the first study (paper I), the loads were between 20-50% of one repetition maximum (1RM) and in study II & III (papers II-IV), the loads were ~30% of 1RM. In the first two studies (paper I and II), each leg performed exercise to concentric torque failure; i.e., the subject could no longer complete the lifting phase through the required range of motion. In the third study, the BFR leg trained to concentric torque failure and the free-flow leg repeated the exact same number of repetitions and sets as the BFR leg (i.e., the amount of work was matched between the two conditions).

Each repetition took approximately 1 second for the concentric phase and 1 second for the eccentric phase in the first study (paper I) and approximately 1,5 second for the concentric phase and 1,5 second for the eccentric phase in the second and third studies (papers II-IV). The tempo was checked with a metronome. The range of motion was between 105°and 5° of knee flexion (0° here defined as full extension) in the first study, and between 100° and 20° of knee flexion in the second and third experiments.

6.4 Monitoring changes in force-capacity, contractility and resting tension

In the third study (paper III), changes in MVC were measured. The subjects performed maximum voluntary isometric contractions in the same knee-extension machine as in the

exercise protocol, but with the lever arm of the machine fixed at an angle corresponding to 90° of knee flexion (full knee extension is here defined as 0°). Isometric force was measured by a strain gauge (HBM U2AC2, Darmstadt, Germany) connected to the lever arm of the knee extension machine, and the signal was amplified and recorded at 1000 Hz using a 16-bit data acquisition card (MP150, BioPac Systems Inc., CA). The back rest was set at a hip joint angle of ~80° of flexion (fully extended hip here defined as 0°), and belts were secured across the subjects hips and shoulders to reduce any movements of the hips, and to minimise assistance from other muscle groups. The subjects were instructed to apply force as rapid and hard as possible and to maintain maximal effort for at least 4 seconds.

Contractility of the vastus medialis was evaluated with neuromuscular electrical stimulation (NMES). For NMES, two self-adhesive electrodes (5 x 10 cm, Polartrode, Medi-Stim, Oslo, Norway) were placed longitudinally on the vastus medialis and the positions of the electrodes were marked on the skin with indelible ink to ensure the same positioning in each trial. The stimulation protocol comprised two trains of stimuli with duration of 200 milliseconds each and with a frequency of 20 Hz, and two trains of stimuli of 200 ms duration and 50 Hz frequency. Each square-wave pulse lasted 0.5 ms, and the voltage was fixed at 120 V. One minute separated the last isometric MVC attempt and the NMES testing, and 5-10 seconds separated each NMES-induced contraction. From the forces generated at 20 and 50 Hz, the ratio between 20 and 50Hz (20/50 Hz ratio) was calculated and used as an index of low frequency fatigue.

While the ankle was strapped to the lever arm in the NMES testing, passive force against the lever arm was also measured as an indirect index of passive muscle tension.

6.5 Muscle biopsy sampling

In the third experiment (papers III & IV), biopsies were obtained from m. vastus lateralis in the non-occluded leg before exercise and from both legs 1, 24 and 48 hours after exercise, and care was taken to ensure that the time points of the post-exercise biopsies were respected, with reference to the end of the training protocol for each leg. The reason for this design was that we wished to minimise potential artifacts resulting from the biopsy procedure itself when assessing the first post-exercise muscle sample from the occluded leg. During the biopsy, subjects laid supine, and the procedure was performed under local anesthesia (Xylocain® adrenaline, 10 $mg \cdot ml^{-1}$ + 5 $\mu g \cdot ml^{-1}$; AstraZeneca, Södertälje, Sweden).

The first muscle sample from each leg was taken from the vastus lateralis approximately midway between the origin and the insertion, from a location which was distal to the part that was compressed by the tourniquet during training (occluded leg). In the non-occluded leg, the first incision was taken from the corresponding location and this incision was also used for the second biopsy (1 hour post-exercise) but the needle was angled in a different direction so that the sample was taken about 5 cm from the first. The second (24 h) and third incisions (48 h) were placed approximately 3 and 6 cm proximally to the first incision. Care was taken to avoid affected tissue from earlier biopsies.

Originally, biopsies were taken from a further three participants, i.e., ten subjects in total, but unfortunately samples were lost for some of the subjects because of a freezer failure during transport, and because of technical difficulties with the biopsy sampling. Complete sets of biopsies (including both limbs and all time points) were achieved in 7 of the subjects for satellite cell numbers. For protein signaling, N = 7 from pre to 24 hours post-exercise, and N = 6 for 48 hours post-exercise. For tetranectin, N=8.

6.6 Tissue processing for protein signaling, immunoblot analysis

Muscle biopsy specimens were freeze-dried, freed of blood and connective tissue, and then homogenized in ice-cold buffer (80 µl/mg dry weight) containing 2 mM HEPES, pH 7.4, 1 mM EDTA, 5 mM EGTA, 10 mM MgCl2, 50 mM β-glycerophosphate, 1% TritonX-100, 1 mM Na3VO4, 2 mM dithiothreitol, 20 µg/ml leupeptin, 50 µg/ml aprotinin, 1% phosphatase inhibitor cocktail (Sigma P-2850), 40 µg/µl PMSF. Homogenates were then centrifuged at 10,000 g for 10 min at 4°C in order to remove cellular debris and the resulting supernatant was stored at -80°C. Protein concentrations were determined in aliquots of supernatant diluted 1:10 in distilled water using a bicinchoninic acid assay (Pierce Biotechnology, Rockford, IL, USA). Samples were diluted in Laemmli sample buffer (Bio-Rad Laboratories, Richmond, CA, USA) and homogenizing buffer to obtain a final protein concentration of 1.5 µg/µl. Following dilution, the samples were heated at 95°C for 5 min to denature proteins present in the supernatant. Samples were then kept at -20°C until further analysis.

Samples containing 30 µg total protein were separated by SDS-PAGE on Criterion cell gradient gels (4-20 % acrylamide; Bio-Rad Laboratories) for separation of all proteins investigated. Electrophoresis was performed on ice at 200 V for 120 min, following which, the gels were equilibrated in transfer buffer (25 mM Tris base, 192 mM glycine, and 10%

methanol) for 30 min in order to optimize transfer. The proteins were then transferred to polyvinylidine fluoride membranes (Bio- Rad Laboratories) at a constant current of 300 mA and 100 V for 3 h at 4ºC, after which the membranes were stained with MemCodeTM Reversible Protein Stain Kit (Pierce Biotechnology) (Antharavally et al., 2004) to confirm equal loading of the samples. All samples were run simultaneously on multiple gels, however, with all samples from each subject loaded on the same gel always beginning with the preexercise sample followed by the free-flow leg and BFR leg samples for each time-point. Membranes were blocked for 1 h at room temperature in Tris-buffered saline (TBS; 20 mM Tris base, 137 mM NaCl, pH 7.6) containing 5% non-fat dry milk. Subsequently, the membranes were incubated overnight with commercially available primary polyclonal phosphospecific antibodies (mTOR Ser2448; p70S6k Thr389; eEF2 Thr56; AMPK Thr172; ERK1/2 Thr202/Tyr204; p38 Thr180/Tyr182; Akt Ser473; p90RSK Thr573; Cell Signaling Technology Inc., Danvers, MA, USA).

For further details regarding the dilution, incubation and visualisation of the primary antibodies, please refer to Paper IV

6.7 Immunohistochemistry

6.7.1 Dystrophin and Tetranectin

The muscle samples were rinsed in physiological saline and visible fat and connective tissue were removed. From one part of the biopsy, 8 µm thick cross sections were cut and later stained with antibodies against dystrophin's COOH-terminus (Dys 2, Novocastra Laboratories Ltd, Newcastle, UK) and diluted in blocking solution [1% BSA, and 0.05% Tween-20 in TBS (TBST)] for 2 h at room temperature or overnight at 4°C. As a marker for sarcolemmal permeability, an antibody against tetranectin was used (Hyb-130-11, AntibodyShop, Gentofte, Denmark). Tetranectin is a plasma protein which like albumin and Evans Blue Dye is a good marker for muscle fiber damage resulting from eccentric exercise (Kalhovde, Paulsen, Raastad, et al., in press). The sections were incubated for 45–60 min with an appropriate secondary antibody and mounted with coverslips (Fluoromount-G, Chemi-Teknik AS, Oslo, Norway, or ProLong Gold Antifade Reagent with DAPI, Invitrogen-Molecular Probes, Eugene, OR). The muscle sections were washed in TBST between each step.

Images were captured using a digital camera (Olympus DP72, Olympus-Europa GmbH, Hamburg, Germany) connected to a light microscope (Olympus BX61) with appropriate filters. Pictures were taken with 4x, 10x, 20x, and 40x magnification objectives, where 4x was used for overview images and 10x was used for evaluations of the number of positive fibers. Intact fibers on each sample were counted, but the outermost layer of muscle fibers in the samples was excluded in order to avoid areas damaged by sectioning, as were other areas and fibers with visible artifacts (folds, tears, etc.). The mean number of counted fibers for each time point per individual was 238 (range 43-882). The program Axiovision (Zeiss, Oberkochen, Germany) was used to analyse the images and the degree of staining for tetranectin was determined with the measure mean green density (MGD). The stainings for the COOH-terminus were inspected to help identification of the borders of the muscle fibers.

6.7.2 Evaluation of tetranectin staining

McNeil & Khakee (1992) and Clarke et al. (1993) used threshold values for determining the number of albumin-positive fibers in the sections. A somewhat similar threshold-based model was adopted for the purposes of the present investigation. The contours of each counted fiber were outlined just beneath the sarcolemma, and the MGD value of the cytoplasm was measured with the Axiovision program. Pilot studies indicated that subjectively determined positive fibers had an intracellular staining (as indicated by MGD) at least 50% stronger than presumably negative fibers. This corresponded to about three standard deviations above the mean of negative fibers in a baseline sample. Therefore, this threshold was chosed for MGD.

Four presumably negative fibers from each section, two peripheral and two centrally located fibers, were chosen based on having the lowest MGD values of the peripheral and central fibers, respectively. Central fibers were within the middle third of the length and the middle third of the width of the picture, and peripheral fibers were consequently located outside these limits. The mean MGD of these four fibers was calculated and fibers with a staining ≥50% stronger than this mean were counted as tetranectin-positive. Values for positive fibers are given as percentage (%) of the total number of counted fibers.

6.7.3 Satellite cells

Serial cross-sections (8 μm) were incubated with antibodies (ab). In order to visualize satellite cells/myoblasts, sections were analyzed for immunoreactivity against CD56/NCAM (monoclonal ab, ab9018, Abcam; 1:200). Alexa-488 (FITC) (goat anti-rabbit or goat anti-mouse; Invitrogen-Molecular Probes, Eugene, Oregon, USA) was used as a secondary antibody. The sections were finally counterstained with DAPI (for nuclear staining) and mounted under coverslips (ProLong Gold Antifade Reagent with DAPI, P36935, Invitrogen-Molecular Probes). Images of the stained cross-sections were captured using an Axiocam camera (Zeiss, Oberkochen, Germany) mounted on a Axioskop-2 light microscope (Zeiss). Multiple images (10x, x20, and x40 objectives) were taken so that the whole muscle biopsy cross-section was captured.

To quantify the number of cells positive for NCAM, a cell that contained both DAPI and NCAM antibody staining and was inside the membrane of the muscle fiber was considered as positive. Specifically, the NCAM marking on a satellite cell is typically seen as a ring around the DAPI staining, and cells with this appearance were counted as SC. No marker for the basal lamina was used, so the contours of each fiber had to be detected via nonspecific staining from the antibodies used. However, no differences are seen in the number of NCAM-positive cells in muscle fibers between this method and measurements performed with laminin (Professor Fawzi Kadi, personal communication to T.S. Nilsen). Data are presented as number of positive cells per 100 myofibers.

6.8 Ratings of DOMS, RPE and acute pain

Delayed onset muscle soreness (DOMS) was self-rated by the subjects pre-exercise and at 24-120 hours postexercise using the visual analog scale (VAS). The 0 value for the VAS was designated as ‘‘no soreness at all,’’ and the maximum value of 10 was ‘‘extreme muscle soreness’’. The subjects were asked at the end of both the occluded and the nonoccluded protocols to use the Borg ratings of perceived exertion (RPE) and CR-10 scales to rate the perceived exertion and acute pain, respectively, in their quadriceps. The 10 on the Borg CR-10 pain scale was anchored as ‘‘the previously worst exercise-induced pain experienced in your quadriceps,’’ and the subjects were allowed to use even higher values (up to 12) if they thought that they had exceeded this during the tests.

6.9 Electromyography (EMG) and goniometry

The skin above the quadriceps of each subject was shaved with a hand razor and carefully cleaned with ethanol before electrode placement. Bipolar surface self-adhesive Ag/AgCl monitoring electrodes (model 2223, 3M Medical, Germany) with a 4.0 cm inter-electrode distance were placed on the medial portion of the vastus lateralis (VL), and the vastus medialis (VM) muscles of the quadriceps femoris muscle group approximately 15 and 10 cm above the proximal border of the patella. These placings were chosen to avoid having the electrodes passing over the innervation zones of the muscles during the exercise movement, while still leaving enough room for the tourniquet.

Knee joint angle was measured with an electrogoniometer, which was positioned laterally to the knee joint. Calibration of the goniometer signal was performed at anatomical knee joint angles of 0 and 90 degrees using a geometric retractor. A linear encoder was used to measure the position and displacement of the weight stack. The goniometer and encoder data were used to identify the concentric and eccentric phases of each repetition.

The EMG and goniometer/encoder data were collected using the MuscleLab 3010e recording and acquisition system (Ergotest Technology, Langesund, Norway), employing the accompanying software program on a lap-top computer. The MuscleLab EMG leads had built in preamplifiers and the pre-amplified signal was filtered through a 8-1200Hz band pass filter before it was root mean square (RMS) converted. The RMS converted signal was then sampled at 100Hz, synchronized with the other devices connected. EMG was normalized to a MVC at 70 degrees of knee flexion, using the normalization function of the MuscleLab unit.

A display with the EMG RMS curve was placed in front of the leg extension machine for monitoring purposes and to provide real time feedback to the subject. Because the normalization value was chosen automatically by the MuscleLab, the first of the two MVC attempts was saved and used as the reference value (100%) if the two attempts did not differ by more than ~5% in RMS amplitude, in which case further attempts were made until a plateau in amplitude was noted.

During the 30% of 1RM endurance tests with and without occlusion, as in the MVC trials, the EMG RMS curve was displayed in real-time in front of the subject for motivation and loud verbal encouragements were also used to motivate the subjects to continue to the point of concentric torque failure. The average RMS-values for complete concentric and eccentric phases, respectively, were used for analysis. The repetitions with the lowest and highest EMG during the concentric and eccentric phases were identified. The first repetition

of each set was discarded, since the subjects generally had not found the proper tempo. During the final two or three repetitions in each set, most subjects also had difficulty following the correct tempo due to the accumulating fatigue, but these repetitions were considered for analysis as long as they were in the stipulated range of motion.

6.10 Blood flow restriction

The same 135 mm cuff (width of inflatable bladder, 100 mm) and automatic tourniquet system (Zimmer ATS 2000) was used in all experiments in this thesis. In Paper I, a pressure of 200 mm Hg was used. This level of pressure in combination with our cuff size has been shown to be more than sufficient to completely occlude blood flow in most subjects when lying in a supine position (Crenshaw et al., 1988).

In separate experiments, we found that this pressure was also sufficient to completely occlude blood flow in the seated position in the vast majority of subjects (Wernbom, Hisdal, Raastad, unpublished results). This does not mean that the blood flow was completely occluded during exercise, because of the rise in mean arterial pressure that accompanies strenuous exercise. However, the present author believes that the restriction was most likely still quite severe, and this was attested by the experiences of many of the subjects. During early pilot studies, 250 mm Hg was also tested during exercise, but this level of pressure was somewhat painful in itself, and it was felt that the pain could influence the results of the endurance tests. Therefore, we settled for 200 mm Hg in the first experiment.

In papers II-IV, pressures of 90-100 mm Hg were used, in order to induce partial but not complete occlusion. Most of the previous literature had used a partial restriction model, where the pressure was applied throughout the exercise bout including rest periods. Notably, the training pressures we used are considerably lower than those that have typically been reported in many of the studies on BFR exercise, where a narrow cuff (33 mm in width) has often been used. With a 33 mm cuff and pressures of 160-180 mm Hg, Takano et al. (2005) reported a ~70% reduction of the femoral blood flow at rest in the seated position. However, it is important to note that wider cuffs are generally more effective in occluding the blood flow than more narrow ones (Crenshaw et al., 1988; Graham et al., 1993).

For example, Crenshaw et al. (1988) determined that with a 45 mm thigh cuff, the pressure needed for complete occlusion of blood flow in the lower limb (measured with the subjects in supine position) was on average ~280 mm Hg, and in some cases more than 400

mm Hg; while with a 120 mm cuff, the pressure needed for full occlusion was ~150 mm Hg. Thus, we decided on a relatively wide cuff and a low pressure during training to minimise compressive and shear forces on soft tissues (Crenshaw et al., 1988; Graham et al., 1993).

It is interesting that when expressed as a percentage, both our present model and the narrow "Kaatsu" model seems to involve training pressures of slightly above 50% of the complete occlusion pressure during resting conditions.

Figure 6.1. Experimental set-up for blood flow restriction during the knee extension exercise with a pressure cuff connected to an automatic tourniquet system (Zimmer ATS 2000). The same cuff (outlined in red in the picture) and tourniquet system was used in all experiments in this thesis. Photo by Jesper Augustsson, RPT PhD. Model: Camille Neeter, RPT PhD.

6.11 Statistics

Of the variables monitored, there were two types of data sets:

1. Muscle function, contractility, soreness, passive tension, tetranectin straining, intracellular signaling, and satellite cells were assessed before and repeatedly after exercise.
2. Data for muscle endurance, RPE, acute pain (CR-10) and EMG were only collected during exercise (BFR leg vs free flow leg).

In paper I, because different loads were used, differences in the number of repetitions between the BFR and the free-flow test conditions were computed by using paired-samples t-tests for each load. For the Borg RPE, Borg CR-10, and DOMS ratings in papers I and II, the Friedman test and the Wilcoxon signed rank test were used to determine differences. For EMG and number of repetitions in paper II, differences between the BFR and the free-flow test conditions were computed with paired t-tests.

In paper III, because the same subjects were evaluated repeatedly for changes in muscle function variables and tetranectin from baseline, one-way repeated-measures ANOVA with Dunnett's post hoc test was performed for both the BFR and the free-flow leg. A two-way ANOVA (with Bonferroni's post hoc test) was used to assess differences between the legs.

In paper IV, one-way repeated-measures ANOVA with Dunnett's post hoc test was performed for both the BFR and the free-flow leg to identify statistically significant changes in protein signaling and satellite cells from baseline. A two-way ANOVA (with Bonferroni's post hoc test) was used to assess differences between the legs.

The level of significance for all statistical analyses was $p \leq 0.05$.

PART 7: Results and discussion

7.1 Blood flow restriction reduces muscle endurance at low exercise loads in the knee extension exercise (papers I and II)

Prior to this study, blood flow restriction with pressure cuffs had been demonstrated to reduce time to fatigue in static exercise in various limb muscles (e.g., Bonde-Petersen et al., 1975) but little was known about the effects of BFR on dynamic resistance exercise. Our results demonstrated that BFR reduced endurance at 20-40% of 1RM in the knee extension exercise, but not at 50% of 1RM. The knee extension exercise was performed in a conventional variable resistance knee extension machine, and with both concentric and eccentric muscle actions. The occlusion pressure was set at 200 mm Hg (see section 6.10).

An important detail in the interpretation of the results is that the exercise was performed without relaxation between the repetitions; i.e., the muscle was constantly under tension during the whole set. The rationale for performing the exercise in this manner was to eliminate the hyperemic blood flow that occurs primarily when the muscle relaxes (Walløe & Wesche, 1988), in order to investigate the effects of BFR during the actual exercise as opposed to studying mainly the effects of restricting the blood flow in the recovery periods between repetitions. In this way, we could also compare our results on the effects of BFR during dynamic exercise with the previous literature on BFR in static/isometric exercise.

Interestingly, our finding of no difference in endurance with versus without BFR at 50% of 1RM corresponds quite well with data showing no effects of BFR on static exercise endurance at 50% of MVC in the quadriceps (Hisaeda et al., 2001), although it should be noted that the muscle tension at 50% of 1RM is somewhat lower than at 50% of MVC.

The mean intramuscular pressure (IMP) in the quadriceps is sufficiently high at forces ≥50% of MVC to severely limit or completely occlude the circulation in the rectus femoris and vastus lateralis (Sadamoto et al., 1983; Sjøgaard et al., 1986), which together with the metabolic demands result in relative ischemia in the working muscle. The cessation of blood flow occurs when IMP exceeds the mean arterial pressure, thus resulting in no perfusion pressure (Sjøgaard et al., 1988). At 100% MVC, mean IMP in excess of 200 mm Hg have been reported in the rectus femoris, the vastus medialis and the vastus lateralis (Sadamoto et al., 1983; Sejersted et al., 1984; Sjøgaard et al., 1986), and peak values of up to 600 mm Hg have been noted (Sejersted & Hargens, 1995). Generally, the IMP increases with the depth in

the muscle at which the IMP is recorded, so that the highest levels are recorded deep in the muscle (Sadamoto et al., 1983; Sejersted et al., 1984; Sjøgaard et al., 1986).

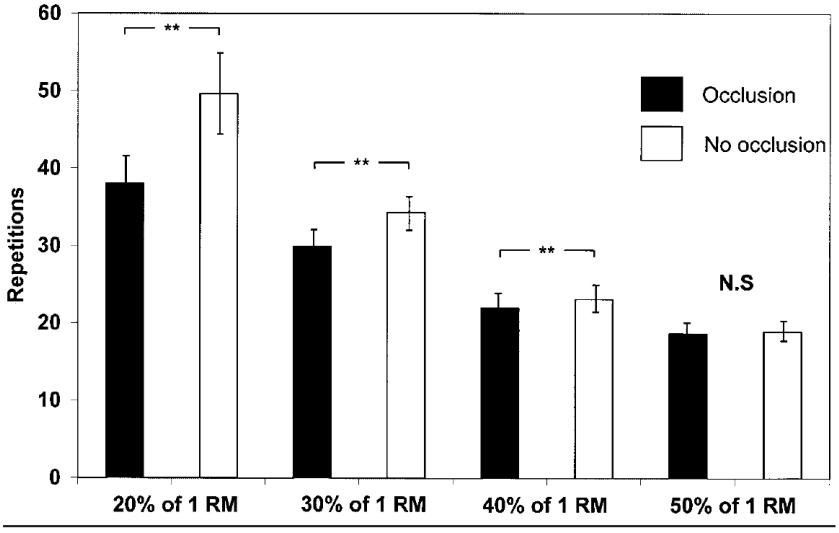


Figure 7.1. Effects of BFR (200 mm Hg) on the number of repetitions performed in the knee extension exercise in the 1st set at different loads. Figure from Paper I, reprinted with permission.

However, reactive hyperemia, a sign of insufficient blood flow, is seen in the quadriceps already after static exercise at 15% of MVC (Sjøgaard et al., 1986) and after continuous dynamic (concentric-eccentric) knee extensions at 10% of MVC (Shoemaker et al., 1994). Further evidence that relative ischemia can occur at surprisingly low intensities was provided by de Ruiter et al. (2007), who showed that the oxygenation level in the vastus lateralis and medialis during isometric knee extensions reached values similar to total cuff occlusion already at torques of ~25% of MVC. In this context, one should keep in mind that the pressures in the smaller blood vessels are considerably lower than in the large arteries (Guyton & Hall, 2000).

Collectively, these observations may well explain why we found only minor differences in endurance between knee extensions performed with severe BFR at 200 mm Hg versus without occlusion at 40% and even at 30% of 1RM (refer to Figure 7.1 above). In fact, even 20% of 1RM was associated with sensations of exercise-induced ischemia in the free-flow leg. Pilot experiments indicated that the blood flow in the femoral artery almost ceased during

the concentric phase in dynamic knee extensions at 35-40% of 1RM, while during the eccentric phase there was still some blood flow (Wernbom, unpublished observations).

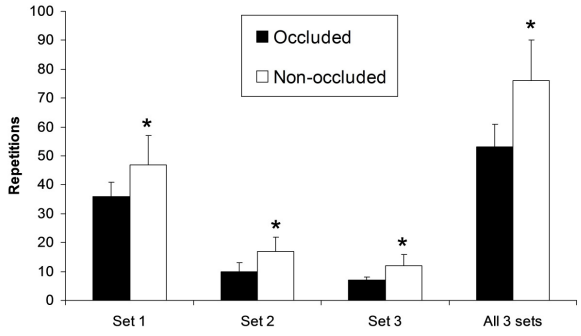


Figure 7.2. Effects of continuous partial blood flow restriction (occlusion pressure 100 mm Hg) on the number of repetitions performed in the knee extension exercise in sets #1-3 and in total at 30% of 1RM. Figure from Paper II, reprinted with permission.

7.2 Fatiguing low-load resistance exercise both with and without BFR induces DOMS (papers I-III)

With paper I, we were the first to report that low-intensity resistance exercise combined with blood flow restriction with pressure cuffs can induce DOMS. A caveat was that the training model in this paper was one with intermittent occlusion, while much of the literature related to training effects has been conducted with continuous partial BFR training models. However, our finding of DOMS with low-load BFR resistance exercise was confirmed in papers II and III, with a continuous partial BFR model, and other studies and case reports have also reported DOMS with BFR training (Umbel et al., 2009; Iversen & Røstad, 2010).

In hindsight, the occurrence of DOMS should not be very surprising, as ischemia and hypoxia are well known risk factors for muscle damage and rhabdomyolysis. The occurrence of DOMS in the free-flow leg in papers I-III was likely caused in part by the effects of temporary exercise-induced ischemia, as outline above in section 7.1.

7.3 Low-load resistance exercise to failure both with and without BFR causes marked increases in muscle activity during concentric and eccentric muscle actions (paper II)

Based on the remarkably similar sensations of acute pain in the free-flow leg vs the BFR leg, and also on the occurence of DOMS in both conditions when the exercise was performed without relaxation of the muscle between the repetitions, we were interested to study muscle activity during the exercise. More specifically, we hypothesised that similarly high levels of muscle activation could be induced in the free-flow leg in the knee extension exercise when performed in a "no-relaxation" manner. Previous studies had not controlled for the degree of effort, as they had matched the protocols with and without BFR for the amount of work performed.

The results demonstrated that not only could the "no-relaxation" technique induce similar levels of EMG activity as BFR training, it even tended to result in slightly higher muscle activity, which was significant during the eccentric phase in the last set (refer to table 2 in paper II). This has potential implications for training without BFR at low loads, and these will be discussed later in perspectives in Part 9.

Nevertheless, training to concentric torque failure both with and without BFR resulted in high levels of EMG, although predictably more repetitions were required to reach concentric torque failure without BFR. Moreover, our results show a significant eccentric component with low-load BFR resistance exercise (reaching values up to ~50% of MVC with the last repetitions in each set). The significance of this finding remains to be determined, but it could be speculated that this increase in muscle activity also during the eccentric phase contributes to both the DOMS observed and the overall training effect.

7.4 Multiple sets of low-load resistance exercise to failure with BFR cause both acute and longer-lasting decrements in muscle force generating capacity (paper III)

Based on the observations in papers I & II, we wanted to see if the DOMS with BFR resistance exercise was accompanied by reductions in muscle torque as measured by MVC. Preliminary results for changes in MVC in experiment III were reported at the 6th International Conference of Strength Training in Colorado in late 2008. However, before all data had been collected and treated statistically, a paper by Umbel et al. (2009) appeared which demonstrated that BFR resistance exercise at 35% of MVC resulted in both DOMS and decrements in MVC, the latter lasting up to 48 hours post-exercise. The results of paper III are

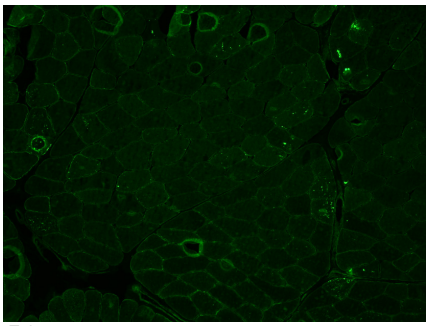
in agreement with those of Umbel et al. (2009), although the magnitude of MVC decrement seems slightly larger and the time-course for recovery somewhat longer in our study.

The results for the acute drop in MVC in the minutes immediately after exercise (while the partial BFR was still maintained) are in agreement with the findings of Yasuda et al. (2008, 2009). Possible mechanisms are discussed in paper III.

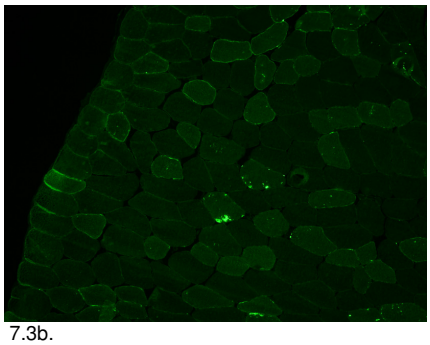
7.5 Multiple sets of low-load resistance exercise to failure with BFR induce signs of increased sarcolemmal permeability and damage at the muscle fiber level (paper III)

Because of the DOMS and decrements in MVC, it seemed reasonable to expect some degree of damage at the muscle fiber level with BFR resistance exercise. At first, we studied antibody staining (by immunohistochemistry) against the COOH-terminal of dystrophin, which has been shown to be affected in eccentric exercise-induced muscle damage (Lovering & De Deyne, 2004). Loss of the COOH-terminal has been linked to sarcolemmal damage (e.g., Lovering & De Deyne, 2004). Our preliminary results with the COOH-antibody staining suggested some interesting changes, but the results were inconclusive; thus the decision was taken to abandon the analysis of COOH-terminal stainings and look for other markers which might be more promising.

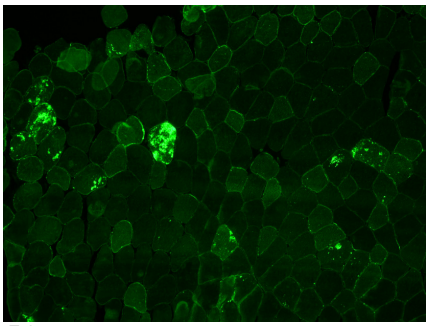
In other projects at the Department of Physical Performance at the NSSS, the muscle damage resulting from maximum eccentric exercise in humans has been extensively studied. In one of these projects, a new marker for muscle fiber damage was tested, the protein tetranectin. In this experiment (Kalhovde et al., in press), high-force eccentric exercise resulted in a large increase in the staining for tetranectin in the myofibrils of the affected muscle fibers. Because tetranectin is a plasma protein like albumin and not normally present in any significant amounts in adult skeletal muscle (see discussion below), this finding suggests altered sarcolemmal permeability, perhaps as a result of damage, allowing influx of tetranectin. Furthermore, tetranectin appears to accumulate especially in areas of myofibrillar disruptions, resulting in a granular-like staining pattern in fibers with myofibrillar damage (Kalhovde et al., in press).Tetranectin, like albumin, may therefore be a useful marker for sarcolemmal permeability, and in addition for myofibrillar disruptions. Some examples of tetranectin staining from experiment III are shown below.



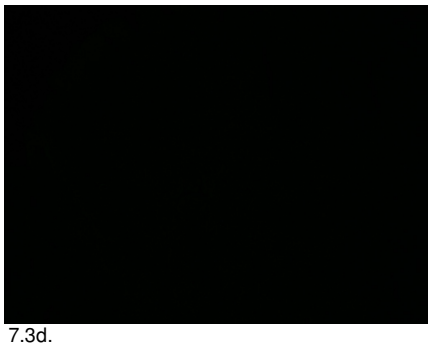
7.3a.



7.3b.

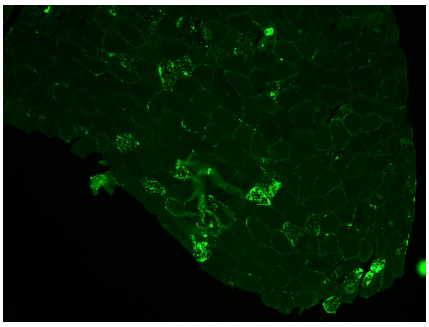


7.3c.



7.3d.

Figure 7.3a. Tetranectin staining, in one subject pre-exercise.
Figure 7.3b. Tetranectin staining in the same subject, 1h post-exercise in the BFR leg. Note the diffuse increase in staining, and occasional granular stainings (e.g., two fibers in the middle of the picture).
Figure 7.3c. Tetranectin staining in the same subject, 48h post-exercise in the BFR leg. Note the diffuse increase in staining, and some fibers with strong granular stainings (bright green).
Figure 7.3d. Secondary antibody staining in the same subject, 48h post-exercise in the BFR leg.



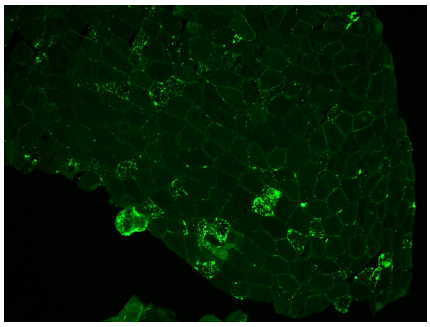


Figure 7.4a (left). Tetranectin staining in one subject,1h post-exercise in the BFR leg.

Figure 7.4b (right). Tetranectin staining in the same subject, muscle section close to the one in the first picture. Note that many of the same fibers are positive in both pictures.

Physiological significance of tetranectin

Tetranectin, originally believed to be a tetramer (hence the number prefix tetra), has been found in human plasma as a homo-trimer, each monomer with a mass of ~20 kDa (Holtet et al., 1997; Thougaard et al., 1998). The molecular weight of tetranectin in humans was reported to be approximately 68 kDa (Clemmensen et al., 1986), making it slightly heavier than albumin (67 kDa). On western blotting, however, human serum tetranectin has been detected as a ~25 kDa band (Wever et al., 1998; Hittel et al., 2003). This may be due to the fact that the three peptide chains of tetranectin are non-covalently bound, thus these bonds may be weak and easily broken, resulting in monomer forms of tetranectin. In addition, the molecular weight of 25 kDa instead of ~20 kDa could be the result of post-translational modifications.

While human skeletal muscle contains mRNA for tetranectin, it does not normally accumulate in detectable amounts in adult human muscle (Albrechtsen & Wewer, unpublished, cited in Xu et al., 2001). However, expression of tetranectin is stimulated and immunostaining has been observed during differentiation as well as regeneration of skeletal muscle after injury (Wewer et al., 1998). These authors suggested a role for tetranectin in wound healing, via interactions with extracellular matrix (ECM) proteins and components. A role in wound healing is supported by Iba et al. (2009) who demonstrated impaired cutaneous wound healing in mice lacking tetranectin. In a previous study, Iba et al. (2001) observed spinal deformities in mice with deletion of tetranectin; and in advanced cases, the growth plates were disorganized and irregular. Although Iba et al. (2001) did not find any apparent muscle pathology in these mice, a role for tetranectin in wound healing, remodelling and adaptation also in skeletal muscle cannot be excluded.

Interestingly, we observed increased heat shock protein (HSP) staining in the same fibers that displayed elevated tetranectin staining (data not shown), and we are currently investigating the HSP response to BFR exercise. Apart from the manuscript of Kalhovde et al. (in press), the present author is aware of only one other study (as yet unpublished) that has convincingly shown the presence of an extracellular protein like tetranectin and albumin inside muscle fibers after damaging exercise (Yu & Thornell, 2003, manuscript in Yu, 2003).

Yu & Thornell (2003) reported an increased staining of the plasma protein fibrinogen, a 340 kDa molecule, inside some muscle fibers from subjects that had run downstairs multiple times from the 10^{th} floor in a building. This mode of exercise is well-known to result in DOMS in the soleus in subjects who are not used to eccentric exercise (Yu, 2003). Judging

from their pictures, the fibrinogen staining in the paper of Yu & Thornell (2003) appeared to be mostly subsarcolemmal, but they reported that the staining varied widely, with some fibers having almost half of their cross-sectional area occupied by fibrinogen.

Considerable variability in the pattern of staining was found in the present study (Paper III) as well, varying from diffuse staining covering the whole cytoplasm to very strong granular staining; where the latter could be both focal and in some fibers cover most of the cytoplasm. Thus, the tetranectin stainings in our study had a different appearance from the fibrinogen staining of Yu & Thornell (2003). It could be speculated that this was due to the differences in molecular weight (68 kDa vs 340 kDa) and/or different functions and fates inside the muscle fibers.

Possible causes of muscle increased sarcolemmal permeability with BFR training

It is postulated that the combination of ischemic conditions and exercise may be a major cause of sarcolemmal permeability and/or damage with BFR exercise, as anoxia in itself can lead to Ca^{2+} influx and subsequent damage to the muscle fibers (Fredsted et al., 2005). Importantly, muscle activation during anoxia shortens the time required for increased Ca^{2+} influx to take place (Fredsted et al., 2005). Also, reoxygenation after anoxia does not readily reverse the Ca^{2+} accumulation and may even temporarily cause further Ca^{2+} influx and damage to the sarcolemma (Fredsted et al., 2005), perhaps due to reactive oxygen species (ROS), as both ischemia and reperfusion are associated with increased levels of ROS (Zuo & Clanton, 2005).

A possible mediator of sarcolemma damage is phospholipase A2 (PLA2), which plays a crucial role in triggering membrane leakiness in skeletal muscle (Duncan & Jackson, 1987; Ørtenblad et al., 2003). Duncan & Jackson (1987) showed that inhibition of PLA2, or lipooxygenase (LOX) downstream of arachidonic acid (AA) in the PLA2-AA-LOX pathway, attenuated damage to the sarcolemma, but not the myofibrillar breakdown consequent to increased influx of extracellular Ca^{2+}. They concluded that there were at least two pathways in damage resulting from excessive Ca^{2+}; one which damaged the sarcolemma and one which caused the destruction of the myofilament apparatus.

Isoforms of PLA2 in skeletal muscle are activated by several stimuli, including ischemia, exercise, stretch and cell swelling (Ørtenblad et al., 2003; Burkholder, 2009). LOX enzymes are major sources of ROS in skeletal muscle fibers (Zuo et al., 2004), which may in part explain the damaging effects of the PLA2-AA-LOX pathway on the sarcolemma. PLA2 also liberates lysophopholipids such as lysophosphatidylcholine (LPC) (Ørtenblad et al.,

2003; Burkholder, 2009). Both the AA-LOX and the LPC routes lead to taurine efflux, and higher levels of LPC cause release of CK from myocytes (Ørtenblad et al., 2003). Thus, both the AA-LOX and the LPC arms of the PLA2 pathway may cause damage to the sarcolemma.

Increased muscle levels of AA and prostaglandins have been noted in studies on cats subjected to electrically induced isometric exercise and ischemia, and greater increases were seen after a combination than after either intervention alone (Rotto et al., 1989; Symons et al., 1991). Of particular note, these changes were observed within a few minutes after the start of the combined ischemia and exercise period, strongly indicating that PLA2 in skeletal muscle can be activated even by relatively brief ischemic exercise. Moreover, low-intensity contractions during ischemic conditions induced elevations in intramuscular levels of prostaglandins, but failed to do so during free-flow conditions (Symons et al., 1991).

Interestingly, Umbel et al. (2009) reported DOMS, muscle swelling (measured by ultrasound) and decreased muscle torque after a bout of pure concentric knee extensions with BFR (3 sets to failure at 35% of MVC). This suggests that ischemia markedly increases the susceptibility of the working muscle to a point where normally not damaging exercise may become damaging, perhaps via activation of PLA2 and downstream pathways.

Notably, the tetranectin uptake appeared to be most pronounced in type I fibers (Figure 6 in paper III). The type I muscle fibers are presumably recruited already from the start, while the type II fibers (first type IIa, and later type IIx) would primarily be recruited when the type I fibers have already been fatigued (Wernbom et al. 2009). As a result, the type I fibers would be subjected to longer durations of stress than the type II fibers. Another factor may be the eccentric muscle actions that are inherent in most BFR strength training regimes (see paper II and also Part 9). As evidenced by the DOMS, the decline in MVC and the increased tetranectin staining, some damage may also have occurred in the non-occluded leg. It is likely that ischemic conditions were temporarily present also in this limb due to the exercise itself.

7.6 Multiple sets of low-load resistance exercise to failure with BFR induce both acute and longer-lasting increases in hypertrophic signaling (paper IV)

There were several notable findings in this paper: firstly, there was a prolonged increase (up to at least 24 hours) in the phosphorylation of p70S6K at Thr389 in the occluded leg. Interstingly, phospho-p70S6K was elevated also in the free-flow leg at 24h, but not at 1h. Secondly, the phosphorylations of Akt, mTOR, ERK1/2 and p90RSK, kinases that have a positive influence on p70S6K, did not change along with the p-p70S6K. On the other hand,

the phosphorylation of p38, another kinase that can positively impact on the mTOR-p70S6K pathway, increased at 1h in the BFR leg but not at the later time points. Thirdly, the phosphorylation of AMPK did not increase, if anything it tended to decrease with the BFR protocol.

The increase in the phosphorylation of p70S6K at Thr389 in the occluded leg (Figure 7.5.) was not surprising given the results of previous studies. The novel finding in the current investigation is that the possible duration of the elevated p-p70S6K (Thr389) with BFR training is extended to at least 24 hours, when studied in the fed state. We do not rule out that an even longer elevation is possible, especially given that we did not investigate time points between 24 and 48 hours. As p-p70S6K (Thr389) is a validated predictor for muscle protein synthesis (Kumar et al., 2009, Burd et al., 2010a) and exercise-induced hypertrophy (Baar & Esser, 1999, Terzis et al., 2008), our observations may help explain why BFR resistance training at 20-50% of 1RM can be effective for hypertrophy even with only 2 sessions per week (Takarada et al., 2002, 2004).

That p-p70S6K was elevated also in the free-flow leg at 24h, but not at all at 1h was a surprising finding. We expected just the opposite, i.e., that some increase would be seen at 1h but not at 24h, due to the fact that the training in the free-flow leg was perceived as quite strenuous (especially during the first two sets) but not to the extent as with the BFR leg. Because of the cross-transfer effects demonstrated by Madarame et al. (2008), it may be that the local training was not the only factor at work, but that a systemic response was involved as well. Since we did not collect blood samples, we do not have information pointing to any systemic effector(s) which may have interacted with the training itself. Nevertheless, possible candidates include, but are not limited to, catecholamines (e.g., noradrenaline), growth hormone and IGF-1 (see Wernbom et al., 2008, for further discussion).

However, even if an interaction between local training effects and systemic factors took place in the present study, one may ask why a p70S6K (Thr 389) response was not seen at 1h post-exercise in the free-flow leg. Some insight may be gained from a study by Spiering et al. (2008), who demonstrated that when heavy knee extensions (5RM load) were preceded by an upper-body strength protocol inducing marked elevations in circulating hormones, the p-p70S6K (Thr389) in the vastus lateralis was not increased at 3 hours post-exercise. When the leg training was preceeded by rest, however, p-p70S6K (Thr389) increased. These authors speculated that the cortisol response may have inhibited p-p70S6K in the high-hormone condition. Indeed, the inhibitory effect of glucocorticoids on muscle protein synthesis is associated with dephosphorylation of p70S6K (Shah et al., 2000).

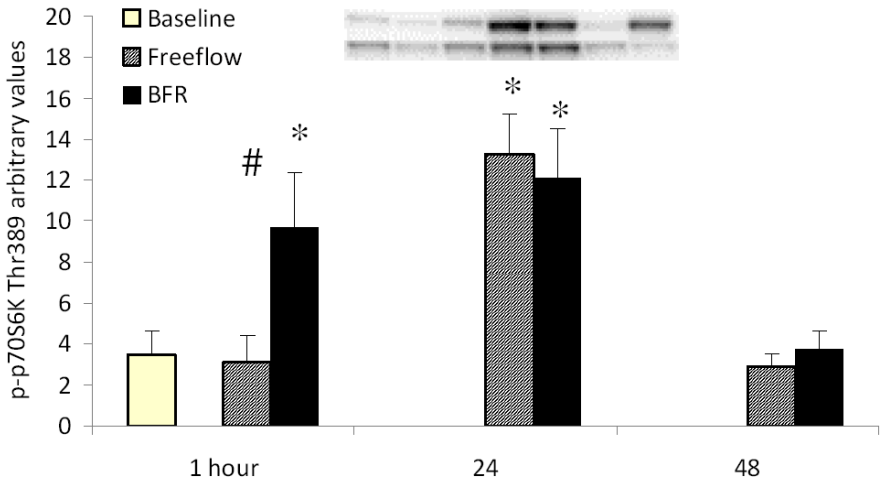


Figure 7.5. Increase in phosphorylation of p70S6K at Thr389 in the BFR resistance trained leg at 1h, and in both the BFR and the free-flow leg at 24h. * = significantly different from baseline ($p<0.05$). # = significantly different from free-flow leg ($p<0.05$). Two examples of the western blots are shown.

Notably, strenuous BFR exercise can be associated with acutely elevated cortisol levels (Fujita et al., 2007), even if performed in a unilateral manner (Viru et al., 1998). One could therefore speculate that a cortisol response triggered by the very strenuous training of the BFR leg could have had an inhibiting effect on subsequent early exercise-induced p70S6K signaling in the free-flow leg, which was later released and reversed.

Alternatively, the response in the BFR leg was simply faster to manifest itself. For example, maximum eccentric exercise was shown to cause an earlier rise in muscle protein synthesis than maximum concentric exercise, even when matched for the training volume (Moore et al., 2005). As discussed earlier, increased muscle protein breakdown may cause the intracellular concentration of the amino acid leucine to rise, which in turn triggers activation of VPS34 and mTOR. It could thus be that the BFR leg experienced more damage and/or greater transport of leucine across the sarcolemma, resulting in an earlier stimulation of the mTOR-p70S6K pathway. The damage may also have been associated with increased p70S6K via other processes than myofibrillar breakdown. In any case, the interactions between local and systemic responses, and exercise order, are areas worthy of further research.

As for kinases and pathways leading to the p70S6K responses in this study, there are several possible candidates, including many which we did not investigate. The ERK1/2

pathway may have been involved in the 1h response, but this seems unlikely given that it was back to baseline (or even slightly below) at this time point, although we may have missed a transient early activation of ERK1/2. The possible involvement of ERK1/2 in the 24h response also seems questionable, because if anything, p-ERK1/2 tended to be below the pre-exercise values also at this time point. Again, it must be emphasised that there may be a redundancy between the MAPK pathways.

The early increase in the phosphorylation of p38 to BFR resistance exercise is a novel and interesting finding, as p38 is involved in differentiation and MHCII synthesis, and also in translation (i.e., protein synthesis) via MNKs and MK2. The early response of p38 could also play a part in the early p70S6K response via mTOR. The relation between p38 and p70S6K is not necessarily linear, however, making it difficult to determine if p38 is indeed involved. Regarding the increased phosphorylation of p70S6K at 24 hours, a role for p38 seems unlikely, as p38 was back to baseline by then. None of our investigated kinases seemed to be able explain the 24 hour increase in p-p70S6K.

We did not investigate JNKs. Other kinases and pathways of interest with regard to mTOR-p70S6K signaling include, but are not limited to, protein kinase A (PKA), the protein kinase C (PKC) family, MAP4K3, VPS34, CaMKII isoforms and splice variants, and the pathways leading to increased PA production such as PLD1, PLD2, and isoforms of LPAAT and DGK.

With regard to AMPK, a trend was noted, where the phosphorylation at Thr172 tended to decrease at 24h and 48h in the BFR leg. As AMPK is a negative regulator of hypertrophy and related signaling, this is an interesting observation, and the possible significance of this remains to be determined.

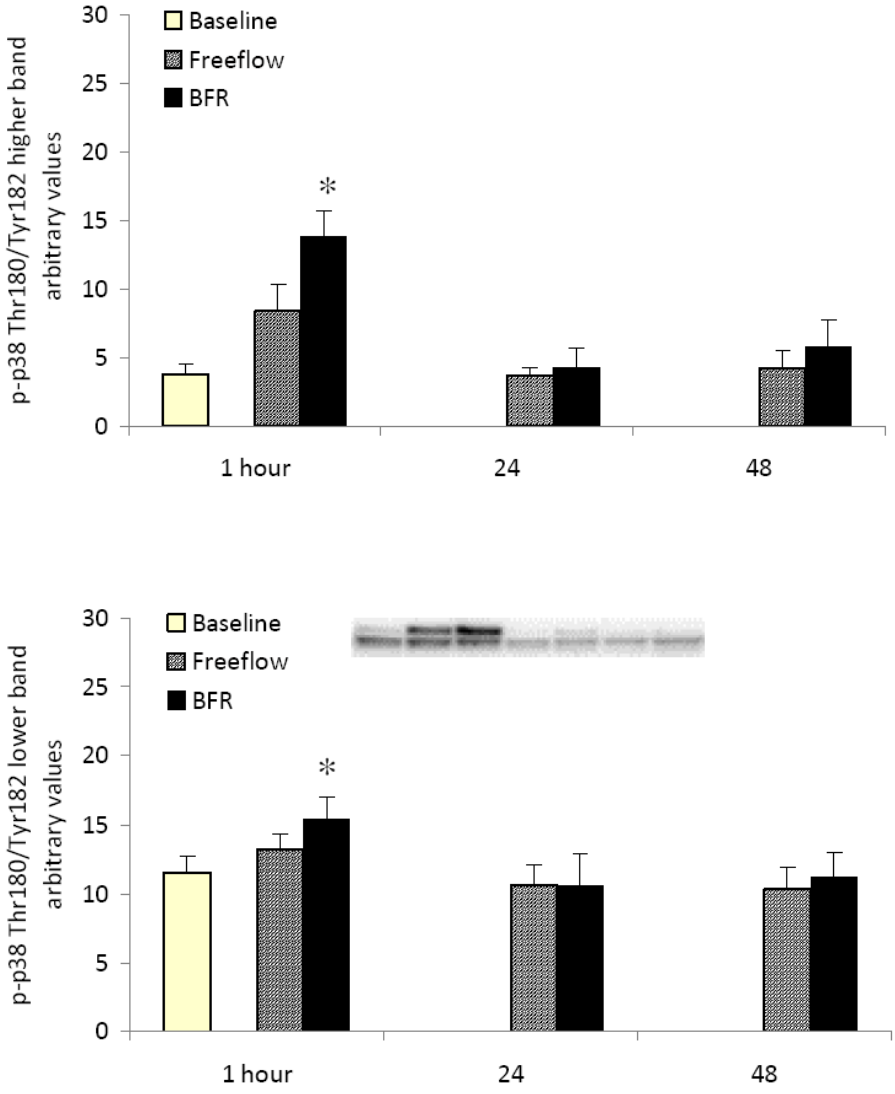


Figure 7.6. Levels of phosphorylated p38 at Thr180/Tyr182 in the BFR resistance exercised leg and the free-flow leg at 1h, 24 hrs and 48 hrs after exercise. Upper panel; upper band, presumably p38gamma, lower panel; lower band, presumably p38alpha. .* = significantly different from baseline levels.

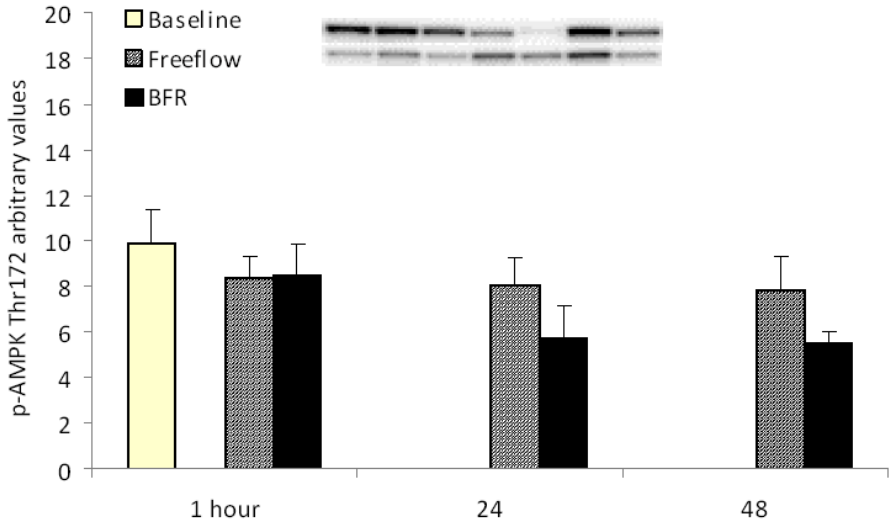


Figure 7.7. Trend towards a decrease in phosphorylation of AMPK at Thr172 in the BFR resistance trained leg at 24h and at 48h post-exercise. Two examples of the western blots are shown.

7.7 Multiple sets of fatiguing low-load resistance exercise both with and without BFR results in rapid increases in satellite cell number per muscle fiber (paper IV)

The 33-53% increase in the number of satellite cells (SC) per muscle fiber post-exercise is remarkable, and quite large in comparison to some of the previous studies. For example, Mackey et al. (2010) reported increases of 25% and 18% in SC number per fiber after 12 weeks of heavy strength training and very light resistance exercise, respectively. However, it is now well established that satellite cell numbers can increase within 24-48 hours in response to an acute bout of maximum eccentric exercise (Dreyer et al., 2006; Mikkelsen et al., 2009; Paulsen et al., 2010) and electrical stimulation (Mackey et al., 2009).

In the study of Dreyer et al. (2006), SC number was elevated by 140% at 24h post-exercise in young men, and in the paper by Paulsen et al. (2010), the SC number was ~73% higher than in the control muscle, averaged over all time points after exercise. Similarly, Mikkelsen et al. (2009) reported a 96% increase in SC number 8 days after eccentric exercise. Thus, it seems that some forms of exercise can indeed induce large increases in SC number. Judging from this study and the abstracts of Nielsen et al. (2011), low-load BFR resistance exercise is a powerful stimulus for increases in satellite cell numbers.

An even more remarkable finding with our current study is the ~33-36% elevation in SC number already 1 hour after exercise. To our knowledge, such an early increase has not been reported in human subjects after a single bout of exercise. We believe that this early increase was not due to an actual elevation in cell number, but an effect caused by the elongation and the swelling of the activated cells. In support of this notion, SCs have been shown to become enlarged with activation already within 10 minutes after injury (Anderson, 2000). The increase took place in both the BFR and the free-flow legs. The reasons for this activation are not clear, but as discussed earlier, even the free-flow leg can be considered to have been "ischemic" due to the no-relaxation manner of the exercise, and some degree of damage appeared to be present in this leg as well (Paper III).

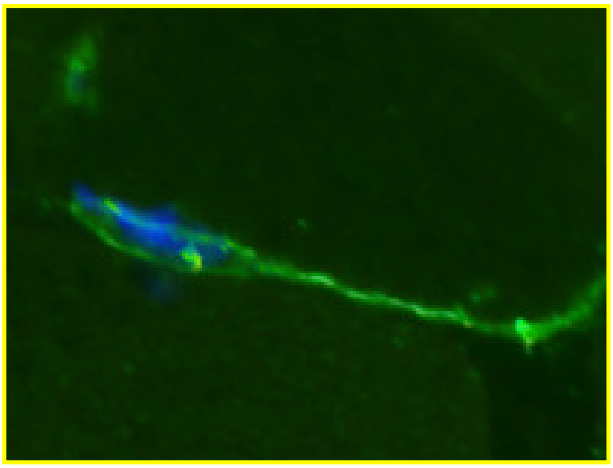


Figure 7.8. Satellite cell with extension, a sign of early activation.

In an electron microscopy study on a primate model of ischemia-reperfusion in skeletal muscle, Gregory & Mars (2004) reported that the quiescent SCs that were found in the pre-ischemia samples had a length of about 6.5 µm, while the length in early activated SCs at 6 hours after tourniquet-induced ischemia (3 hours) was about 10-15 µm (i.e., the length was doubled). Furthermore, they reported a doubling in the cross-sectional area of early activated SCs, as well as reorientation of some SCs to the long axis of the muscle fibers. All these effects would increase the likelihood of detecting SCs in immunohistochemistry sections. In addition, it should be mentioned that NCAM stains a greater part of the satellite cell than Pax7, which is confined to the nucleus. Mackey et al. (2009) reported that NCAM-stained

SCs could be followed through four or five 7 µm sections, while Pax-7-labelled SCs could not be followed through more than two sections.

A very early increase in SCs has been observed also with maximum eccentric exercise (Paulsen et al., unpublished data). Regarding the later increases in SC number in the current study, we presume that these were at least in part caused by proliferation.

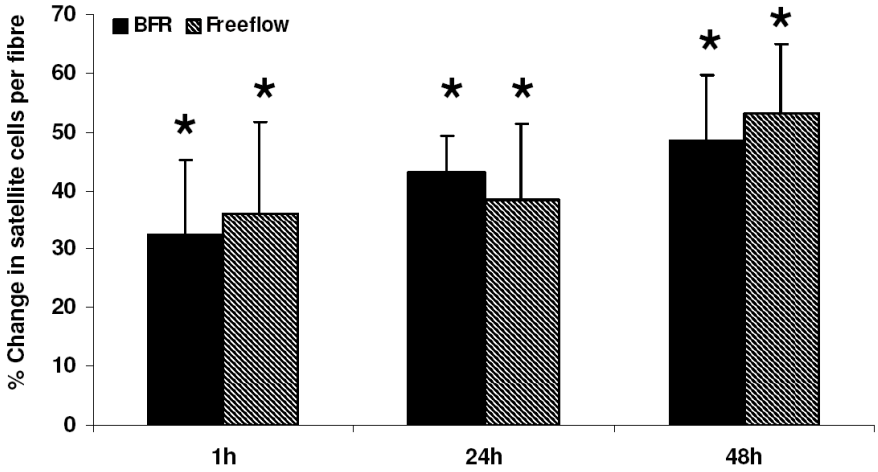


Figure 7.9. Satellite cell numbers per muscle fiber at post-exercise, percentage change in relation to pre-exercise. Note the increase already at 1h, a sign of early activation (see text). .* = significantly different from baseline levels ($p<0.05$).

PART 8: Summary and Conclusions

8.1 Main findings:

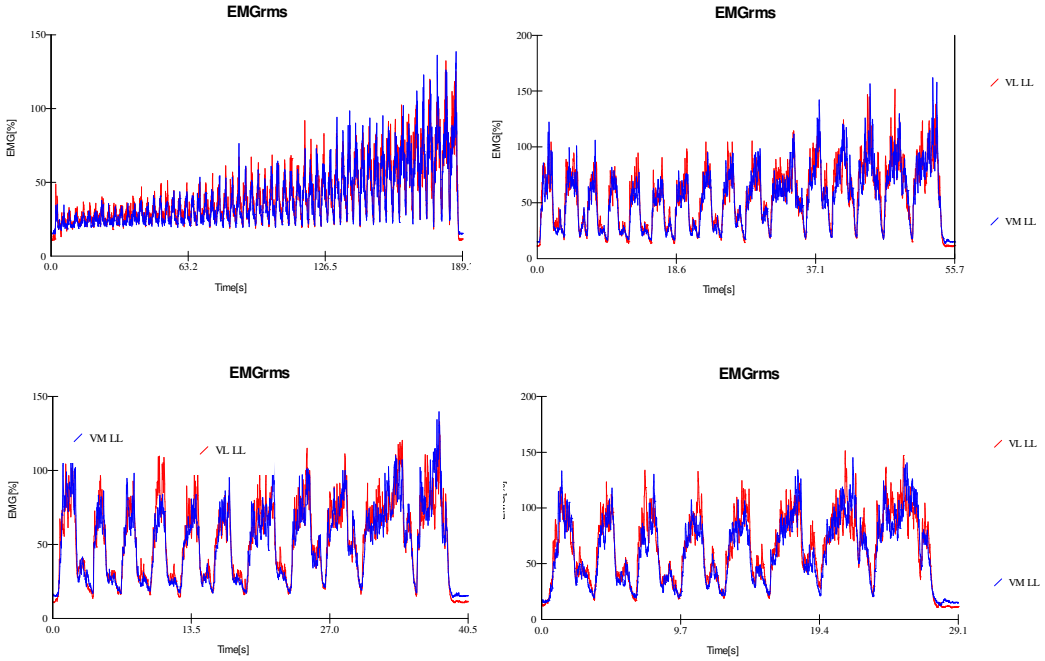
- Blood flow restriction reduces muscle endurance at low exercise loads (20-40% of 1RM) but not necessarily at moderate to higher loads (≥50% of 1RM) in the knee extension exercise. The intramuscular pressures in the quadriceps are probably quite high already at low-to-moderate loads in this exercise, sufficient to induce relative ischemia as long as the muscle is under tension.
- Fatiguing low-load dynamic knee extension exercise both with and without blood flow restriction induces DOMS.
- Fatiguing low-load dynamic knee extension exercise both with and without blood flow restriction induces high muscle activity in the quadriceps. Not only the concentric but also the eccentric muscle activity increases as the point of torque failure draws closer. Very likely, this indicates a high degree of muscle fiber recruitment, possibly also during eccentric muscle actions.
- Multiple sets of low-load resistance exercise to failure with BFR induce decrements in maximum torque capacity and signs of increased sarcolemmal permeability and damage at the muscle fiber level. These phenomena were also observed in the free-flow leg, but to a lesser degree.
- Multiple sets of low-load resistance exercise to failure with BFR appear to induce long-lasting (≥24 hours) increases in hypertrophic signaling, and rapid increases in satellite cell numbers. These phenomena were also observed in the free-flow leg, but to a somewhat lesser degree with regard to signaling.

PART 9: Perspectives, speculations, and suggestions for further research

9.1 Muscle activity, a key factor in low load BFR resistance training?

Our findings in Paper II are in line with previous data suggesting both type I and type II muscle fiber use even with very low load BFR exercise (see section 3.2.1). Some examples from unpublished experiments by the author on EMG during BFR resistance exercise are shown below. As can be seen, high levels of EMG can be reached with as little resistance as 20-25% of 1RM, approaching levels seen during conventional heavy resistance exercise.

Interestingly, type I fibers appear to be very sensitive to blood flow occlusion and their rate of glycolysis increases markedly during ischemic exercise, whereas the increase in glycolysis for type II is only marginal with ischemia compared with normal conditions (Greenhaff et al., 1993). With a decline in force in type I fibers, more type II fibers would have to be recruited for the work to continue. It is therefore possible that the remaining force-producing fibers could be exposed to relatively high tensions near the end of the sets, at least in relation to the nominal load. In addition, there may be a significant eccentric component in BFR resistance exercise (see Paper II, and also figures 9.1a-d).



Figures 9.1a-d. Muscle activity (measured by EMG) during 4 sets to failure of low-load dynamic knee extensions (20-25% of 1RM) with continuous partial BFR (100 mm Hg). Rest between sets: 45 sec. 1st set = 61 repetitions. 2nd set = 16 repetitions, 3rd set = 11 repetitions to failure, 4th set = 8 repetitions. EMG normalised to MVC (100%). VL LL = vastus lateralis, left leg. VM LL vastus medialis, left leg.

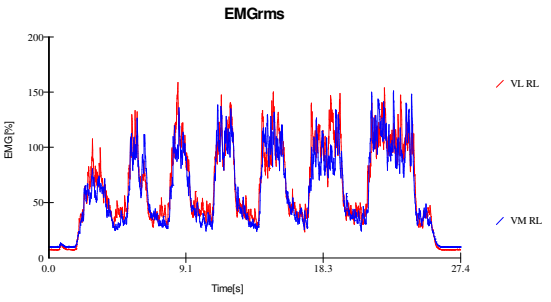


Figure 9.2. EMG during heavy resistance training with dynamic knee extensions (80-85% of 1RM), one set to failure (7 repetitions). Same subject (MW) as in previous figures. EMG normalised to MVC (100%). VL RL = vastus lateralis, right leg. VM RL vastus medialis, right leg.

9.2. Mechanotransduction, growth factors, and other local stimuli in BFR training

The possible roles of mechanotransduction, growth factors and hormones were briefly mentioned earlier. In addition, changes in blood flow (Kraemer & Ratamess, 2004), heat stress (Goto et al., 2003), reactive oxygen species (ROS) (Takarada et al., 2000a), hypoxia–hyperoxia (Kawada, 2005), and nitric oxide (NO) (Kawada & Ishii, 2005) have all been suggested as possible stimuli in BFR exercise. A few of these factors will be discussed below.

Nitric oxide (NO)

Blocking endogenous NO production decreases the hypertrophic response by ~50% in animal models of functional overload (Smith et al., 2002; Sellman et al., 2006). As outlined before, satellite cells can be activated in response to mechanical stretch by the release of hepatocyte growth factor (HGF) in a NO-dependent manner (Tatsumi, 2010). In addition, NO is involved in other mechanotransduction processes such as integrin signaling (Zhang et al., 2007). The isoforms of nitric oxide synthase (NOS) are activated by numerous stimuli, including exercise and possibly also hypoxia (Stamler & Meissner, 2001).

Kawada and Ishii (2005) reported increased levels of NOS-1 mRNA along with an increase in muscle weight in a rat model of chronic partial occlusion. However, changes in other possible regulators of muscle mass were also present, such as a decrease in myostatin. Little is currently known regarding the possible roles of NO in muscle hypertrophy in human models of resistance training, including ischemic strength training. Given the dramatic results of Nielsen et al in terms of satellite cells and myonuclear addition, it is tempting to speculate on an involvement of NO with BFR resistance training.

Prostaglandins

It has been suggested that NO regulates the expression of cyclooxygenase 2 (COX-2) in skeletal mucle (Soltow et al., 2006). The COX enzymes catalyze the production of prostaglandins from arachidonic acid that is released from membrane phospholipids by the enzyme phospholipase A2 (Bos et al., 2004; Shen et al., 2006). Prostaglandins of the COX-2 pathway include prostaglandin E2 (PGE2) and prostaglandin F2 alpha (PGF2a). These were implicated in mechanotransduction and the control of protein synthesis and degradation already in the early 1980s (for a review see Thompson & Palmer, 1998).

Blocking the production of these prostaglandins with nonsteroidal anti-inflammatory drugs (NSAIDs) was reported to blunt the increased protein synthesis after an acute bout of heavy eccentric exercise in young subjects (Trappe et al., 2001, 2002). Furthermore, the COX-2 pathway is important for myonuclear addition in vivo and satellite cell activation and proliferation in vitro (Bondesen et al., 2006), and PGF2a may regulate hypertrophy of skeletal muscle by activating both cell fusion and protein synthesis (Horsley & Pavlath, 2003) and by decreasing cell death via the inhibitor of apoptosis protein (IAP) BRUCE (Jansen & Pavlath, 2008). PGI2, another COX product, was shown to enhance cell fusion between myoblasts (Bondesen et al., 2007). Finally, studies on rodent models have demonstrated the importance of the COX-2 pathway in recovery from atrophy (Bondesen et al., 2006) and for optimal hypertrophy resulting from functional overload (Soltow et al., 2006; Novak et al., 2009).

Interestingly, it was reported that PGF2a induced hypertrophy independent of Akt, possibly via PI3K-ERK-mTOR pathway (Markworth & Cameron-Smith, 2011). However, it should be noted that stimulation of the receptor for PGF2a is also known to activate other pathways, including p38, JNK and PKC (Bos et al., 2004). Also of interest is the recent finding that PGE2 is a potent inducer of MGF (Kravchenko et al., 2011).

The relatively few human data that exists are contradictory, and no training study in humans has been published which has confirmed a role of prostaglandins in skeletal muscle hypertrophy. On the contrary, an enhanced hypertrophic and strength response in older subjects undergoing resistance training was observed when supplemented with ibuprofen or acetaminophen vs placebo (Trappe et al., 2011). These findings should be interpreted with caution, because older individuals often display increased oxidative stress and inflammation. Hence, the administration of these NSAIDs may have dampened the chronic inflammation, which in turn may have made the muscles more anabolic and responsive to the strength training stimulus. Also, the effects of the NSAID were not clear, as both COX-1 protein and mRNA increased with resistance training independent of NSAIDs.

In younger subjects, Krentz et al. (2008) reported no effects of ibuprofen on muscle thickness increase in the elbow flexors after unilateral strength training. However, the dose was low, and ibuprofen was given on the days when one of the arms was trained, but not when the other arm was trained. This design precludes any definitive conclusions. Using a high dose of the NSAID indomethacin infused locally, Mikkelsen et al. (2009) showed total blocking of satellite cell increase after eccentric exercise in comparison to the leg which did not receive indomethacin. Interestingly though, in another paper from the same project, Mikkelsen et al. (2010) reported no effects of the NSAID on muscle protein synthesis.

Similarly, Burd et al (2010c) reported that a COX-2 inhibitor (celecoxib) did not prevent elevations in MPS 24 hour after a bout of eccentric exercise. If anything, there was a tendency for greater elevations in MPS with the COX-2 inhibitor (0.056 ± 0.004 to 0.108 ± 0.014%/h) compared with placebo (0.074 ± 0.004 to 0.091 ± 0.005%/h), although this was not significant. These authors suggested that COX-1 may be more important than COX-2 for MPS, which would be consistent with the earlier reports of Trappe et al. (2001, 2002). This may also explain the results of Paulsen et al. (2010), who showed that celecoxib did not affect recovery after damaging eccentric exercise, or the satellite cell response. Again, variations in the doses and how they were administered could also in part explain discrepant findings.

Regardless, it appears that the COX enzymes and prostaglandins are important for the satellite cell response. This has been shown in C2C12 myoblasts in vitro, where a COX-2 inhibitor totally blocked stretch-induced increases in nuclear number (Otis et al, 2005; Soltow et al., 2010). In the study of Soltow et al. (2010), inhibitors of NO and NF-kappaB also abolished the effects of stretch. These authors concluded that NO, COX-2, and NF-kB are necessary for stretch-induced proliferation of myoblasts. Collectively, one may envision that prostaglandins are not obligatory for muscle protein synthesis, but they are important or even crucial for elevations in satellite cells and myonuclear number and could thus impact on hypertrophy in the longer term. This would have implications also for BFR exercise.

Human studies have shown increased levels of PGF2a after conventional resistance exercise (Trappe et al., 2006) and eccentric resistance exercise (Trappe et al, 2002). Whether prostaglandins are produced in humans also in response to low-load training with vascular occlusion is currently not known. However, as noted in Section 7.5, increased muscle levels of arachidonic acid and prostaglandins have been observed in studies on cats subjected to electrical stimulation combined with brief ischemia (Rotto et al., 1989; Symons et al., 1991).

Moreover, prostaglandins are involved in the sensitization and stimulation of group III and IV muscle afferents, and the responses of these afferents are increased during dynamic exercise with occlusion compared with freely perfused conditions (Hayes et al., 2006).Group IV thin fibers represent the afferent pathway from metaboreceptors and may mediate the GH response during muscle activity (Viru et al., 1998). On the basis of these observations, the potential roles of prostaglandins in ischemic strength training should be investigated.

Other molecules downstream of PLA2: arachidonic acid, LOX and LPC

As previously discussed, activation of PLA2 has been implicated in damage to the sarcolemma by various stimuli, possibly via LOX enzymes, LPC and ROS production (Ørtenblad et al., 2003). Moreover, arachidonic acid (AA) is the precursor not only for the prostaglandins and thromboxanes of the COX pathway as well as metabolites of the LOX enzymes, but also for products of the cytochrome p450 (CYP) pathway. Again, it is interesting to note that PLA2 isoforms, and thus also production of LPC and AA, can be activated in skeletal muscle by a range of stimuli including including exercise, stretch, ischemia and cell swelling (Ørtenblad et al., 2003; Burkholder, 2009).

Intriguingly, LPC, like phosphatidylcholine, has been demonstrated to have IGF-1 like effects on skeletal muscle cells and activate PLD (Rauch & Loughna, 2005). LPC, but not AA, was shown to be involved in the response of ERK1/2 to mechanical stretch in skeletal myotubes (Burkholder, 2009), thus demonstrating a role for LPC in mechanotransduction. Addition of AA to skeletal muscle is known to increase muscle protein synthesis (Smith et al., 1983; Palmer & Wahle, 1987), and AA mimicked the effects of stretch on MPS.

In addition to COX and LOX products, muscle contractions are known to cause accumulation of CYP metabolites (Gao et al., 2008). LOX and CYP, along with COX enzymes, have been implicated in translation initiation in smooth muscle cells (Neeli et al., 2003). Interestingly, the AA-induced increases in MPS in smooth muscle cells appeared to be more dependent on LOX and CYP than on COX (Neeli et al., 2003). Although there may be cell type differences in the roles of AA metabolites in stimulating MPS, it is tempting to speculate that some of the increase in MPS in skeletal muscle may be mediated by LOX and/or CYP, and/or even by AA itself.

However, AA and its metabolites have also been implicated in proteolysis (Rodemann & Goldberg, 1982) and muscle wasting (Tisdale, 2009). A possible explanation of this paradox may be that certain molecules in these pathways are regulated differentially between various conditions. For example, stretch of muscle cells initially increases damage as well as PGF2a and PGE2, but with continued stretch, proteolysis and PGE2 levels decreases while PGF2a and protein synthesis continues to be elevated (Vandenburgh et al., 1990).

Reactive oxygen species (ROS)

Regarding the potential role of ROS in muscle hypertrophy, several interesting observations have been made in both skeletal muscle and cardiac muscle. ROS have been linked with cardiac hypertrophy; an effect which may be mediated via phosphatidic acid, which in turn is

in part regulated by the enzyme phospholipase D (Tappia et al., 2006). As previously noted, a role for PLD activation and production of phosphatidic acid in mTOR signaling in response to mechanical stimulation in skeletal muscle has been suggested (Hornberger et al., 2006), although the relevance of PLD now appears to be in question. Perhaps some redundancy or even compensatory mechanisms exist between the routes to PA production, so that inhibition of one pathway is compensated for by some other pathway(s).

In cardiac muscle, activation of PLD is seen in the early part of the reperfusion after ischemia, whereas following prolonged reperfusion, a reduced activity is seen instead (Tappia et al., 2006). These authors speculated that low levels and brief exposures to ROS may stimulate PLD-mediated signal transduction, whereas prolonged exposure and high levels of ROS result in PLD inhibition. In line with these observations, low levels of hydrogen peroxide (H_2O_2) induces hypertrophy in cardiac myocytes, whereas higher concentrations result in apoptosis (Kwon et al., 2003). H_2O_2 activates p70S6K in different cell types (Bae et al., 1999; Tu et al., 2002; Cully et al., 2010), and it has been suggested that H_2O_2 acts as a second messenger in growth factor-induced p70S6K signaling (Bae et al., 1999).

Seemingly in contrast, growing evidence supports a role for ROS in skeletal muscle atrophy (Powers et al., 2007; Dodd et al., 2010, McClung et al., 2010); but again, this can possibly be explained by the negative effects of prolonged exposures and high levels of ROS, as opposed to brief exposures to low concentrations. In addition, the spatial regulation (or lack of it) of ROS such as H_2O_2 may be very important in determining whether they have beneficial or detrimental effects (Veal & Day, 2011).

Indeed, accumulating evidence suggest that ROS may be associated with growth also in skeletal muscle cells. Recently, it was reported that IGF-1-induced hypertrophy and signaling in C2C12 skeletal muscle cells were dependent on ROS, as the antioxidant N-acetylcysteine (NAC) inhibited both IGF-1 induced anabolic signaling (mTOR, p70S6K, Akt, ERK1/2) and myotube hypertrophy (Handayaningsih et al., 2011). Furthermore, H_2O_2 at low levels stimulated cell growth (Caporossi et al., 2003) and protein synthesis (Orzechowski et al., 2002) in L6 myoblasts. In the study of Orzechowski et al. (2002), the maximal effect on MPS (+90%) at 10µM H2O2, but already at 1µM H_2O_2 MPS was increased by 52%. At 100µM H_2O_2, MPS was still increased by 54%, but mitogenicity was decreased by 22% at this level of oxidative stress.

Given these findings, studies are warranted on the possible involvement of ROS in BFR resistance exercise and other modes of strength training.

Growth factors

No definitive evidence currently exists on the possible involvement of growth factors in BFR resistance training. Drummond et al. (2008) found no upregulation of MGF mRNA. In contrast, Gualano et al. (2010) observed an upregulation of MGF mRNA in a case study, but the subject suffered from inclusion body myositis. Given the response of MGF to PGE2, hyperthermia and low pH (Kravchenko et al. (2008, 2011), it is possible that there is an upregulation of MGF in response to BFR resistance exercise also in healthy individuals, if the exercise is sufficiently strenuous and/or damaging.

In support of a connection between muscle damage and MGF, upregulation of MGF mRNA has been detected as early as 2.5 hours after conventional heavy resistance exercise (Hameed et al., 2003) and MGF has been shown to be induced by muscle damage per se (Hill & Goldspink, 2003). Studies are needed to determine if increased MGF can occur in healthy individuals with occlusion training. Similarly, the possible roles of HGF and FGFs (see further below) should also be explored.

A damage-sensor mechanism in BFR resistance exercise?

Hypoxia, in particular in combination with exercise, as well as cell swelling, have been shown to increase membrane permeability in muscle fibers both in vitro and in vivo (Ørtenblad et al., 2003; Zuo et al., 2004, Fredsted et al., 2005, Xu et al., 2008). Thus, a "damage-sensor" mechanism could be operative also with BFR resistance training.

Interestingly, training in a hypoxic chamber was recently shown to enhance the gains in strength and muscle cross-sectional area resulting from conventional resistance training (Nishimura et al., 2010). Moreover, 7-9 days at high altitude (4559 m) resulted in a doubling of the muscle protein synthesis (along with an increased protein breakdown) in humans (Holm et al., 2011). Also, BFR enhanced the strength gains in response to an isokinetic resistance training program (Sakuraba & Ishikawa, 2009). It was not clear from the latter paper whether the program involved only concentric muscle actions or both concentric and eccentric muscle actions. Regardless, because the isokinetic training involved maximal muscle actions in both the free-flow and the BFR groups, this strongly suggests that the BFR potentiated the training stimulus.

In other words, hypoxia and ischemia appears to sensitise skeletal muscle to the effects of exercise in terms of both damage and hypertrophy, suggesting a link between these two

processes. It is possible that ischemic metabolites such as LPC and arachidonic acid (and downstream products) play crucial parts in both processes. These effects could be mediated via influx/efflux and production of various molecules and factors, and/or a result of more direct actions of these metabolites on signaling pathways associated with protein turnover, via e.g. spatially increased ROS production. Whether the end result will be hypertrophy or breakdown and atrophy may well in part depend on the levels, durations and locations of these metabolites (and subsequent ROS production), and the presence of antioxidants and other defense mechanisms necessary to restrain potentially damaging effects.

9.3 Systemic responses

A study on strength training with blood flow restriction provided evidence for a potentiating effect of systemic factors (Madarame et al., 2008). In this study, performing additional leg exercise with vascular occlusion after arm training resulted in greater increases in both muscle area and isometric strength for the elbow flexors compared with an identical arm training regime supplemented with leg exercise without occlusion. Madarame et al. (2008) suggested that noradrenaline was responsible for the enhanced hypertrophy, based on the differences in the acute response between the two regimes, while GH and testosterone did not differ significantly. They also remarked that several beta-adrenergic agonists have anabolic effects on skeletal muscle.

However, a causative role for catecholamines was not proven in their study and it should be noted that the increase in GH was significant for the occluded regime, while the response for the non-occluded regime was not. Hence, a role for GH, synergistically with the catecholamine response, cannot be ruled out at present. Also, local factors such as FGF1 & FGF2 can increase in the circulation after various stimuli (e.g. exercise) have made the sarcolemma more permeable to the passage of molecules. This possibility should not be ignored. Interestingly, unilateral wrist exercise was shown to cause changes not only in the exercised arm but also in the contralateral resting arm in the levels of FGF2, GH and IGF-1 (Eliakim et al., 2000; Nemet et al., 2002).

From the description, it appears that the arm training routine in the study of Madarame et al. (2008) was submaximal in terms of the effort required, and the ~3–4% increase (not significant) in muscle area in the trained arm in the non-occluded group compared with the ~11–12% increase in the occluded group suggests that it was largely unsuccessful in itself. Therefore, it is presently unclear whether an additive effect of an enhanced systemic response

would occur in the presence of a more effective local training stimulus. Furthermore, while the amplifying effect was present in the trained arm, the untrained arm did not increase in muscle area (~1%). Thus, as the authors themselves noted, a local exercise stimulus is indispensible for muscle hypertrophy.

9.4 Integration of responses with low-load BFR training

As argued by Widegren et al. (2001), it is unlikely that exercise responses are caused by one single mechanism and more likely that they depend on the integration of multiple local and systemic factors. As is obvious from the above discussion, numerous mechanisms may contribute to the hypertrophic effects seen after ischemic strength training.

Current evidence suggests that the high degree of motor unit recruitment is a major factor, and there is also some data indicating that the systemic response may enhance the effects of the mechanical tension during certain circumstances (e.g., at low-to-moderate loads). Growing evidence suggests additive and/or independent effects of ischemia–reperfusion and anoxia in the training situation, but definitive demonstrations are still lacking. Further investigations are obviously needed to study both the impact of individual factors and the interplay between them.

Nevertheless, it is tempting to speculate that to a certain extent, the same underlying processes may in part be responsible for both the local and the systemic responses. For example, activation of PLA2 isoforms in skeletal muscle with exercise during ischemic conditions may not only lead to damage or permeability but also to mechanotransduction signaling (e.g. ERK1/2, p38, p70S6K), growth factor release (FGFs) and production (MGF), and to increased GH (via sensitisation of groups III and IV afferents by arachidonic acid metabolites and lactic acid).

This scenario could help explain why some studies have reported associations and correlations between the gain in muscle CSA and the acute GH response with hypertrophy-oriented strength training (Goto et al., 2005). In this context, it deserves to be noted that typical “hypertrophic” strength training (e.g., 3 x 8-10RM) often has a significant ischemic component, as evidenced by the marked deoxygenation that takes place during the exercise (Tamaki et al., 1994; Tanimoto & Ishii, 2006).

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PAPER I

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EFFECTS OF VASCULAR OCCLUSION ON MUSCULAR ENDURANCE IN DYNAMIC KNEE EXTENSION EXERCISE AT DIFFERENT SUBMAXIMAL LOADS

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ABSTRACT. Wernbom, M., J. Augustsson, and R. Thomeé. Effects of vascular occlusion on muscular endurance in dynamic knee extension exercise at different submaximal loads. *J. Strength Cond. Res. 20(2):372–377. 2006.*—Strength training with low load under conditions of vascular occlusion has been proposed as an alternative to heavy-resistance exercise in the rehabilitation setting, when large forces acting upon the musculoskeletal system are unwanted. Little is known, however, about the relative intensity at which occlusion of blood flow significantly reduces dynamic muscular endurance and, hence, when it may increase the training effect. The purpose of this study was to investigate endurance during dynamic knee extension at different loads with and without cuff occlusion. Sixteen subjects (20–45 years of age) with strength-training experience were recruited. At 4 test sessions, the subjects performed unilateral knee extensions to failure with and without a pressure cuff around the thigh at 20, 30, 40, and 50% of their 1 repetition maximum (1RM). The pressure cuff was inflated to 200 mm Hg during exercise with occlusion. Significant differences in the number of repetitions performed were found between occluded and nonoccluded conditions for loads of 20, 30, and 40% of 1RM ($p < 0.01$) but not for the 50% load ($p = 0.465$). Thus, the application of a pressure cuff around the thigh appears to reduce dynamic knee extension endurance more at a low load than at a moderate load. These results may have implications regarding when it could be useful to apply a tourniquet in order to increase the rate of fatigue and perhaps also the resulting training effect. However, the short- and long-term safety of training under ischemic conditions needs to be addressed in both healthy and less healthy populations. Furthermore, the high acute pain ratings and the delayed-onset muscle soreness associated with this type of training may limit its potential use to highly motivated individuals.

KEY WORDS. ischemia, quadriceps, low-intensity strength training, cuff occlusion, delayed-onset muscle soreness

INTRODUCTION

Strength training is an important tool in the prevention and rehabilitation of injuries (17). Intensity or load is generally regarded as the most important variable in the prescription of strength training (18). It is commonly agreed among researchers and authors (4, 6, 10, 17, 18, 22) that the intensity has to reach levels of at least 60% of the maximum in order to stimulate increases in strength and that the largest strength gains occur when the training load is between 80 and 100% of the maximum weight that can be lifted only once (1 repetition maximum [1RM]). Training programs aiming to stimulate muscle hypertrophy generally prescribe a load of 6–12RM, corresponding to about 70–85% of 1RM (4, 10, 17, 18, 22).

In the therapeutic setting, however, it is often difficult and sometimes even contraindicated to use such heavy loads as 70–85% of 1RM or more (e.g., early rehabilitation after a sports injury). This is a problem because of the atrophy that often takes place in the muscles in the injured area. For example, differences in quadriceps strength and volume on the order of ~10% between the injured and the uninjured sides may persist for several years after reconstruction of the anterior cruciate ligament (ACL) (5); thus, there is a need for effective therapeutic alternatives to heavy-resistance exercise to counteract the atrophy and the impairment in muscle function that occurs during the early postoperative phase after ACL reconstruction.

Two groups of scientists from Japan (37, 46) tested the hypothesis that training with a relatively low resistance under conditions of reduced blood flow (i.e., ischemia) would stimulate increases in strength. They used tourniquet cuffs to partially restrict the blood flow to the working muscles and thus to increase the rate of fatigue. Their studies (1, 2, 37, 45–47) have shown that low- to moderate-intensity (20–50% of 1RM) resistance training under conditions of vascular occlusion can lead to considerable increases in strength and muscle volume, similar to conventional resistance training with heavy loads.

In light of these findings, ischemic strength training appears well worth further investigation in rehabilitation and other contexts. Indeed, Ohta and colleagues (31) demonstrated superior results for low-intensity training with pressure cuffs compared with performing the same exercises without occlusion in patients who had undergone reconstruction of the ACL.

Studies (12, 24) investigating the static endurance of various muscle groups have shown that the difference in endurance between conditions of cuff occlusion and nonocclusion disappears when the force level reaches ~40–60% of maximal voluntary isometric action (MVIA). This can be explained by the intramuscular occlusion of the blood flow that occurs because of the high pressure in the muscle (35, 36, 38). To our knowledge, no study has investigated the effects of cuff occlusion on endurance during conventional dynamic resistance training with coupled concentric-eccentric actions. Hence, it is difficult to say anything about the load below which it may be an advantage to use a pressure cuff in order to increase the rate of fatigue and possibly also the training effect. Therefore, the purpose of the present study was to investigate dynamic endurance of the quadriceps muscle during external occlusion of the blood flow by a tourniquet cuff and to compare it with the endurance without a cuff at different submaximal loads. During pilot studies, we observed that training performed to exhaustion with multiple sets

TABLE 1. Subject characteristics.

	n	Mean	*SD*
Women	3		
Men	13		
Age (y)		27.9	6.1
Height (cm)		181.3	7.9
Weight (kg)		86.5	11.8

of low to moderate loads gave rise to acute ischemic muscle pain as well as delayed-onset muscle soreness (DOMS) regardless of whether a cuff was used. Thus, a secondary purpose was to collect data regarding acute pain and DOMS ratings and to compare pain and soreness ratings between occluded and nonoccluded conditions.

The null hypothesis was that there would be no significant difference in endurance, pain ratings, and DOMS between conditions of cuff occlusion and nonocclusion.

METHODS

Experimental Approach to the Problem

To examine the effects of vascular occlusion on dynamic knee extension endurance, a within-subjects study design was used in which one leg was tested with cuff occlusion and the other leg was tested without occlusion in a randomized fashion. The order of the conditions was also randomized and then remained the same throughout the study.

Subjects

Sixteen healthy persons (13 men and 3 women, 20–45 years of age) who exercised on a regular basis in a local fitness center volunteered for the study (Table 1). The participants had several years of experience in strength training, but none competed in bodybuilding, weightlifting, or power lifting. They were considered to be in a relatively steady state in their strength development, and no dramatic changes in their strength could be expected during the time course of the study. The subjects were permitted to train their quadriceps as usual except for 4 days before each test and for 3 days afterward, for ratings of DOMS were part of the study. They were informed about the procedures and potential risks of the tests before their informed consent was obtained. The Human Ethics Committee at the Faculty of Medicine, Göteborg University, Göteborg, Sweden, approved the study.

Strength-Testing Procedures

The subjects were tested for their unilateral 1RM strength for each leg in a variable resistance knee extension machine (Leg Extension FL130, Competition Line, Borås, Sweden). Because they were all familiar with the dynamic knee extension exercise, only 1 session was devoted to strength testing. The 1RM was established according to the procedures of Staron et al. (40).

Endurance-Testing Procedures

At 4 subsequent sessions after the 1RM testing, with at least 10 days between each session, the subjects were tested for their endurance in the knee extension exercise. The loads were 50, 40, 20, and 30% of 1RM for the first, second, third, and fourth sessions. This test order was chosen to allow for gradual familiarization to the pain level. A pressure cuff of 135 mm in width, with a 100-mm wide pneumatic bag inside, was connected to a surgical tourniquet system (Zimmer A.T.S. 2000, Zimmer Patient Care, Dover, OH) with automatic regulation of the pressure. The cuff was wrapped around the proximal part of the thigh and inflated to a pressure of 200 mm Hg just before exercise with external occlusion and deflated to 0 during the rest periods in between. After a warm-up, the subjects performed as many repetitions as possible for a total of 4 sets for each leg. The rest period between each set was 45 seconds for both the nonoccluded and the occluded conditions. The range of motion was between 105 and 5° of knee flexion (0° = full extension) and the tempo was set at 30 repetitions per minute (1 second each for the concentric and the eccentric action) with a metronome. This rate was based on the subjects' self-selected cadence during pilot trials. No rest was permitted between the repetitions, and the weight stack was allowed to touch down only very lightly to avoid relaxation of the quadriceps. The number of repetitions performed in the first set was noted as a measure of endurance. Hoeger et al. (25) showed a test-retest reliability of $r = 0.86$–0.96 for knee extension endurance tests at loads of between 40 and 60% of 1RM when performed in a similar continual cadence as in our study and $r = 0.98$ for the 1RM test.

Ratings of Pain, Perceived Exertion, and DOMS

At the end of both the occluded and the nonoccluded endurance protocols, the subjects were asked to use the Borg CR-10 scale (13) to rate the pain in their quadriceps at its worst. The value of 10 was anchored as "the worst lactic acid pain you have ever experienced in your quadriceps," and the subjects were allowed to mark even higher values if they felt that they had exceeded this. The maximum possible value was denoted as 12. The subjects were also asked to use a Borg RPE scale (13) to rate their maximum local perceived exertion. For the 40 and 20% of 1RM tests, the subjects were given a visual analog scale (VAS) to rate their soreness at baseline (before the tests) and every 24 hours thereafter until any eventual DOMS had subsided. The 0 value for the VAS was designated as "no soreness at all," and the maximum value of 10 was "extreme muscle soreness." The VAS has been used in several studies on DOMS (7, 39, 48, 49).

Statistical Analyses

Power analysis on data from pilot studies revealed that in order to detect a 3% difference in endurance, at least 13 participants were required to achieve a power of 0.90.

Differences in the number of repetitions between the occluded and the nonoccluded test conditions were computed by using a paired-samples *t*-test. The Friedman test and the Wilcoxon signed rank test were used to determine differences in Borg RPE, Borg CR-10, and DOMS ratings. Means and *SD*s were calculated for the number of repetitions, whereas median and interquartile ranges were computed for the Borg RPE, Borg CR-10, and DOMS ratings (ordinal). Significance was accepted at the alpha level of $p \leq 0.05$.

RESULTS

Significant differences in the number of repetitions performed were found between the occluded and the nonoccluded conditions for loads of 20, 30, and 40% of 1RM ($p < 0.01$) but not for the 50% load ($p = 0.465$) (Figure 1). Pain ratings were significantly higher for the occluded

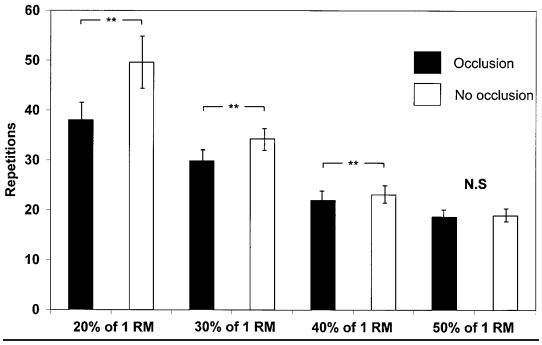

FIGURE 1. Maximal number of repetitions performed under occluded and nonoccluded conditions for loads of 20, 30, 40, and 50% of 1 repetition maximum. Values are expressed as mean ± *SD*. ** Difference ($p < 0.01$) from occluded condition.

condition compared with the nonoccluded condition for all loads ($p < 0.05$) except for the 20% load ($p = 0.112$) (Table 2). There were no significant differences between loads for either the occluded or the nonoccluded condition. Borg RPE values were not different between occluded and nonoccluded conditions for any load. For the DOMS ratings, no significant differences were noted between occluded and nonoccluded conditions for any load (Table 2).

DISCUSSION

The major finding of the current study was that severe restriction of blood flow with a thigh tourniquet cuff had no influence on the endurance in the dynamic knee extension exercise at an intensity of 50% of 1RM. In contrast, the external compression had a markedly negative impact on the endurance at the 20% load. The most simplistic explanation for these results is that the intramuscular pressure, which rises in proportion to the force developed by the muscle (35), may reach levels high enough to cause serious impediment of the blood flow during dynamic resistance exercise with moderate loads (23).

Isokinetic data from Dudley et al. (16) suggest that the maximum torque capability of the quadriceps during slow concentric actions (40–90°·s^{-1}) is on the order of ~70–90% of the isometric maximum. A 1RM may thus represent up to 70–90% of the MVIA in terms of the force developed by the muscle. Because the ratio of force to intramuscular pressure is the same for concentric and isometric actions (42), the force level at which a state of occlusion is reached may also be similar for these types of actions. The results from the current study therefore seem to agree with previous studies that have shown that the blood flow to the quadriceps is insufficient from force levels of ~15–25% of MVIA and upwards, approaching 0 at ~50% of MVIA (19, 28, 38). The results are also in line with a recent study by Hisaeda et al. (24), who showed no significant differences in endurance for static knee extension between conditions of external occlusion and without occlusion at 50% of MVIA.

During exercise, blood flow occurs mainly in the relaxation period between muscle actions (41, 50). In the current study, the rest periods between the repetitions were eliminated and each set was thus performed in a nonstop manner until failure. If rest periods even as short as 1–2 seconds had been included, the results would probably have been different, because the muscle blood flow becomes maximal almost instantly after relaxation of the working muscle (8).

A certain degree of caution should be taken when interpreting the results from the current study. First, the subjects were trained. This may have important implications for the degree of intramuscular pressure, which depends on the absolute force and the architectural features of the muscle (35, 36). Thick, bulging muscles generate higher levels of pressure than do long, slender ones with parallel fibers, and the more curved the muscle fiber is, the greater is the force component directed toward the center of the muscle (36, 42). Because hypertrophied muscles have a larger degree of pennation as well as greater thickness and volume (27), it is likely that a muscle of a strong person occludes its own blood flow at a lower relative force level than that of a weaker person (9, 26). Second, the results could vary with different knee extension machines because of the unique torque characteristics of each machine.

As expected, low- to moderate-load strength training with restricted blood flow was associated with considerable pain because of the ischemia and accumulation of metabolites in the quadriceps. The pain ratings were generally higher for the occluded condition. When questioned, however, most subjects reported that they experienced the most severe pain immediately after the end of each set in the occluded condition, just before the cuff was deflated. During the exercise itself, there appeared to be little or no difference between exercising with and without a tourniquet cuff. The Borg RPE values were generally high, as expected considering that the subjects performed all-out sets.

To our knowledge, no studies have reported DOMS after resistance exercise at such low intensities as 20% of 1RM. The soreness ratings for the 20% load are comparable with values reported by Sorichter et al. (39), whose subjects performed 7 sets of 10 pure eccentric actions in

TABLE 2. Median and interquartile ranges for delayed-onset muscle soreness (DOMS), pain, and exertion ratings for the occluded and the nonoccluded test conditions.

	Occluded				Nonoccluded			
Variables	20%	30%	40%	50%	20%	30%	40%	50%
DOMS	5.5 (2.6)		5.5 (6.0)		7.0 (3.8)		6.0 (4.8)	
Pain	9.5 (3.5)	10.0 (1.5)*	10.0 (3.0)†	9.0 (3.0)‡	8.5 (3.5)	8.0 (3.0)	8.0 (4.0)	7.0 (2.0)
Exertion	19.0 (2.0)	18.0 (2.2)	19.0 (3.0)	17.0 (2.0)	18.0 (2.0)	17.5 (2.2)	19.0 (1.0)	18.0 (2.2)

* Difference ($p = 0.043$) from the 30% nonoccluded condition.
† Difference ($p = 0.001$) from the 40% nonoccluded condition.
‡ Difference ($p = 0.037$) from the 50% nonoccluded condition.

the knee extension exercise at 150% of MVIA, and by Vincent and Vincent (48), whose subjects performed a total of 15 sets of 12RM for the quadriceps. The fact that the subjects in our study were adapted to heavy-resistance exercise, which includes a considerable eccentric component, makes the observation of DOMS even more intriguing. Vincent and Vincent (48), however, showed that strength-trained subjects experienced even greater DOMS compared with untrained subjects after a protocol of the same relative intensity and volume.

We speculate that in our model, fast muscle fibers were recruited toward the end of each set because of the anaerobic conditions. Greenhaff et al. (21) showed a greatly increased rate of glycogenolysis in type I fibers and a marked decline in force and near total depletion of phosphocreatine in both fiber types during intermittent electrical stimulation of the quadriceps with the blood flow occluded. In contrast, the decline in force during the same protocol of stimulation but with intact circulation was ascribed almost solely to fatigue in type II fibers. Although Greenhaff et al. (21) used electrical stimulation instead of voluntary activation, their findings are of relevance for the development of fatigue and soreness in the present study. With a decline in force in type I fibers, more type II fibers would have to be recruited for the work to continue (3). Near the end of the sets, the remaining force-producing fibers could be exposed to relatively high tensions. It is also possible that some fast fibers are preferentially recruited and slow fibers are derecruited during eccentric actions and that this may occur already at loads as low as 25% of MVIA (30). Fast muscle fibers are generally regarded as more susceptible to perturbations in their function after eccentric actions than are slow fibers (29). Because of the intense metabolic demands during ischemic exercise, reactive oxygen species may also have played a part in the etiology of DOMS (49).

It is important to note that although there are no published reports on any incidents or serious adverse effects as a result of training with cuff occlusion, there are very little data available regarding the short- and long-term effects of this type of exercise on the soft tissues beneath and distal to the tourniquet. As discussed by Takarada et al. (46), training with cuff occlusion may have the potential to create muscle damage and even more serious adverse effects such as thrombosis and damage to blood vessels. In another study (44), the same research group reported no evidence of muscle damage or oxidative stress as judged by the low levels of creatine phosphokinase and lipid peroxide after their protocol. To our knowledge, the authors have not reported any direct morphological data regarding effects of occlusion training on soft tissues. Because low-intensity resistance exercise with partial occlusion has been suggested as a possible countermeasure against sarcopenia (46), it is important that safety issues of this type of training in healthy as well as in less healthy populations be clarified. For example, diabetes is associated with increased risk for venous thromboembolism (33) and neuropathy (34), which is of relevance for the use of pressure cuffs.

In the context of limb surgery, studies with animal models have shown that the deleterious effects of long-duration cuff occlusion on soft tissues can at least in part be ascribed to the effects of compression of the underlying tissue, which compounds the effects of ischemia (32). However, the compounding effects of compression appear to be dependent on the degree of pressure. Pedowitz et al. (32) showed no necrosis in muscle samples after 2 hours of tourniquet application at 125 mm Hg but showed several necrotic samples after the same duration of application at 350 mm Hg. Absence of arterial pulsations was confirmed in both cases during the occlusion period. Thus, the necrosis was likely a result of the additional effects of the high degree of compression. The authors recommended the use of wide tourniquets during limb surgery, for wide cuffs have been shown to achieve occlusion at considerably lower pressures than have narrower cuffs (15, 20). Therefore, it could be argued that if pressure cuffs should be used during training, they should be wide so that the pressure necessary to achieve a partial occlusion will be low.

It is also worth noting that the studies published to date comparing strength training with and without cuff occlusion (1, 2, 14, 45–47) have controlled only for load and volume, not for effort. In these studies, narrow cuffs or cuffs with low pressures causing partial occlusion were applied throughout the entire workout, including rest periods. The occlusion groups generally trained to failure, whereas the groups training without cuff occlusion were instructed to perform the same number of repetitions as the groups training with cuffs. Because partial occlusion prolongs recovery, it could be argued that the occlusion groups performed multiple all-out sets, whereas the controls (no cuff) performed few, if any, all-out sets.

The results of the present study show that for strength-trained subjects performing the knee extension exercise at low to moderate loads (~40–50% of 1RM), there is little or no difference between training with and without a thigh tourniquet in terms of endurance if the repetitions are performed in a nonstop manner with no relaxation of the quadriceps muscle. Hence, it may be hypothesized that by performing all-out sets and using short rest periods (e.g., 15–45 seconds) between sets during low- to moderate-intensity training, thus creating a relatively ischemic state, similar training effects could be induced as when training with cuff occlusion. Although no studies testing this hypothesis have been published yet, several studies (3, 11, 43) have shown muscle hypertrophy as a result of conventional training with a high work:rest ratio at intensities of ~30–50% of maximum. Intuitively, a training model that is based on a muscle's own internal occlusion would have advantages from both a safety point of view and a practical point of view. On the other hand, at very low loads, such as 20% of 1RM and below, external occlusion will have a marked effect on the rate of fatigue and possibly also on the resulting adaptations.

PRACTICAL APPLICATIONS

Occlusion of the blood flow of the quadriceps muscle by means of a thigh tourniquet appears to reduce the dynamic knee extension endurance more at very low loads than at moderate loads. These results may have applications regarding when it could be advantageous to use a pressure cuff in order to increase the rate of fatigue and perhaps also the resulting training effect. However, the results regarding the pain and the soreness associated with all-out strength training during ischemia suggest that this type of exercise is mainly for highly motivated individuals and should be introduced carefully and with a low volume initially (e.g., 1–2 sets). It is recommended

that future studies focus on the issue of whether cuff occlusion has any additive effects on adaptations in strength and muscle volume after low- to moderate-load strength training when the training is performed to muscular failure. Also, the minimum intensity that still results in strength gains and muscle hypertrophy or, equally important, preserves the existing muscle mass from atrophy deserves further investigation. Finally, the short- and long-term safety of training during ischemic conditions, especially concerning the use of pressure cuffs and their potential effects on soft tissues (i.e., nerves, blood vessels, and muscles), needs to be addressed regarding both healthy and less healthy populations.

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PAPER II

Acute Effects of Blood Flow Restriction on Muscle Activity and Endurance During Fatiguing Dynamic Knee Extensions at Low Load

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Abstract

Wernbom, M, Järrebring, R, Andreasson, MA, and Augustsson, J. Acute effects of blood flow restriction on muscle activity and endurance during fatiguing dynamic knee extensions at low load. *J Strength Cond Res* 23(8): 2389–2395, 2009—The purpose of this study was to investigate muscle activity and endurance during fatiguing low-intensity dynamic knee extension exercise with and without blood flow restriction. Eleven healthy subjects with strength training experience performed 3 sets of unilateral knee extensions with no relaxation between repetitions to concentric torque failure at 30% of the 1 repetition maximum. One leg was randomized to exercise with cuff occlusion and the other leg to exercise without occlusion. The muscle activity in the quadriceps was recorded with electromyography (EMG). Ratings of perceived exertion (RPE) and acute pain were collected immediately, and delayed onset muscle soreness (DOMS) was rated before and at 24, 48, and 72 hours after exercise. The results demonstrated high EMG levels in both experimental conditions, but there were no significant differences regarding maximal muscle activity, except for a higher EMG in the eccentric phase in set 3 for the nonoccluded condition ($p = 0.005$). Significantly more repetitions were performed with the nonoccluded leg in every set ($p < 0.05$). The RPE and acute pain ratings were similar, but DOMS was higher in the nonoccluded leg ($p < 0.05$). We conclude that blood flow restriction during low-intensity dynamic knee extension decreases the endurance but does not increase the maximum muscle activity compared with training without restriction when both regimes are performed to failure. The high levels of muscle activity suggest that performing low-load dynamic knee extensions in a no-relaxation manner may be a useful method in knee rehabilitation settings when large forces are contraindicated. However, similarly to fatiguing blood flow restricted exercise, this method is associated with ischemic muscle pain, and thus its applications may be limited to highly motivated individuals.

Key Words strength training, ischemia, vascular occlusion, blood flow restricted exercise, muscle hypertrophy, muscle activation

Introduction

In the last 8 to 10 years, low-load training with blood flow restriction has attracted a lot of attention, both as a possible alternative to heavy resistance exercise in the rehabilitation setting and as a training method to increase muscle strength and size in healthy individuals. Several studies have shown that blood flow restriction by pressure cuffs in combination with low-intensity resistance exercise induces muscle mass increases at rates comparable with those seen with conventional strength training (17,26–28) and sometimes at even higher rates (2,12). In the studies that have included control groups that have trained at the same intensities and volumes but without cuff occlusion, the nonoccluded groups have generally made little if any gains in muscle size and strength (1,2,12,27,28).

However, because vascular occlusion reduces the muscle endurance at the low-to-moderate loads typically used in training with blood flow restriction (33), and because the occlusion has usually been applied continuously (including rest periods) throughout the training session, the effort required to complete a given amount of work in an exercise bout should be substantially less with free flow conditions. Therefore, one might argue that previous studies have not controlled for the degree of effort when comparing training with and without cuff occlusion. Furthermore, it has been reported that there was little difference in the acute pain ratings between the occluded and nonoccluded legs when dynamic knee extensions were performed in a continuous nonstop manner to fatigue, indicating that ischemic conditions were induced by the exercise itself (33). Therefore,

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the purpose of this study was to investigate whether there were any differences in muscle activity, as measured by electromyography (EMG), and endurance during low-intensity dynamic knee extension exercise performed to fatigue with and without cuff occlusion.

Methods

Experimental Approach to the Problem

To examine the effects of blood flow restriction on muscle activity and endurance during dynamic knee extensions, a within-subjects study design was used in which 1 leg (dominant or nondominant) was tested with cuff occlusion, and the other leg was tested without occlusion in a randomized fashion. The order of the conditions (with or without cuff occlusion) was also randomized. All subjects participated in a familiarization session (1 repetition maximum [1RM] testing, practice of maximal isometric efforts for EMG normalization, and dynamic knee extensions combined with vascular occlusion) 1 week before the main experiment. Normalization of the EMG amplitude with respect to the EMG obtained during a maximal isometric activation was performed to provide an index of the level of neuromuscular activation during the exercises and also because this procedure increases the reliability of the measurements (4).

Subjects

Eleven healthy persons (8 men and 3 women, 20–39 yr of age) volunteered for the study (Table 1). All participants had several years of experience with strength training. Some of the subjects were track and field athletes (sprinters and jumpers) and soccer players, and the others trained for their own strength and conditioning purposes, but none competed in strength sports. From a strength point of view, the subjects were classified as "trained" on the continuum between untrained and elite, as outlined by Deschenes and Kraemer (10), and they were tested in the beginning of a new training year cycle. The participants were instructed not to train their quadriceps during the last 3 days before the main test. The subjects were informed of the experimental risks and signed an informed consent document before the investigation. The study was approved by the Human Ethics Committee at the Faculty of Medicine, University of Gothenburg, Sweden.

Table 1. Subject characteristics.

	n	Mean	*SD*
Men	8		
Women	3		
Age (yr)		25	5
Height (cm)		178	10
Weight (kg)		77	13

Procedures

Strength Testing and Familiarization. One week before the main test, the subjects were tested for their unilateral 1RM strength for each leg in a variable resistance knee extension machine (Leg Extension FL130, Competition Line, Borås, Sweden). Because the participants were all familiar with the dynamic knee extension exercise, only 1 session was devoted to strength testing. The 1RM was determined according to the procedures of Staron et al. (22). After the 1RM for each leg had been established, the subjects practiced maximum isometric efforts in the same knee-extension machine, with the lever arm of the machine fixed at an angle corresponding to 70° of knee flexion (full knee extension is here defined as 0°). After 5 minutes of rest, a load of 30% of 1RM was chosen, and the subject was familiarized with the partial blood flow restriction and the cadence of repetitions to be used during the main test. A curved tourniquet cuff of 135 mm in width with a 100 mm wide pneumatic bag inside of it was connected to a surgical tourniquet system (Zimmer A.T.S. 2000, Zimmer Patient Care, Dover, OH, USA) with automatic regulation of the pressure. The cuff was wrapped around the proximal part of the thigh and inflated to a pressure of 100 mm Hg just before exercise with partial occlusion and kept inflated during the rest periods in between the sets. For each leg, 1 set of knee extensions to concentric torque failure was performed with cuff occlusion, followed by a second set of 5 repetitions after 45 seconds of rest, during which the partial occlusion was maintained. This was done to confirm that the subjects tolerated the pressure to be used during the main experiment 1 week later.

Endurance Testing. At a subsequent session after the 1RM testing, with 6 to 10 days between the sessions, the subjects were tested for their endurance in the unilateral knee extension exercise at 30% of 1RM. The same tourniquet system as in the familiarization session was used and inflated to a pressure of 100 mm Hg just before exercise with cuff occlusion, and this pressure was maintained for the occluded leg during the exercise, including rest periods between sets. After a warm-up, the subjects performed as many repetitions as possible for a total of 3 sets for each leg. The rest period between each set was 45 seconds for both the nonoccluded and the occluded leg. The range of motion was between 100° and 20° of knee flexion (0° = full extension), and the cadence was 20 repetitions per minute (1.5 s each for the concentric and the eccentric muscle actions), which was controlled with a metronome set at 40 beats per minute. No rest was permitted between the repetitions, and the subjects were instructed to stop the eccentric phase just before the weight stack touched down to avoid relaxation of the quadriceps. The number of repetitions performed in each set was noted as a measure of endurance, and the muscle activity during the exercise was measured by EMG, as described below. After all 3 sets for the occluded leg had been completed, the pressure cuff was deflated. The subjects

remained seated in the machine during the rest periods between the sets.

Continuous partial occlusion rather than intermittent severe occlusion was chosen because most of the previous studies have used a partial occlusion training model and because partial blood flow restriction is more tolerable than severe or complete occlusion (8). Hoeger et al. (15) demonstrated a test-retest reliability of $r = 0.86$ to 0.96 for knee extension endurance tests at loads of between 40% and 60% of 1RM when performed in a similar continuous fashion as in our study. Tagesson and Kvist (23) reported intraclass correlation coefficient values of 0.90 to 0.96 for the unilateral knee-extension 1RM test.

Electromyography and Goniometry. The skin was shaved with a hand razor and carefully cleaned with ethanol before electrode placement. Bipolar surface self-adhesive Ag/AgCl monitoring electrodes (model 2223, 3M Medical, Neuss, Germany) with a 4.0 cm interelectrode distance were placed on the medial portion of the vastus lateralis (VL) and the vastus medialis (VM) muscles of the quadriceps femoris muscle group approximately 15 and 10 cm above the proximal border of the patella. These placings were somewhat similar to those used by Andersen et al. (4), and they were chosen to avoid having the electrodes passing over the innervation zones of the muscles during the exercise movement while still leaving enough room for the tourniquet. The approximate locations of the innervation zones were based on the anatomic guidelines provided by Rainoldi et al. (20). Knee joint angle was measured with an electrogoniometer, which was positioned laterally to the knee joint. Calibration of the goniometer signal was performed at anatomic knee joint angles of 0° and 90° using a geometric retractor. A linear encoder was used to measure the position and displacement of the weight stack. The goniometer and encoder data were used to identify the concentric and eccentric phases of each repetition.

The EMG and goniometer/encoder data were collected using the MuscleLab 3010e recording and acquisition system (Ergotest Technology, Langesund, Norway), using the accompanying software program on a laptop computer (3). The MuscleLab EMG leads had built-in preamplifiers, and the pre-amplified signal was filtered through a 8 to 1,200 Hz band pass filter before it was root mean square (RMS) converted. The RMS converted signal was then sampled at 100 Hz and synchronized with the other connected devices. The EMG was normalized to a maximum voluntary isometric activation (MVIA) using the normalization function of the MuscleLab unit. After a warm-up with gradually increasing force developments, 2 MVIA trials were performed at a knee angle of 70°. The subjects were instructed to smoothly increase force, and once the maximum was reached, the effort was sustained for at least 3 to 4 seconds, during which time EMG was collected for normalization. Alkner et al. (3) reported a coefficient of variation between trials for EMG to be in the range of 4.7–9.2% for the MuscleLab system.

A display with the EMG RMS curve was placed in front of the leg extension machine for monitoring purposes and to provide real-time feedback to the subject. Because the normalization value was chosen automatically by the MuscleLab, the first of the 2 MVIA attempts was saved and used as the reference value (100%) if the 2 attempts did not differ by more than approximately 5% in RMS amplitude, in which case further attempts were made until a plateau in amplitude was noted. During the 30% of 1RM endurance tests with and without occlusion, as in the MVIA trials, the EMG RMS curve was displayed in real-time in front of the subject for motivation, and loud verbal encouragements were also used to motivate the subjects to continue to the point of concentric torque failure. The average RMS values for complete concentric and eccentric phases, respectively, were used for analysis. The first repetition of each set was discarded because the subjects generally had not found the proper tempo. During the final 2 or 3 repetitions in each set, most subjects also had difficulty following the correct tempo because of the accumulating fatigue, but these repetitions were considered for analysis as long as they were in the full range of motion.

Ratings of Pain, Perceived Exertion, and Delayed Onset Muscle Soreness. The subjects were asked at the end of both the

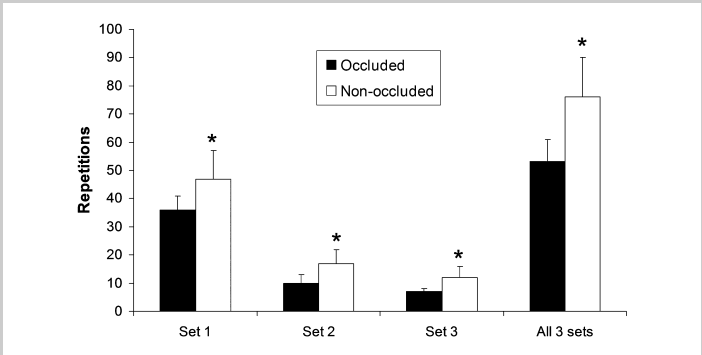

Figure 1. Number of repetitions with and without partial occlusion. * = $p < 0.05$ between the nonoccluded and occluded conditions.

Table 2. Lowest and highest electromyographic (EMG) activation (% of maximum voluntary isometric activation [MVIA]) for complete concentric and eccentric phases for vastus medialis (VM) and vastus lateralis (VL) during occluded and nonoccluded conditions, set 1 to 3 (means ± *SD*).

	Concentric EMG (% of MVIA)				Eccentric EMG (% of MVIA)			
	Lowest	Highest	Lowest	Highest	Lowest	Highest	Lowest	Highest
	Occluded set 1		Nonoccluded set 1		Occluded set 1		Nonoccluded set 1	
Muscle								
VM	34 ± 10	101 ± 22	37 ± 12	107 ± 24	24 ± 8	50 ± 13	26 ± 8	57 ± 18
VL	36 ± 11	86 ± 19	34 ± 11	97 ± 17	23 ± 7	45 ± 10	23 ± 7	44 ± 12
VM+VL	35 ± 10	94 ± 18	36 ± 11	102 ± 19	24 ± 7	48 ± 10	24 ± 7	50 ± 14
	Occluded set 2		Nonoccluded set 2		Occluded set 2		Nonoccluded set 2	
Muscle								
VM	73 ± 18	98 ± 27	72 ± 22	107 ± 27	29 ± 9	52 ± 16	32 ± 9	61 ± 16
VL	66 ± 19	91 ± 26	63 ± 19	96 ± 20	26 ± 9	46 ± 12	28 ± 10	53 ± 15
VM+VL	70 ± 18	94 ± 23	68 ± 20	102 ± 21	28 ± 8	49 ± 13	30 ± 9	57 ± 15
	Occluded set 3		Nonoccluded set 3		Occluded set 3		Nonoccluded set 3	
Muscle								
VM	77 ± 21	93 ± 23	78 ± 21	102 ± 20	34 ± 11	47 ± 12	37 ± 12	64 ± 17*
VL	70 ± 19	85 ± 24	67 ± 19	93 ± 24	31 ± 10	40 ± 11	33 ± 13	53 ± 15*
VM+VL	74 ± 19	89 ± 21	72 ± 20	98 ± 20	32 ± 10	44 ± 11	35 ± 12	58 ± 15*

*$p < 0.05$ between the nonoccluded and occluded conditions; $n = 11$.

occluded and the nonoccluded protocols to use the Borg CR-10 and ratings of perceived exertion (RPE) scales (6) to rate the pain and perceived exertion in their quadriceps. The 10 on the Borg CR-10 pain scale was anchored as "the previously worst exercise-induced pain experienced in your quadriceps," and in line with the recommendations of Borg (6), the subjects were allowed to use even higher values (up to 12) if they thought that they had exceeded this during the tests. Delayed onset muscle soreness (DOMS) was rated at 24, 48, and 72 hours postexercise using the visual analog scale (VAS). The 0 value for the VAS was designated as "no soreness at all," and the maximum value of 10 was "extreme muscle soreness" (33).

Statistical Analyses

Power analysis on data from pilot studies revealed that, to detect a 33% difference in the number of repetitions in the first set, at least 7 participants were required to achieve a power of 0.90. Differences in EMG activity and in the number of repetitions between the occluded and the nonoccluded test conditions were computed with paired *t*-tests, whereas comparisons regarding Borg RPE, Borg CR-10, and DOMS ratings were performed with the Wilcoxon signed rank test. Means and *SD*s were calculated for EMG and for the number of repetitions, whereas median and interquartile ranges were computed for the Borg RPE, Borg CR-10, and DOMS ratings (ordinal). Statistical Package for Social Science (SPSS, Chicago, IL, USA), version 14.0, was used for the analyses. Significance was accepted at the alpha level of $p \leq 0.05$.

Results

Significant differences in the number of repetitions performed were found between the occluded and the nonoccluded legs for each of the 3 sets and for all 3 sets combined (Figure 1). No differences in minimum and maximum muscle activity were noted between the conditions, except for the third set, in which the activation in the eccentric phase during the repetitions with the highest activity was significantly greater in the nonoccluded leg ($p = 0.005$ for VL and VM combined) (Table 2). Acute pain (Borg CR-10) and RPE ratings were not

Table 3. Median values and interquartile ranges for local perceived exertion (Borg rating of perceived exertion [RPE] 6–20 scale), pain (Borg CR-10), and delayed onset muscle soreness (DOMS; visual analog 0–10 scale).

Variables	Occluded	Nonoccluded
Borg RPE	18,9 (1,3)	19,0 (1,2)
CR-10	9,7 (2,1)	9,2 (1,5)
DOMS (24 hr post)	2,9 (2,8)	4,0 (2,0)*
DOMS (48 hr post)	3,3 (2,8)	5,0 (2,5)*
DOMS (72 hr post)	2,5 (2,4)	3,5 (4,1)*

*$p < 0.05$ between nonoccluded and occluded conditions; $n = 11$.

significantly different between the conditions (Table 3). For DOMS, significantly greater ratings on the VAS scale were noted for the nonoccluded leg ($p < 0.05$) (Table 3).

Discussion

The major finding of the current study was that although partial restriction of blood flow with a thigh tourniquet did influence the endurance in the dynamic knee extension exercise at an intensity of 30% of 1RM, it did not increase the maximum level of muscle activity in the quadriceps either in the concentric or the eccentric phase compared with no cuff occlusion when both regimes were performed to the point of concentric torque failure. If anything, there was a tendency for greater activity in both of the vastus muscles during the nonoccluded condition for both the concentric and the eccentric phases, which became significant for the eccentric phase in the third set. Generally, the lowest activity was seen in the beginning of the sets and the highest during the last few repetitions of each set, and this was true for both conditions and for both the concentric and the eccentric phases. Also, the muscle activity during the first repetitions, especially during the concentric phases, was clearly greater during the second and the third sets compared with the first set.

At first glance, our findings may appear to contradict those of previous studies that have compared training with and without cuff occlusion in terms of muscle activity and in which occlusion was demonstrated to yield higher levels of muscle activation. However, in these studies (25,26,34,35), the degree of effort most likely differed between the conditions, in contrast with the current study where the effort was maximal in both conditions. Furthermore, in the present study, high levels of activation (86–107% of MVIA) were seen in both conditions in the last few repetitions during the concentric phases, and shorter bursts (lasting a few tenths of a second) of approximately 120–160% of MVIA were not uncommon. Therefore, our results confirm the findings of Takarada et al. (25,27) of high EMG levels during low-intensity (20–40% of 1RM) training with blood flow restriction. The novel finding in our study is that a high level of activation is possible also without cuff occlusion if the repetitions are performed in a no-relaxation manner.

The high EMG levels suggest, but do not directly prove, type II fiber recruitment. However, Krustrup and colleagues (16) recently demonstrated that blood flow restriction during pure concentric knee extensor exercise at a low workload (29 Watts) resulted in a lowered creatine phosphate content in 93% of the fast-twitch fibers, which strongly supports that the majority of type II fibers can be recruited at low loads during ischemic conditions.

It is well known that muscle blood flow during both static and dynamic exercise occurs mainly during the relaxation period between contractions (5,18). By eliminating the relaxation of the muscle between repetitions in our protocol, the muscle blood flow likely became insufficient to meet the metabolic demands, and consequently not only the cuff-occluded leg but also the nonoccluded leg were submitted to relative ischemia. We therefore suggest that because of the ischemic conditions, the type I fibers became highly fatigued during the first set, thus necessitating the recruitment of an increasing number of motor units containing type II fibers as the set progressed, as indicated by the increase in EMG (Table 2). In addition, the EMG data indicate that the type II fibers were recruited earlier in the second and third sets than in the first set, presumably because of residual fatigue in type I fibers.

The gradual increase in the EMG during the eccentric phase also deserves comment. During the first few repetitions of each set, the activity was low, as expected (approximately 25–30% of MVIA), but as the point of concentric torque failure drew nearer, the eccentric activity showed a marked increase, ending at levels of approximately 50–60% of MVIA, which suggests the possibility that motor units containing fast fibers were increasingly recruited also during the eccentric phase. To the best of our knowledge, this increase in activation during eccentric muscle actions has not been reported before in the literature on training combined with blood flow restriction. Therefore, we propose that fatiguing low-intensity dynamic training during ischemic conditions includes a significant eccentric component and that this could potentially contribute to the training effects observed with this type of training.

Not surprisingly, fatiguing low-load strength training was associated with a significant degree of pain because of the ischemia and accumulation of metabolites in the quadriceps. The Borg RPE values were also generally high, as expected, considering that the subjects performed all-out sets. Interestingly, the pain ratings were similar between conditions. In contrast, in our previous study (33), occlusion was associated with greater pain levels. However, in that study (33), the pain levels were maximal in the moments immediately after the exercise, when the cuff had not yet been deflated. The high pressure (200 mm Hg) used in combination with the relatively wide cuff probably imposed a nearly complete restriction of blood flow. In the current study, the lower pressure used (100 mm Hg) most likely allowed for some washout of metabolites, and therefore the pain did not increase in the rest periods between the sets. Nevertheless, it is possible that metabolites accumulated to such a degree during and after the exercise with cuff occlusion that it may have impacted on the activation of the muscle, thus explaining the differences observed in the third set.

Although we did not measure blood flow in this study, the endurance data, along with feedback from the subjects, suggested that the occlusion was indeed affecting the muscle. In fact, several of the subjects experienced difficulty in starting the first repetition in the second and third sets with the occluded leg, but once they had managed to do the first repetition, they were usually able to do several more. Furthermore, we determined in separate pilot experiments

that 100 mm Hg in combination with our relatively wide cuff reduced blood flow in the femoral artery at rest by approximately 50–60% in the seated position, as measured by Doppler ultrasound. Takano et al. (24) used a narrow tourniquet (33 mm in width) and pressures of 160 to 180 mm Hg and reported an approximately 70% reduction of the femoral blood flow at rest in the seated position. Wider cuffs are generally more effective in occluding the blood flow than are more narrow ones (9,14). Consequently, we were able to use a relatively low pressure to minimize compressive and shear forces on soft tissues, in particular nerves and blood vessels (9,14).

As in our previous study (33), multiple sets of low-intensity training to fatigue induced DOMS. However, the soreness in the nonoccluded leg was significantly greater than for the occluded leg, which may be explained by the greater number of repetitions completed for the leg that trained without blood flow restriction. An important underlying factor behind the DOMS may be the relatively high increase in activation observed during the eccentric phases from the beginning to the end of each set, as discussed previously. The ischemia-reperfusion and the formation of reactive oxygen species (ROS) occurring during exercise could also play a role (29–31). Intriguingly though, Goldfarb and colleagues (13) reported that plasma protein carbonyl levels, a marker of ROS-mediated damage, were significantly less elevated after blood flow restricted exercise compared with conventional resistance training.

Because studies have demonstrated hypertrophy (1,2,12,17,28), and increases in muscle protein synthesis (MPS) (11), with low-intensity (20% of 1RM) occlusion training, and because the femoral blood flow may become insufficient already at low loads during continuous dynamic knee extensions (21), we previously speculated that it is possible to induce hypertrophy also without cuff occlusion at low intensities (approximately 20–30% of 1RM) in this exercise if performed in a no-relaxation manner (32). In line with this hypothesis, Burd et al. (7) recently reported in a preliminary study that 4 sets to failure at 30% of 1RM induced increases in mixed MPS to a similar extent as 4 sets at 90% of 1RM, whereas performing 4 sets at 30% of 1RM with submaximal effort resulted in markedly less stimulation of MPS. Future studies should investigate whether the acute MPS increases induced by fatiguing low-intensity exercise without occlusion will translate into increases in muscle mass and strength after a training period.

The major limitation of the present study is that this was an acute experiment and not a training study. Therefore, some caution is obviously warranted when interpreting the findings. Furthermore, we recognize that we measured only a few variables (muscle activity and work) of the many that may influence the hypertrophic response with this type of training, as reviewed by Wernbom et al. (32) and Manini and Clark (19). For example, there may have been a greater build-up of local metabolites and a larger response of growth factors and hormones in the blood flow restricted condition. On the other hand, the greater amount of work and the slightly higher muscle activity during the eccentric phase could in theory lead to greater adaptations for the non-occluded regime. Finally, the findings of the present study concern the dynamic knee extension exercise with variable resistance and may not necessarily be generalizable to other exercises or other muscle groups.

Practical applications

Cuff occlusion decreases the muscle endurance at low loads but does not necessarily result in greater maximal muscle activity in the quadriceps in the knee extension exercise if performed to the point of concentric torque failure. The knee extension exercise in a variable resistance machine appears to be sufficient in itself to induce relative ischemia already at low loads if performed in a continuous manner without relaxation of the muscle between the repetitions. The high degree of muscle activation suggests that these methods may be useful in rehabilitation (e.g., after sports injuries), in which large loads sometimes may be contraindicated. However, because it is rather painful to train close to concentric torque failure at these low loads regardless of whether a pressure cuff is used or not, the applicability of these methods may be limited to highly motivated individuals. Furthermore, because multiple sets of fatiguing dynamic knee extensions at these low loads often lead to DOMS, the volume or level of effort should probably be limited initially.

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PAPER III

Contractile function and sarcolemmal permeability after acute low-load resistance exercise with blood flow restriction

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Abstract

Conflicting findings have been reported regarding muscle damage with low-intensity resistance exercise with blood flow restriction (BFR). Therefore, this study investigated muscle function and muscle fibre morphology after a single bout of low-intensity resistance exercise with and without BFR. Twelve physically active subjects performed unilateral knee extensions at 30% of their one repetition maximum (1RM), with partial occlusion on one leg and the other leg without occlusion. With the BFR leg, five sets were performed to concentric torque failure, and the free-flow leg repeated the exact same number of repetitions and sets. Biopsies were obtained from vastus lateralis before and 1, 24 and 48 hours after exercise. Maximum isometric torque (MVC) and resting tension were measured before and after exercise and at 4, 24, 48, 72, 96 and 168 hours post-exercise. The results demonstrated significant decrements in MVC (lasting ≥48 hours) and delayed onset muscle soreness in both legs, and increased resting tension for the occluded leg both acutely and at 24 hours post-exercise. The percentage of muscle fibres showing elevated intracellular staining of the plasma protein tetranectin, a marker for sarcolemmal permeability, was significantly increased from 9% before exercise to 27-38% at 1h, 24h and 48h post-exercise for the occluded leg. The changes in the free-flow leg were significant only at 24h (19%). We conclude that an acute bout of low-load resistance exercise with BFR resulted in changes suggesting mild to moderate muscle damage. The moderately increased membrane permeability may have implications both for safety aspects and for the training stimulus in BFR exercise.

Key words: occlusion training, blood flow restricted exercise, sarcolemma damage, remodeling, mechanotransduction.

Introduction

In the last ~10-12 years, the method of low-load strength training with blood-flow restriction (BFR, also often referred to as "vascular occlusion") has attracted a lot of attention. Several studies have demonstrated that BFR by pressure cuffs in combination with low-intensity training (20-50% of 1 RM) induces muscle mass increases at rates comparable to those seen with conventional strength training (Takarada et al., 2000b, 2002, 2004; Kubo et al., 2006). Notably, this mode of training has been shown to produce strength gains and hypertrophy not only in untrained individuals ranging from young to elderly (Takarada et al., 2000b, 2004; Kubo et al., 2006, Karabulut et al., 2009), but also in well trained athletes (Takarada et al., 2002), as well as in patients recovering after anterior cruciate ligament surgery (Ohta et al., 2003). Taken together, these findings suggest that low-intensity strength training with cuff occlusion has a broad application potential.

In some studies, regimes with a remarkably high training frequency of two BFR training sessions per day for up to 6 days per week (i.e. a total of 12 training sessions per week) have been tried successfully over short periods (1-2 weeks), resulting in significant increases in muscle cross-sectional area (CSA) and strength despite the very short training periods (Abe et al., 2005; Fujita et al., 2009). Indirect measures of muscle damage such as creatine kinase (CK) and myoglobin did not increase appreciably in these and other studies (e.g., Takarada et al., 2000a), thus supporting that a fast recovery is possible.

In contrast, Wernbom et al. (2006, 2009) observed moderate to severe delayed onset muscle soreness (DOMS) in the quadriceps after acute bouts of low-intensity strength training with cuff occlusion, which were performed with multiple sets to concentric torque failure. Similarly, Umbel et al. (2009) observed mild DOMS after an acute bout of BFR training involving the knee extensors, as well as significant decrements (~10-15%) in maximum

voluntary isometric torque and increases in vastus lateralis muscle CSA at 24-48 hours post-exercise. Moreover, a recent case report revealed very high serum levels of CK (> 12000 U/L) and extreme muscle soreness in a subject 48-96 hours after an acute bout of low-load BFR training (Iversen & Røstad, 2010). CK values in excess of 10000 U/L are considered diagnostic for rhabdomyolysis (Clarkson et al., 2006). Collectively, these observations indicate a potential for muscle damage with the first bout of BFR strength training even at low loads (20-30% of 1RM).

Prolonged anoxia causes calcium (Ca^{2+}) influx and loss of cell membrane integrity both in myotube culture (Ørtenblad et al., 2003) and in muscle preparations (Fredsted et al., 2005). Interestingly, Fredsted et al. (2005, 2007) noted that these changes occurred much faster when the anoxia was combined with electrical stimulation. Furthermore, hypoxic exercise (12.7% O_2) resulted in increased uptake of Evans Blue Dye (EBD) in rat skeletal muscle, suggesting compromised membrane integrity (Xu et al., 2008). EBD is known to bind to albumin (Straub et al., 1997; Hamer et al., 2002), hence any increase in intracellular EBD staining presumably reflects increased albumin uptake by the muscle. Albumin has been used as a marker for skeletal muscle damage caused by eccentric exercise (McNeil & Khakee, 1992). Thus, not only high mechanical stress but also hypoxia, especially in combination with exercise, can result in damage to the muscle cell membrane.

A few studies on the acute effects of training with cuff occlusion have been published (Cook et al. 2007; Yasuda et al. 2008, 2009; Umbel et al. 2009); but except for the study of Umbel et al. (2009), muscle function was studied for a limited time period, from pre-exercise to the first few minutes following the bout. Furthermore, no morphological data at the muscle fibre level following an acute BFR exercise bout has been reported in the literature to date. If a first-time bout can induce muscle damage, this would potentially have implications for the prescription and safety of BFR resistance exercise. This concern may be especially relevant to

disused muscle. For example, unloading is known to markedly increase the vulnerability of human skeletal muscle to relatively light eccentric exercise (Ploutz-Snyder et al., 1996). Given that BFR resistance exercise may be used not only in the training of healthy individuals but also in rehabilitation settings, it is important to establish whether or not low-load BFR resistance exercise can induce symptoms of muscle damage.

Therefore, the aim of the present investigation was to study neuromuscular fatigue and recovery as well as fibre morphology following an acute bout of low-load resistance exercise with and without BFR. Specifically, we hypothesised that resistance exercise with BFR would result in signs of muscle damage and that these would be greater than those induced by exercise at the same volume and load without BFR.

Materials and methods

Subjects

Eight male subjects (mean $\pm$ SD, 26 $\pm$ 3 yr, height 180 $\pm$ 6 cm, body mass 80 $\pm$ 10 kg) and four female subjects (24 $\pm$ 2 yr, height 171 $\pm$ 6 cm, body mass 66 $\pm$ 8 kg) were recruited from the student population at the Norwegian School of Sports Science. All subjects were exercising on a regular basis (e.g., running, cycling, cross-country skiing, alpine skiing, skating, terrain marching and field exercises) and had previous experience with resistance training, but some were not performing heavy strength training at the time of the study while others were very well trained in this regard. The participants were instructed not to perform any strenuous activities involving their quadriceps during the last 72 hours before the main test. They were informed of the experimental risks and signed an informed consent document prior to the investigation. The study complied with the standards set by the Declaration of Helsinki and was approved by the Regional Ethics Committee of Southern Norway.

Experimental Design

To examine the acute effects of dynamic low-intensity strength training with BFR on muscle function in the quadriceps, a within-subjects study design was used in which one leg (dominant or non-dominant in a randomised fashion) was tested with cuff occlusion and the other leg was tested without occlusion. To match the volume of work, and because BFR reduces the dynamic endurance at low loads (Wernbom et al., 2006, 2009), the BFR/occluded leg was always trained before the free-flow/non-occluded leg.

All subjects participated in a familiarisation session one week (range 4-11 days) before the main experiment. In the main trial, the subjects were tested for maximal voluntary isometric strength immediately before the exercise bout, at 1 and 2 minutes post-exercise, and at 4, 24, 48, 72, 96 and 168 hours post-exercise. Muscle contractility was also tested with neuromuscular electrical stimulation (NMES) of the vastus medialis at the same test occasions following the isometric testing, except at 168 hours post-exercise testing, where no NMES was performed.

Muscle Function Testing and Familiarisation Protocols

A week before the main exercise test, the subjects were tested for their unilateral 1RM strength for each leg in a variable resistance knee extension machine (Leg Extension model 66478, Gym2000, Geithus, Norway). The subjects warmed up 5 minutes on a stationary bicycle at a light load (~75 watts) and then moved to the knee extension apparatus. The 1RM was determined according to the procedures of Staron et al. (1990). Tagesson & Kvist (2007)

demonstrated high inter and intra-rater intraclass correlation (ICC) values for the unilateral knee extension 1RM test (ICC = 0.90-0.96), supporting that this test is clinically reliable.

After the 1RM for each leg had been established, the subjects practiced maximum voluntary isometric contractions (MVC) in the same knee-extension machine, with the lever arm of the machine fixed at an angle corresponding to 90° of knee flexion (full knee extension is here defined as 0°). Isometric force was measured by a strain gauge (HBM U2AC2, Darmstadt, Germany) connected to the lever arm of the knee extension machine, and the signal was amplified and recorded at 1000 Hz using a 16-bit data acquisition card (MP150, BioPac Systems Inc., CA). The back rest was set at a hip joint angle of ~80° of flexion (fully extended hip here defined as 0°), and belts were secured across the subjects hips and shoulders to reduce any movements of the hips, and to minimise assistance from other muscle groups. The subjects were instructed to apply force as rapid and hard as possible and to maintain maximal effort for at least 4 seconds.

After the MVC practice, the subjects were familiarised with NMES. Two self-adhesive electrodes (5 x 10 cm, Polartrode, Medi-Stim, Oslo, Norway) were placed longitudinally on the vastus medialis and the positions of the electrodes were marked on the skin with indelible ink to ensure the same positioning in each trial. The stimulation protocol comprised two trains of stimuli with duration of 200 milliseconds each and with a frequency of 20 Hz, and two trains of stimuli of 200 ms duration and 50 Hz frequency. Each square-wave pulse lasted 0.5 ms, and the voltage was fixed at 120 V. One minute separated the last isometric MVC attempt and the NMES testing, and 5-10 seconds separated each NMES-induced contraction.

After resting for 5 minutes after the NMES testing, a load of 30% of 1RM was chosen and the subject was then familiarised with the partial vascular occlusion and the cadence of repetitions to be used during the main test. For each leg, one set of 15 coupled concentric-

eccentric knee extensions was performed with cuff occlusion, followed by a second set of 5 repetitions after 45 seconds of rest, during which the partial occlusion was maintained.

Main exercise and testing protocols

At a subsequent session after the 1RM testing, with ~7 days between the sessions, the subjects reported to the laboratory for the main test. The subjects warmed up 5 minutes on a stationary bicycle at a light load (~75 watts) and then moved to the knee extension apparatus. After warming up with three submaximal isometric contractions with gradually increasing effort for each contraction, the subjects performed two MVCs, with 30 seconds of rest between them. The highest value of the two attempts was used as baseline MVC. After the MVC, the skin above the vastus medialis muscle was washed with isopropanol and the self-adhesive electrodes were applied for the NMES, which proceeded as described above. From the forces generated at 20 and 50 Hz, the ratio between 20 and 50Hz (20/50 Hz ratio) was calculated and used as an index of low frequency fatigue. While the ankle was strapped to the lever arm in conjunction with the NMES testing, passive force against the lever arm was also measured as an indirect index of passive muscle tension.

After 5 minutes of rest, the subjects were tested in the unilateral knee extension exercise at 30% of 1RM, during partial blood flow restriction (see further below). After inflation of the pressure cuff, the subjects performed as many repetitions as possible for a total of five sets for the occluded leg. The subjects remained seated in the machine during the rest periods, and the rest period between each set was 45 seconds for both the non-occluded and the occluded leg. The range of motion was between 100° and 20° of knee flexion (0° = full extension) and the cadence was 20 repetitions per minute (1.5 seconds each for the concentric and the eccentric muscle actions) which was controlled with a metronome set at 40 bpm. No rest was permitted

between the repetitions, and the subjects were instructed to stop the eccentric phase just before the weight stack touched down to avoid relaxation of the quadriceps. The number of complete repetitions performed in each set was noted.

When the five sets for the occluded leg had been completed, and the muscle function tests (1 and 2 minute post-exercise MVC, as well as the 20 and 50Hz NMES at ~3 minutes post-exercise) had been performed, the pressure cuff was deflated (total time under partial occlusion: ~9 minutes). After 10 minutes rest, the subjects performed exactly the same number of sets and repetitions and muscle function testing for the other leg as with the occluded leg, but without the pressure cuff.

Blood flow restriction

A curved tourniquet cuff of 135 mm in width with a 100 mm wide pneumatic bag inside of it was connected to a surgical tourniquet system (Zimmer A.T.S. 2000, Zimmer Patient Care, Dover, OH) with automatic regulation of the pressure. The cuff was wrapped around the proximal part of the thigh and inflated to a pressure of 90-100 mm Hg just before exercise with BFR, and the pressure was maintained throughout the session until both the exercise and the muscle function tests had been completed, after which the cuff was deflated.

Using the same type of cuff, we previously demonstrated that this level of pressure results in decreased muscle endurance at ~30% of 1RM (Wernbom et al., 2009), thus confirming that the degree of occlusion was sufficient to affect muscle performance. In addition, we determined in separate experiments with Doppler ultrasound that the pressure at which the femoral artery blood flow ceased (measured in the seated position) was in the range of 150-205 mm Hg for the subjects. Generally, the male subjects required higher pressures (170-205 mm Hg) than the female subjects (150-170 mm Hg) for complete occlusion. Based

on this information and our experiences regarding the tolerability of different pressures during exercise, it was decided on a cuff pressure of 100 mm Hg for the male subjects and 90 mm Hg for the female subjects in the exercise part of the study.

Notably, these training pressures are considerably lower than those that have typically been reported in many of the studies on BFR exercise, where a narrow cuff (33 mm in width) has often been used. With a 33 mm cuff and pressures of 160-180 mm Hg, Takano et al. (2005) reported a ~70% reduction of the femoral blood flow at rest in the seated position. However, it is important to note that wider cuffs are generally more effective in occluding the blood flow than more narrow ones (Crenshaw et al., 1988; Graham et al., 1993). For example, Crenshaw et al. (1988) determined that with a 45 mm thigh cuff, the pressure needed for complete occlusion of blood flow in the lower limb (measured with the subjects in supine position) was on average ~280 mm Hg, and in some cases more than 400 mm Hg; while with a 120 mm cuff, the pressure needed for full occlusion was ~150 mm Hg. Thus, we decided on a relatively wide cuff and a low pressure during training to minimise compressive and shear forces on soft tissues (Crenshaw et al., 1988; Graham et al., 1993).

Muscle soreness ratings

Muscle soreness was self-rated by the subjects before the exercise bout and at 4, 24, 48, 72 and 96 hours post-exercise on a 100 mm visual analog scale (VAS), where 0 represented “no soreness” and 10 represented “extreme soreness” (Wernbom et al., 2006). Subjects were asked to place a mark on the line on the paper according to the general level of perceived soreness at the time of assessment. A blank scale was used each time to avoid bias from preceding ratings.

Muscle biopsy sampling

Biopsies were obtained from m. vastus lateralis in the non-occluded leg before exercise and in both legs 1, 24 and 48 hours after exercise. Hence, the number of biopsies was limited to three for the BFR leg and four in the free-flow leg. The rationale for this design was to minimise any impact of the procedure itself on the muscle samples (particularly those from the BFR leg), as multiple biopsies can inflict muscle damage and negatively influence recovery around the sampled area (Constantin-Teodosiu et al., 1996). During the biopsy, subjects laid supine, and the procedure was performed under local anesthesia (Xylocain® adrenaline, 10 $mg \cdot ml^{-1}$ + 5 $\mu g \cdot ml^{-1}$; AstraZeneca, Södertälje, Sweden).

The first muscle sample from each leg was taken from the vastus lateralis approximately midway between the origin and the insertion, from a location which was distal to the part that was compressed by the tourniquet during training (occluded leg). In the non-occluded leg, the first incision was taken from the corresponding location and this incision was also used for the second biopsy (1 hour post-exercise) but the needle was angled in a different direction so that the sample was taken about 5 cm from the first. The second (24 h) and third incisions (48 h) were placed approximately 3 and 6 cm proximally to the first incision. Care was taken to avoid affected tissue from earlier biopsies.

Biopsies were taken from 10 out of the 12 participants, i.e., two subjects completed the exercise protocol without any biopsies. Due to some technical difficulties with the biopsy sampling, complete sets of biopsies (including both limbs and all time points) were achieved in 8 of the subjects, therefore N = 8 for tetranectin stainings.

Light microscopic immunofluorescence on cross sections

The muscle samples were rinsed in physiological saline and visible fat and connective tissue were removed. From one part of the biopsy, 8 µm thick cross sections were cut and later stained with antibodies against dystrophin's COOH-terminus (Dys 2, Novocastra Laboratories Ltd, Newcastle, UK) and diluted in blocking solution [1% BSA, and 0.05% Tween-20 in TBS (TBST)] for 2 h at room temperature or overnight at 4°C. As a marker for sarcolemmal permeability, an antibody against tetranectin was used (Hyb-130-11, AntibodyShop, Gentofte, Denmark). Tetranectin is a plasma protein which like albumin and EBD is a good marker for muscle fibre damage resulting from eccentric exercise (Kalhovde, Paulsen, Raastad, et al., in press). The sections were incubated for 45–60 min with an appropriate secondary antibody and mounted with coverslips (Fluoromount-G, Chemi-Teknik AS, Oslo, Norway, or ProLong Gold Antifade Reagent with DAPI, Invitrogen-Molecular Probes, Eugene, OR). The muscle sections were washed in TBST between each step.

Images were captured using a digital camera (Olympus DP72, Olympus-Europa GmbH, Hamburg, Germany) connected to a light microscope (Olympus BX61) with appropriate filters. Pictures were taken with 4x, 10x, 20x, and 40x magnification objectives, where 4x was used for overview images and 10x was used for evaluations of the number of positive fibres. Intact fibres on each sample were counted, but the outermost layer of muscle fibres in the samples was excluded in order to avoid areas damaged by sectioning, as were other areas and fibres with visible artifacts (folds, tears, etc.). The mean number of counted fibres for each time point per individual was 238 (range 43-882). The program Axiovision (Zeiss, Oberkochen, Germany) was used to analyse the images and the degree of staining for tetranectin was determined with the measure mean green density (MGD). The stainings for the COOH-terminus were inspected to help identification of the borders of the muscle fibres.

Evaluation of tetranectin staining

McNeil & Khakee (1992) and Clarke et al. (1993) used threshold values for determining the number of albumin-positive fibres in the sections. A somewhat similar threshold-based model was adopted for the purposes of the present investigation. The contours of each counted fibre were outlined just beneath the sarcolemma, and the MGD value of the cytoplasm was measured with the Axiovision program. Pilot studies indicated that subjectively determined positive fibres had an intracellular staining (as indicated by MGD) at least 50% stronger than presumably negative fibres. This corresponded to about three standard deviations above the mean of negative fibres in a baseline sample. Therefore, this threshold was chosed for MGD.

Four presumably negative fibres from each section, two peripheral and two centrally located fibres, were chosen based on having the lowest MGD values of the peripheral and central fibres, respectively. Central fibres were within the middle third of the length and the middle third of the width of the picture, and peripheral fibres were consequently located outside these limits. The mean MGD of these four fibres was calculated and fibres with a staining ≥50% stronger than this mean were counted as tetranectin-positive. Values for positive fibres are given as percentage (%) of the total number of counted fibres.

Statistical analyses

To identify statistically significant changes in muscle function variables and tetranectin from baseline, one-way repeated-measures ANOVA with Dunnett's post hoc test was performed for both the BFR and the free-flow leg. A two-way ANOVA (with Bonferroni's post hoc test) was used to assess differences between the legs. Unless otherwise indicated, data are presented as means ± SE. The level of significance for all statistical analyses was $p \leq 0.05$.

Results

Exercise Protocols and Muscle Function Testing

The number of repetitions performed before concentric torque failure gradually decreased from 28±5 in the 1st set, to 10±2 in the 2nd set and to 6±1 in the 5th set (means ± SD). MVC was reduced more in the occluded leg than in the non-occluded leg when measured one and two minutes after exercise (-62±15% vs. -22±4%, and -61±14% vs. -21±4%, $p<0.01$, means ± SD) when partial BFR was still maintained, but not at later time points (Figure 1). At 4 hours post-exercise, MVC was reduced by 15±4% in the occluded leg and 12±3% in the nonoccluded leg. At 72 hours post-exercise, the MVC was no longer significantly different from baseline, although a trend was noted in the BFR leg (10±4% reduced MVC, $p<0.10$).

The 20/50Hz ratio decreased in the occluded leg at 3 minutes post-exercise, but was no longer significantly lowered at 4 hours post. In the non-occluded leg, no significant changes were noted at any time point. Only at 3 minutes post-exercise was the difference between the conditions significant ($p<0.01$).

The passive tension increased for the occluded leg (8.5±2.3%, $p<0.01$) and also for the free-flow leg (5.3±2.3%, $p<0.05$) at 3 minutes post-exercise (Figure 2). Thereafter, passive tension declined gradually in both legs with the last significant time point for the occluded leg at 24 hours post. At no time point was the difference between the conditions significant.

Muscle soreness was increased at 24-72 hours after exercise in both legs ($p<0.01$ for all three time points in both legs, Figure 3) and was not significantly different between the conditions, but when a t-test was used to compare the highest values irrespective of the time points, peak DOMS was greater for the BFR leg ($p<0.05$).

Tetranectin immunofluorescence on cross sections

The percentage of muscle fibres showing elevated tetranectin staining increased from 9% before exercise to 31% at 1h and 38% at 24h ($p<0.01$) and 27% at 48h post-exercise ($p<0.05$) in the occluded leg (Figure 4). For the non-occluded leg, tetranectin-positive fibres were significantly increased only at 24h post-exercise (18%, $p<0.05$). At 24h, the percentage of muscle fibres with elevated tetranectin staining was significantly greater in the occluded leg ($p<0.05$). Control markings with a secondary antibody only showed very faint staining and no changes with exercise. Figure 5 shows examples of the tetranectin stainings.

To gain insight into whether one or both of the major fibre types (type I and II) showed increased tetranectin, a subsample of muscle sections was stained with SC-71, which marks type IIa myosin and also to a lesser extent type IIx (Smerdu & Soukup, 2008). They were then compared with neighbouring sections stained with tetranectin. These comparisons indicated that the majority of the tetranectin-positive muscle fibres were type I fibres (Figure 6).

Discussion

There were three major findings of the present study: firstly, there were markedly larger acute reductions (at 1 and 2 minutes post-exercise) in MVC in the occluded leg compared to the non-occluded leg. Secondly, part of the decline in MVC was longer-lasting (at least 48 hours), especially in the occluded leg. Thirdly, an increased occurence of fibres with elevated tetranectin was observed after exercise in both legs, especially in the occluded leg.

Acute force decrements

A large part of the acute fatigue after exercise in the blood flow-restricted leg was probably of a metabolic origin, as the isometric force improved vastly between the release of the pressure cuff and the 4 hour post-exercise MVC measurements. Low-load coupled concentric-eccentric knee extensions to exhaustion with complete cuff occlusion have been demonstrated to lead to depletion of phosphocreatine (PCr) levels in muscle fibres from the vastus lateralis (Ingemann-Hansen et al., 1981). Similarly, Krustrup et al. (2008) reported that when submaximal concentric knee extensor exercise was combined with complete restriction of blood flow, both type I and type II fibres were depleted of PCr. With PCr breakdown, there is an increase in inorganic phosphate (P_i). Because P_i is an important factor in muscle fatigue (Allen et al., 2008), accumulation of P_i is likely contributing to the decline in MVC as well as the slow recovery observed while the cuff was still inflated.

When the blood flow is totally occluded, almost no recovery of PCr levels and force production capability takes place (Harris et al., 1976; Colliander et al., 1988; Meyer et al., 2008). Meyer et al. (2008) reported further that partial occlusion (~120 mm Hg, cuff width not stated) in combination with exercise resulted in a slower recovery of PCr compared to free-flow conditions. Similarly, the partial occlusion in the current experiment impacted on the recovery, as demonstrated by the lack of increase in MVC between 1 and 2 minutes post-exercise. In contrast, as little as 1 minute of free circulation results in a marked recovery of MVC after BFR exercise (Yasuda et al., 2008, 2009).

In addition to P_i, hydrogen ions caused by production of lactic acid may also play a role. Although reduced pH alone has little effect on force and velocity (Allen et al., 2008), data from Karatzaferi et al. (2008) suggests that low pH and phosphorylation of myosin regulatory light chains have additive effects to that of P_i on the velocity of shortening and the isometric force of single muscle fibres at close to physiological temperatures. As a bout of fatiguing low-load occlusion training results in large increases in intramuscular lactate (Ingemann-

Hansen et al., 1981), lowered pH in plasma (Fujita et al., 2007) and in depletion of phosphocreatine (Krustrup et al., 2008), we suggest that accumulation of phosphate ions and hydrogen ions are at least in part responsible for the acute fatigue in occlusion training.

To some extent, a decrease in muscle activation could also be involved. Using a similar BFR exercise protocol as in the current study, we have noted reduced electromyography (EMG) levels (~72% of baseline) during an MVC at the 1 minute post-exercise time point while the cuff was still inflated. In contrast, the EMG of the non-occluded leg was almost unchanged (~95%) at the same time point, even though both the non-occluded and occluded limbs were exercised to the point of concentric torque failure (Wernbom et al., unpublished). Kent-Braun (1999) has suggested that pH has a role in feedback to the central nervous system, which would be a plausible mechanism for a lower EMG at a post-exercise MVC with maintained partial occlusion versus with free circulation. However, other potential causes, such as direct effects of cuff occlusion on the nerves and/or the muscles, cannot be ruled out.

Prolonged force decrements and increased resting tension

Regarding the longer-lasting part of the force decrements, we believe that this reflects some degree of muscle damage, and the increases in tetranectin staining and resting tension lend support to this view. Although much of the immediate post-exercise increase in resting tension was probably due to acute muscle swelling, at the later time points (from 4 hours up to at least 24 hours) it may in part have been caused by injury contractures in damaged muscle fibres, similarly to the proposed scenario for eccentric exercise (Whitehead et al., 2001). Alternatively, the effects of muscle damage on resting tension were indirect and mediated by muscle swelling.

The decrease in MVC in the present study appeared to be smaller than what is seen after acute bouts of severe high-force eccentric exercise (e.g., Hellsten et al., 1997, Brown et al., 1997; Paschalis et al., 2005; Raastad et al., 2010). The BFR exercise protocol was predominantly concentric, and submaximal eccentric muscle actions are known to induce less damage and decline in muscle function than maximal eccentric muscle actions (Paschalis et al., 2005). Further explanations include the comparatively low volume and that the duration of ischemia may not have been long enough to induce major damage.

Nevertheless, at least one "high-responder" could be identified in the present study based on MVC decrements of 46-49% at 48-72 hours post-exercise, and we have observed previous cases of high-responders as judged from extreme DOMS and long-lasting (1-2 weeks) decreases in muscle function after a bout of multiple sets to failure (Wernbom, unpublished observations). Therefore, we believe that this mode of training should be introduced carefully so as to not produce excessive damage in the initial stages of training.

Elevations in tetranectin staining

The molecular weight of tetranectin in humans was reported to be approximately 68 kDa (Clemmensen et al., 1986), making it slightly heavier than albumin (67 kDa). New data from our laboratory (Kalhovde et al., in press) suggests that severe eccentric exercise causes markedly increased intracellular tetranectin staining. Interestingly, strong granular-like staining appeared at the same locations as myofibrillar disruptions, suggesting that this pattern may be a good indication of myofibrillar damage and subsequent remodelling.

In the current study, fibres with a strong granular-like staining were also observed and quite frequently so in some samples, suggesting that low-load BFR resistance training can induce myofibrillar disruptions. Nevertheless, in other samples, such staining patterns were

almost absent despite visibly elevated intracellular tetranectin fluorescence in the muscle fibres. Thus, the dominant pattern of tetranectin staining was a general and diffuse increase in the cytoplasm.

The cytoplasmic presence of tetranectin suggests an increased sarcolemmal permeability. We base this on the following arguments: firstly, tetranectin does not normally accumulate in any appreciable amounts in adult human muscle tissue (Albrechtsen & Wever, unpublished, cited in Xu et al., 2001). Secondly, not only the early but also the later increases in immunostaining seem too rapid to be accounted for by any elevations in de novo synthesis. Wewer et al. (1998) found no staining in injured myofibres from mice until at 3 days post-injury. Thirdly, in several of the tetranectin-positive fibres, accumulation was visible not only in the sarcoplasma but even more so at and just beneath the sarcolemma (Figures 5 and 7). Finally, in situ hybridization suggests that tetranectin is not produced intracellularly in human skeletal muscle after exercise-induced damage (Kalhovde et al, in press).

The increase in staining intensity in tetranectin-positive fibres was typically in the range of 50-80%, suggesting mild damage; but some fibres exceeded 100% (and even 200-500% for strongly granular fibres). Similarly, variations in the staining of albumin, EBD and Procion Orange are evident in the literature; e.g., in the papers of McNeil & Khakee (1992), Petrof et al. (1993), Zhu et al. (2000), Xu et al. (2008) and Ojima et al. (2010). The elevated tetranectin staining at the later time points is consistent with observations on EBD, which can be detected in muscle fibres for several days after exercise-induced damage (Xu et al., 2008).

Possible causes of muscle damage with BFR resistance exercise

We postulate that the combination of exercise and ischemic conditions in the working muscle may be a major cause of damage with BFR resistance exercise. Importantly, muscle activation

during anoxia shortens the time required for increased Ca^{2+} influx to take place (Fredsted et al., 2005). A possible mediator of sarcolemma damage is phospholipase A2 (PLA2) (Duncan & Jackson, 1987). Isoforms of PLA2 in skeletal muscle are activated by several stimuli, including ischemia, exercise, stretch and cell swelling (Ørtenblad et al., 2003; Burkholder, 2009). Activation of PLA2 results in the production of arachidonic acid (AA) and metabolites downstream of AA such as prostaglandins and leukotrienes, and in lysophopholipids such as lysophosphatidylcholine (LPC) (Ørtenblad et al., 2003; Burkholder, 2009). Both the AA and the LPC arms of the PLA2 pathway may cause increased production of reactive oxygen species and taurine loss from myofibres (Ørtenblad et al., 2003).

Interestingly, Umbel et al. (2009) reported DOMS, muscle swelling and decreased muscle torque after a bout of pure concentric knee extensions with BFR (3 sets to failure at 35% of MVC). This suggests that ischemia, like anoxia, increases the susceptibility of the working muscle so that normally not damaging exercise may become damaging. Another factor may be the eccentric muscle actions that are inherent in most BFR strength training regimes. We have previously shown that muscle EMG increases not only during the concentric phase but also during the eccentric phase as the point of concentric torque failure draws closer (Wernbom et al. 2009).

The tetranectin uptake appeared to be most pronounced in type I fibres (Figure 6). We speculate that being recruited already from the start, the type I fibres may have subjected to longer durations of stress than the type II fibres. The data on MVC, DOMS and tetranectin indicate that some damage occurred also in the free-flow leg, which is consistent with studies on fatiguing resistance exercise at 40-50% of 1RM (Volek et al., 2002; Pullinen et al., 2010). We estimate that during the first two or three sets in the free-flow leg, our subjects had only 3-5 repetitions left before failure, based on their reports and our previous data.

Relevance to previous BFR resistance exercise studies

In this study, all five BFR exercise sets were performed "all-out" to concentric torque failure, whereas in some of the high-frequency training studies (Abe et al., 2005; Fujita et al., 2009), it is unclear whether subjects had performed all-out in any set during training. For example, using an identical protocol to that of Fujita et al. (2007; 2009), Fry et al. (2010) noted that at the end of the BFR exercise bout, their subjects reported values of 7-8 on a 0-10 scale of perceived exertion, indicating a high but still submaximal level of effort. This is an important point, as the muscle activity is at its highest in the last few repetitions in each set (Wernbom et al., 2009), and these may also coincide with the lowest oxygenation and energy levels.

With regard to increased membrane permeability, it should be noted that serum CK is a less sensitive marker than myoglobin (Virtanen et al., 1993), possibly due to its higher molecular weight (88 kDa vs 18 kDa). Indeed, serum myoglobin, but not CK, increased ~5-6 times over baseline at 30-120 minutes after a strenuous squat protocol (15-20 repetitions at 50% of 1RM) known to cause exercise-induced hypoxia (Volek et al., 2002). However, delayed elevations (48-96 hours) of myoglobin and CK have been shown after acute bouts of fatiguing resistance exercise at 40-50% of 1RM (Volek et al., 2002; Pullinen et al., 2010).

It is thus possible that previous studies on BFR resistance exercise may have missed both early and late elevations of serum markers, and/or that the markers employed have not been sufficiently sensitive. Alternatively, the protocols were not severe enough to induce marked increases in membrane permeability. Future studies should investigate multiple markers of muscle damage over an extended time-course after acute bouts of BFR exercise.

Limitations

Because we used threshold values as a basis for the evaluation of the stainings, we may have missed more subtle changes in membrane permeability, especially in the type II fibres. Furthermore, we obtained samples only from the vastus lateralis, and the biopsies were taken from areas distal to the tourniquet. Thus, we cannot exclude that greater damage may have occurred in the other muscle bellies, and/or in the muscle tissues beneath the pressure cuff. The potential for local muscle damage is further underscored by the recent observations of Kacin & Strazar (2011) of attenuation of muscle hypertrophy in the quadriceps and even muscle atrophy of the vastus intermedius at the levels beneath the cuff after short-term low-load resistance exercise with BFR at high occlusion pressures (>230 mm Hg)

Conclusions and perspectives

Based on the prolonged torque decrements (≥48 hours) and the indications of increased membrane leakiness, there appears to be a potential for muscle damage with a first-time bout of BFR resistance exercise, especially with multiple sets of all-out efforts. However, temporary elevations in sarcolemmal permeability are not necessarily only associated with damage; mild to moderate changes could also contribute to mechanotransduction processes (Clarke et al., 1993; Grembowicz et al., 1999; Miyake & McNeil, 2003), and thus potentially provide insights into the mechanisms of muscle hypertrophy with BFR exercise.

Nevertheless, the potential for damage to local tissues, as well as the still relatively unexplored systemic responses of BFR resistance exercise (possibly including both beneficial and harmful effects), need to be addressed further. For example, repeated bouts of high venous pressure may constitute one factor underlying the development of varicosities and valvular insufficiencies in superficial veins (Kölegård & Eiken, 2011).

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Figure Legends

Figure 1. MVC before and after the exercise protocol. *, $p<0.05$; significantly different from pre, **, $p<0.01$. ##, $p<0.01$; significantly different from free-flow leg.

Figure 2. Resting tension before and after the exercise protocol. *, $p<0.05$; significantly different from pre.

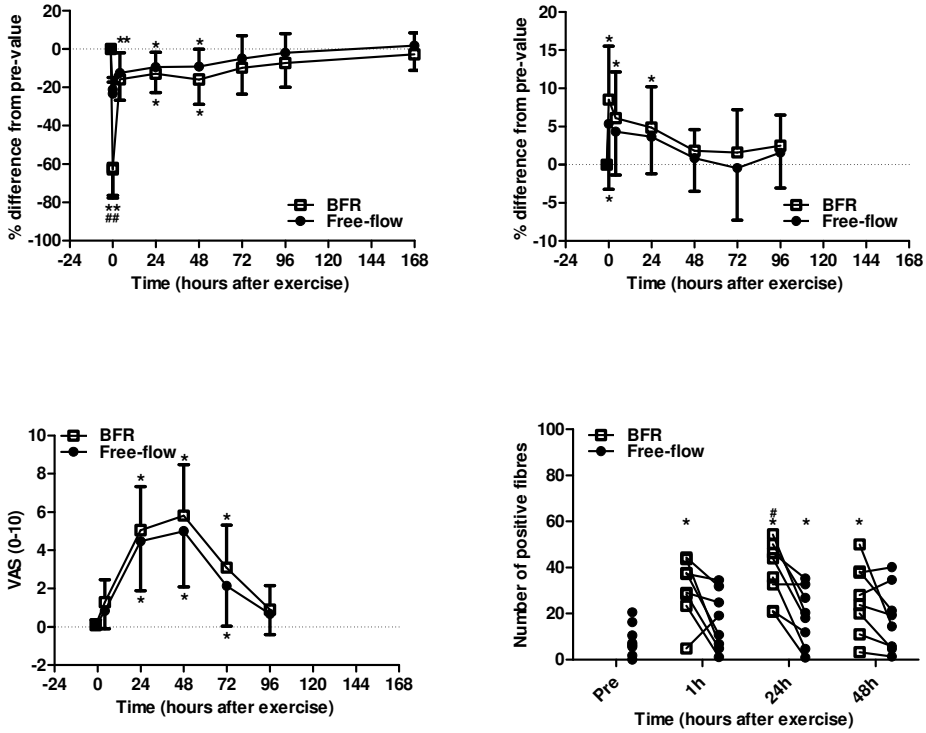
Figure 3. Soreness (VAS-scale) before and after the exercise protocol. *, $p<0.05$; significantly different from pre.

Figure 4. Tetranectin-positive fibers before and after the exercise protocol. *, $p<0.05$; significantly different from pre, #, $p<0.05$; significantly different from free-flow leg.

Figure 5, a-d. Examples of tetranectin stainings from the same subject, before and 1 and 48 hours after BFR-exercise, and control staining with secondary antibody only. Note the increased number of diffusely stained fibers, as well as some granular fibers, particularly at 48 hours post-exercise in this example.

Figure 6, a-d. Examples of tetranectin stainings vs fiber types from two subjects, 1 hour postexercise. In both these cases, two sections were stained with SC-71 in order to identify the tetranectin-positive fibers, but only one section is shown here. Note that type I fibers show a generally greater response than type II fibers in these examples.

Figure 7, a-c. Examples of tetranectin and dystrophin stainings at 40x magnification, from the same subject as in Figure 6a & b,, 1 hour after BFR-exercise. Note the tetranectin staining at the membrane in some of the positive fibers, resulting in yellow/orange colours in the merged picture. Note also that some of the type II fibers (refer to Figure 6a & b) have weak tetranectin staining at the membrane and very fine granular staining in the cytoplasm.



Figures 1-4. MVC, resting tension, DOMS (VAS-scale) and tetranectin-positive fibers.

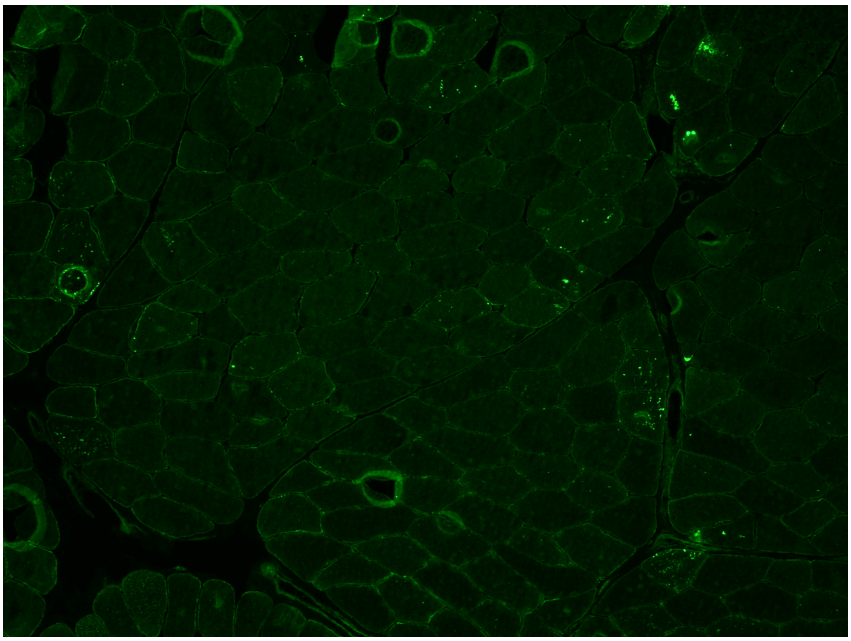


Figure 5a, pre-exercise, tetranectin

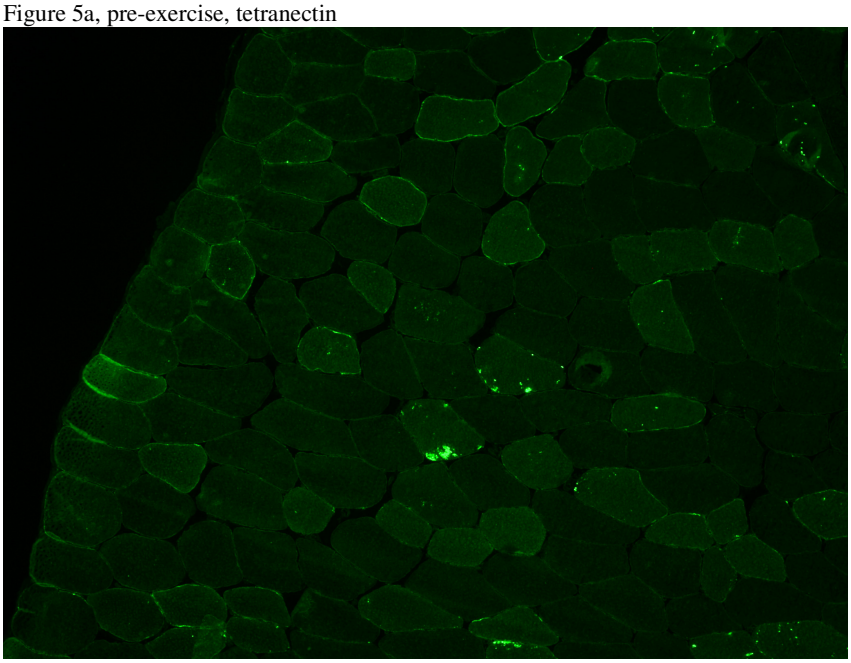


Figure 5b, 1h post-BFR-exercise, tetranectin

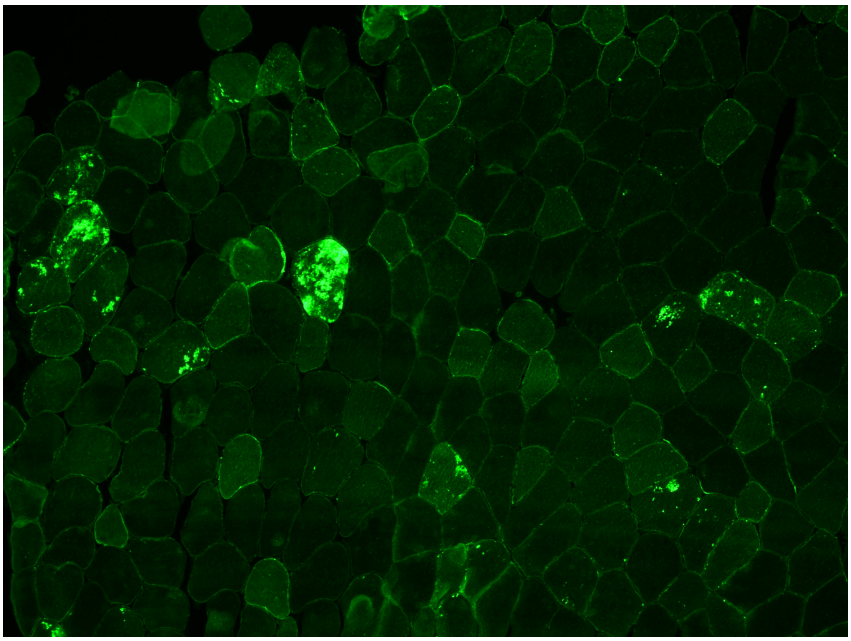


Figure 5c, 48h post-BFR-exercise, tetranectin

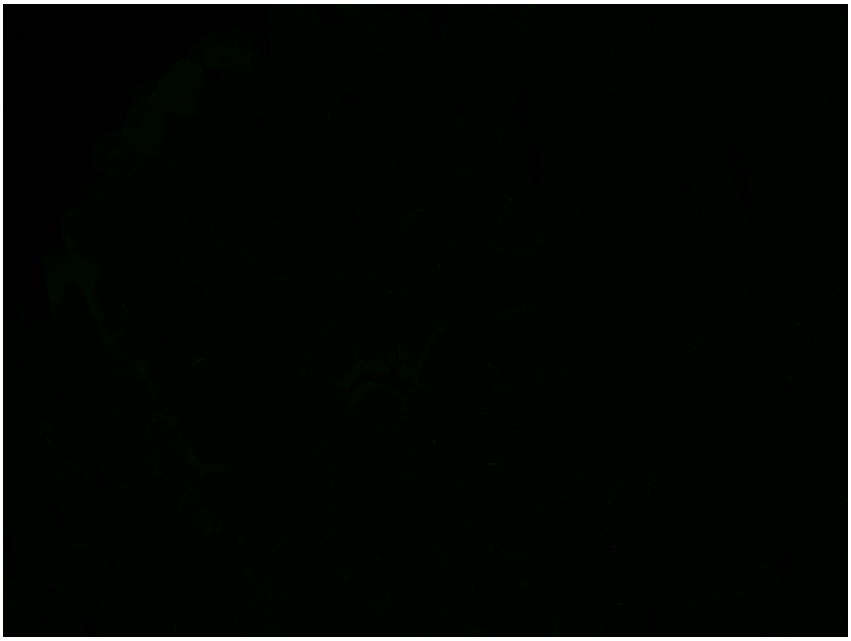


Figure 5d, 48h post-BFR-exercise, secondary antibody

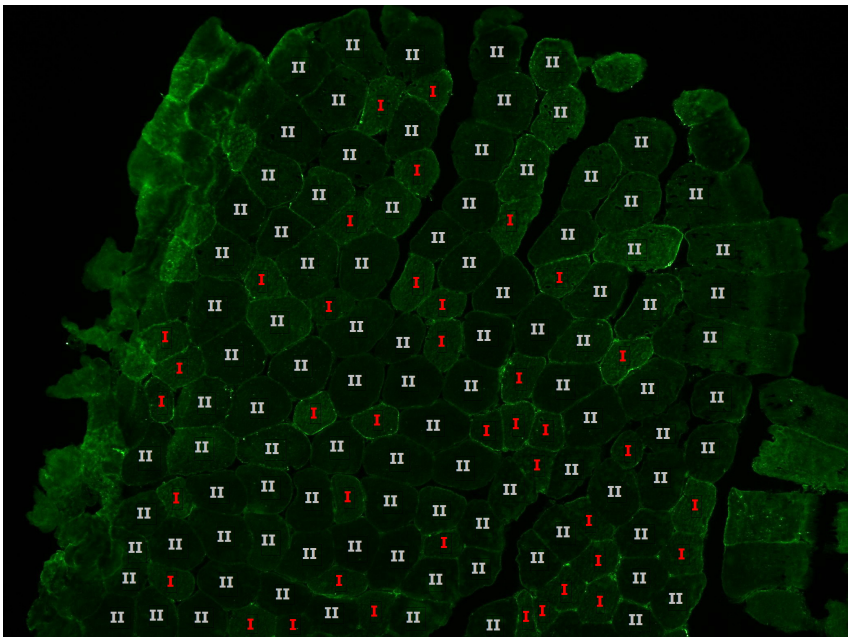


Figure 6a. Subject #2, 1h post-BFR-exercise, tetranectin staining vs fiber type.

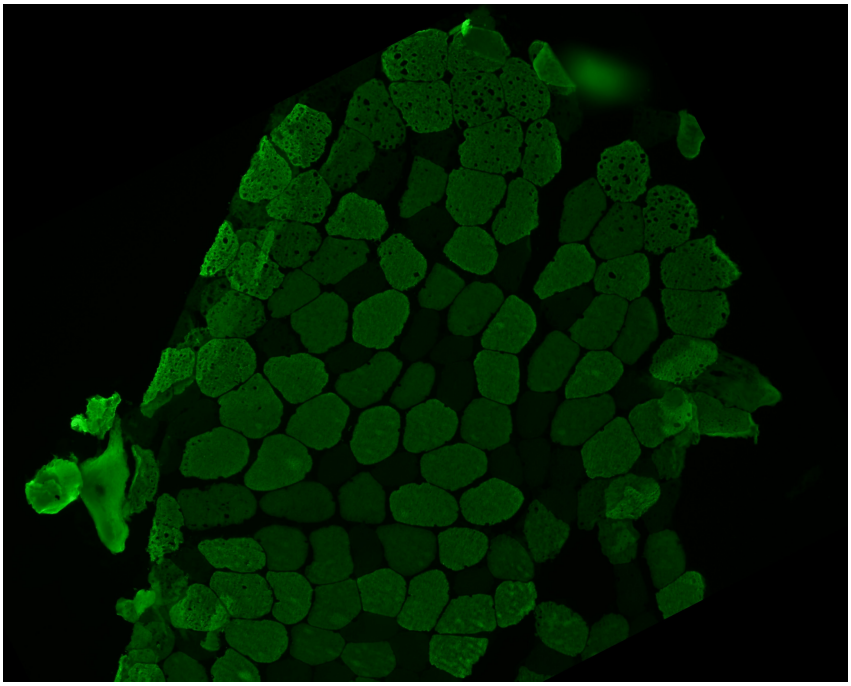


Figure 6b. Subject #2, 1h post-BFR-exercise, SC-71 staining.

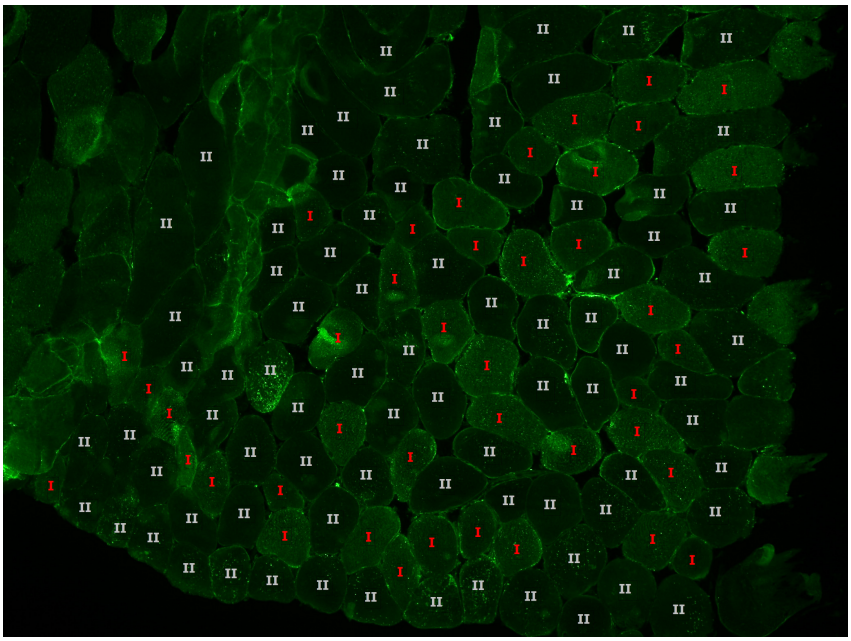


Figure 6c. Subject #3, 1h post-BFR-exercise, tetranectin staining vs fiber type.

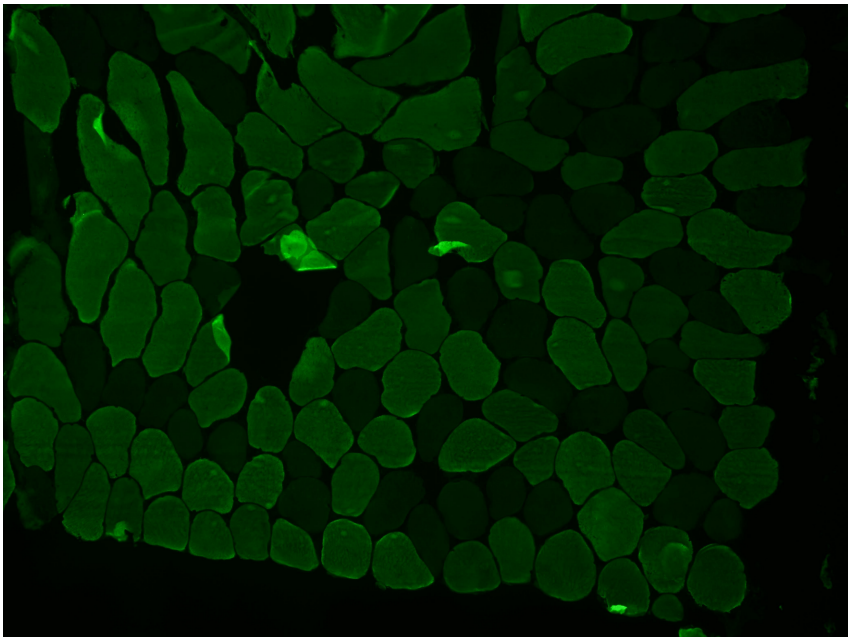


Figure 6d. Subject #3, 1h post-BFR-exercise, SC-71 staining.

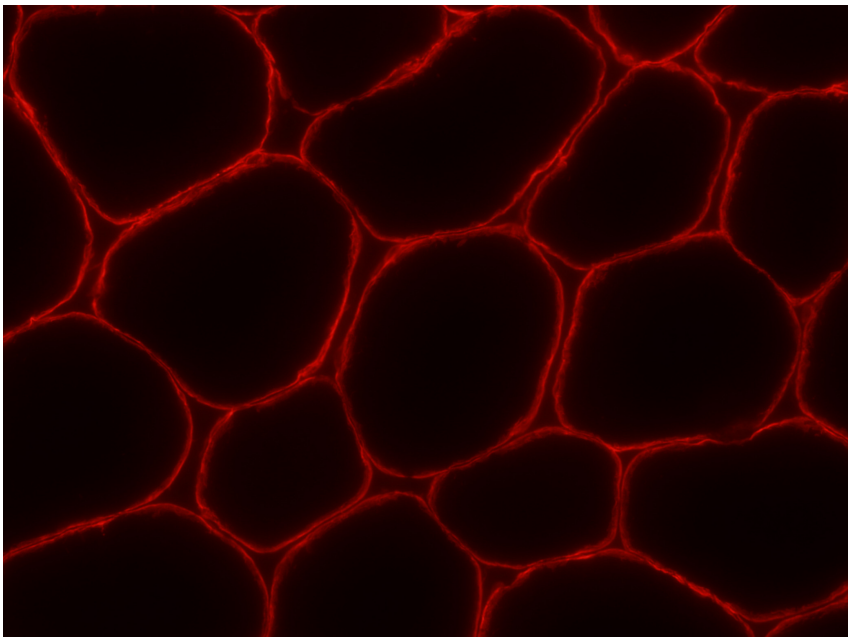


Figure 7a. Subject #2, 1h post-BFR-exercise, dystrophin staining.

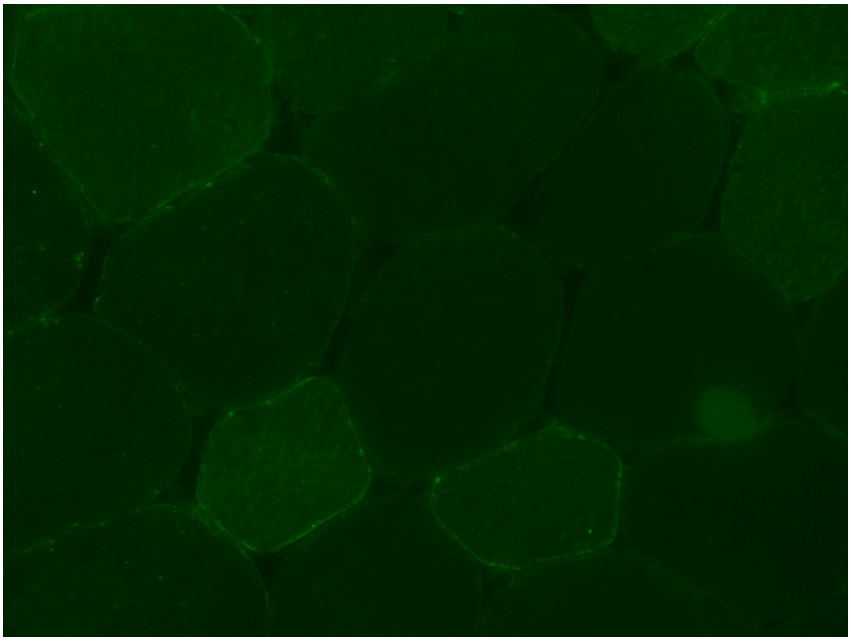


Figure 7b. Subject #2, 1h post-BFR-exercise, tetranectin staining.

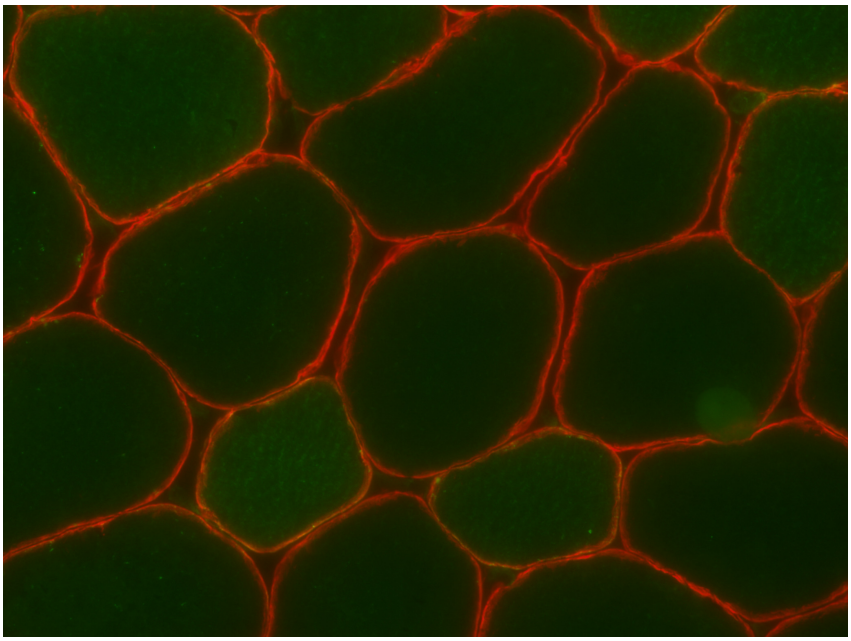


Figure 7c. Subject #2, 1h post-BFR-exercise, dystrophin + tetranectin staining (merged).

PAPER IV

Resistance exercise with blood flow restriction increases protein signaling and satellite cell numbers in human skeletal muscle

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Abstract

The purpose of this study was to investigate hypertrophic signaling at multiple time points after a single bout of low-intensity resistance exercise with and without blood flow restriction (BFR). Seven healthy subjects performed unilateral knee extensions at 30% of their one repetition maximum (1RM), with partial BFR on one leg and the other leg with free blood flow. With the BFR leg, five sets were performed to concentric torque failure, and the free-flow leg repeated the exact same number of repetitions and sets at the same session. Biopsies were obtained from vastus lateralis before and 1, 24 and 48 hours after exercise. At 1 hour post-exercise, phosphorylation of p70S6k (p-p70S6k) at Thr^{389}, a key marker for muscle protein synthesis, was elevated for the BFR leg, whereas in the free-flow leg, p-p70S6k (Thr^{389}) was unchanged. At 24 hours post-exercise, both legs had similarly elevated p-p70S6k (Thr^{389}), while at 48 hours p-p70S6k (Thr^{389}) was essentially back to baseline in both legs. The phosphorylation of p38 was significantly increased in the BFR leg at 1 hour post-exercise. The number of visible satellite cells per muscle fibre on cross-sections was increased from baseline for all post-exercise time points and in both legs (33-53%). Multiple sets to failure of unilateral low-load resistance exercise with BFR can result in an enhanced phosphorylation of p70S6k (Thr^{389}) up to a least 24 hours post-exercise, an early phosphorylation of p38, and a greater number of visible satellite cells per muscle fibre, all of which may have implications for the hypertrophic response to BFR resistance exercise. However, the responses in the free-flow leg for p70S6K and satellite cells suggest that fatiguing low-load resistance exercise can be effective even without BFR.

Key words: occlusion training, blood flow restricted exercise, ischemic strength training, anabolic signaling.

Introduction

During the past decade, low load resistance exercise (20-50% of 1 RM) in combination with blood-flow restriction (BFR) by the use of pressure cuffs has repeatedly been shown to induce muscle hypertrophy in untrained individuals (reviewed by Wernbom et al., 2008). Resistance exercise with BFR, often also referred to as strength training with vascular occlusion, can also induce gains in muscle strength and size in the elderly (Karabulut et al., 2009) as well as in trained athletes (Takarada et al., 2002). Furthermore, low-load BFR training can counteract disuse muscle atrophy in healthy individuals (Cook et al., 2010) and in patients recovering after anterior cruciate ligament surgery (Ohta et al., 2003). Taken together, these findings suggest that low-intensity strength training with BFR has broad potential applications.

In some studies, BFR training regimes with a total of 12 sessions per week have been tried successfully over short periods (1-2 weeks), resulting in significant increases in muscle cross-sectional area (CSA) and strength despite the very short training periods (Abe et al., 2005; Fujita et al., 2009). However, marked hypertrophy has been noted even with only 2 sessions per week of strength training with multiple sets to failure during partial vascular occlusion (Takarada et al., 2002, 2004), and the rates of quadriceps CSA gains in these studies (0.18-0.22% per day) were equal to or even higher than the rates typically resulting from multiple-set heavy resistance training at the same frequency of training (reviewed by Wernbom et al., 2008). Collectively, these findings suggest that fatiguing low-load strength training with partial BFR can induce long-lasting elevations in muscle protein synthesis (MPS) and anabolic signaling, similarly to conventional heavy resistance training.

Also similarly to heavy resistance exercise, low-load BFR training appears to increase MPS primarily through the mTOR-p70S6k pathway (Fujita et al., 2007, Fry et al., 2010). However, studies on acute anabolic signaling with BFR training have to date mainly focused

on the early time period (0-3 hours) after exercise, thus little is known about the time course of hypertrophic signaling after an acute bout of strength training with BFR.

In addition to protein synthesis, successful long-term muscle hypertrophy appears to require myonuclear addition, which in turn is dependent on satellite cell (SC) activation. In a recent study of functional overload in rats, it was even reported that myonuclear addition preceded gains in muscle mass (Bruusgaard et al., 2010). Furthermore, there is good evidence that in humans, large increases in myofibre area in response to strength training are dependent on myonuclear addition (Kadi et al, 1999; Petrella et al., 2008).

To date, however, little has been published in terms of the effects of BFR resistance exercise on SCs, myonuclei, and myogenic factors. Drummond et al. (2008) reported increases in the SC activity markers p21 and MyoD and decreased myostatin mRNA at 3 hours after BFR-resistance exercise (4 sets of 15-30 repetitions at 20% of 1RM), while the mRNA for myogenin, cyclin D1 and the insulin-like growth factor 1 (IGF-1) isoform MGF did not change. Interestingly, the control training at the same load without occlusion induced similar changes as the BFR training. In contrast, Manini et al. (2011) found no significant changes in the mRNA for MyoD, myogenin, myostatin, and IGF-1. However, no quantification of the SC numbers was performed in either of these studies.

Therefore, the aim of the current investigation was to study the acute response to a BFR resistance exercise session up to 48 hours post-exercise, with reference to anabolic signaling and the number of SCs.

Materials and methods

Experimental Design

To examine the acute effects of unilateral dynamic low-intensity strength training with cuff occlusion on hypertophic signaling in the vastus lateralis, a within-subjects study design was used in which one leg (dominant or non-dominant in a randomised fashion) was exercised with cuff occlusion and the other leg was trained without occlusion. To match the volume of work, and because BFR reduces the number of repetitions to failure at low loads (Wernbom et al., 2009), the leg that was randomised to cuff occlusion was always trained first.

All subjects participated in a familiarisation session one week (range 4-11 days) before the main experiment. In the main trial, the subjects were tested for maximal voluntary isometric strength (MVC) immediately before the exercise bout, at 1 and 2 minutes post-exercise, and at 4, 24, 48, 72, 96 and 168 hours post-exercise. Detailed strength and recovery data are reported in a separate publication (Wernbom et al., 2011, manuscript in submission).

Biopsies were obtained from vastus lateralis before and 1, 24 and 48 hours after exercise. The pre-biopsy, as well as the 24 hour and the 48 hour biopsies were collected before strength testing commenced, while the 1 hour post-exercise samples were taken after the exercise protocols had been completed. Because of the limited number of maximal isometric muscle actions, we considered any influence of the MVC's on muscle signaling to be negligible. The subjects were studied in a fed state, and they were instructed to consume breakfast ~3 hours before reporting to the laboratory on the days when biopsies were taken. The rationale for this was that overnight fasting is not representative of the typical strength training situation, and that we wanted to see if previous findings of BFR resistance training in the fasted state on muscle signaling would extend also to training in the fed state. Nevertheless, it should be noted that the subjects were studied ≥3 hours after feeding with

regard to the pre-exercise biopsy and the 24 hour and 48 hour biopsies, and ≥4 hours after feeding with regard to the 1 hour post-exercise biopsy.

After the 1 hour, 24 hour and 48 hour post-exercise biopsies had been completed, the participants were fed a meal from the cafeteria. They were instructed not to change their dietary habits during the time course of the study, and were asked to refrain from any drinks that contained caffeine or alcohol, as well as not to take anti-inflammatory drugs and nutritional supplements (e.g. amino acids, creatine, and exogenous anti-oxidants) that could impact on the results of the investigation. They were also instructed not to perform any strenuous activities involving their quadriceps during the last 72 hours before the main test.

Subjects

Six male subjects and one female subject (mean $\pm$ SD, 26 $\pm$ 3 yr, height 179 $\pm$ 7 cm, body mass 80 $\pm$ 12 kg) were recruited from the student populations at the Norwegian School of Sports Science in Oslo and the School of Sports Science at the University of Gothenburg. All of the participants were physically active and had previous experience with weight training, and some of them were performing heavy strength training on a regular basis.

The subjects were informed of the experimental risks and signed an informed consent document prior to the investigation. The study was approved by the Regional Ethics Committee of Southern Norway and complied with the standards set by the Declaration of Helsinki regarding the use of human subjects as research participants.

Muscle function testing and familiarisation protocols

4-11 days before the main exercise test, the subjects were tested for their unilateral 1RM strength for each leg in a variable resistance knee extension machine (Leg Extension model 66478, Gym2000, Geithus, Norway). The subjects warmed up 5 minutes on a stationary

bicycle at a light load (~75 Watts) and then moved to the knee extension apparatus. Because the participants were all familiar with the knee extension exercise, only one session was devoted to strength testing. The 1RM was determined according to the procedures of Staron et al. (1990).

After the 1RM for each leg had been established, the subjects practiced MVC's in the same knee-extension machine, with the lever arm of the machine fixed at an angle corresponding to 90 degrees of knee flexion (full knee extension is here defined as 0 degrees). After resting for 5 minutes after the MVC testing, a load of 30% of 1RM was chosen and the subject was then familiarised with the partial vascular occlusion and the cadence of repetitions to be used during the main test. For each leg, one set of 15 coupled concentric-eccentric knee extensions was performed with cuff occlusion, followed by a second set of 5 repetitions after 45 seconds of rest, during which the partial occlusion was maintained. This was done to confirm that the subjects tolerated the pressure to be used during the main experiment.

Main exercise and testing protocols

At a subsequent session after the 1RM testing, with ~7 days between the sessions (range 4-11), the subjects reported to the laboratory for the main test. The subjects warmed up 5 minutes on a stationary bicycle at a light load (~75 Watts) and then moved to the knee extension apparatus. After warming up with three submaximal isometric contractions with gradually increasing effort for each contraction, the subjects performed two MVCs.

After 5 minutes of rest, the subjects performed unilateral knee extension exercise at 30% of 1RM during partial blood flow restriction (see further below). After inflation of the tourniquet, the subjects did as many repetitions as possible for a total of five sets for the occluded leg. The cuff pressure was maintained during the whole exercise bout, including the rest periods and was released after the post-exercise testing had been completed (total time

under partial occlusion: ~9 minutes). The subjects remained seated in the machine during the rest periods, and the rest period between each set was 45 seconds for both the non-occluded and the occluded leg. The range of motion was between 100° and 20° of knee flexion (0° = full extension) and the cadence was 20 repetitions per minute (1.5 seconds each for the concentric and the eccentric muscle actions) which was controlled with a metronome set at 40 bpm. No rest was permitted between the repetitions, and the subjects were instructed to stop the eccentric phase just before the weight stack touched down to avoid relaxation of the quadriceps. The number of complete repetitions performed in each set was noted.

When the five sets for the occluded leg had been completed, and the muscle function tests had been performed, the pressure cuff was deflated. After 10 minutes rest, the subjects performed exactly the same number of sets and repetitions and muscle function testing for the other leg as with the occluded leg, but without BFR.

Blood flow restriction

A curved tourniquet cuff of 135 mm in width with a 100 mm wide pneumatic bag inside of it was connected to a surgical tourniquet system (Zimmer A.T.S. 2000, Zimmer Patient Care, Dover, OH) with automatic regulation of the pressure. The cuff was wrapped around the proximal part of the thigh and inflated to a pressure of 90-100 mm Hg just before exercise with vascular occlusion and kept inflated during the rest periods in between the sets. Using the same type of cuff, we previously demonstrated that this level of pressure results in decreased muscle endurance at ~30% of 1RM, thus confirming that the degree of blood flow restriction was sufficient to affect muscle performance (Wernbom et al., 2009). Our present results also support this, as MVC was reduced to a much greater degree with BFR exercise than with free-flow conditions at 1 and 2 minutes post-exercise (see the results section).

Muscle biopsy sampling

Biopsies were obtained from m. vastus lateralis in the non-occluded leg before exercise and from both legs 1, 24 and 48 hours after exercise, and care was taken to ensure that the time points of the post-exercise biopsies were respected, with reference to the end of the training protocol for each leg. The reason for this design was that we wished to minimise potential artifacts resulting from the biopsy procedure itself when assessing the first post-exercise muscle sample from the occluded leg. During the biopsy, subjects laid supine, and the procedure was performed with a Bergström needle under local anesthesia (Xylocain® adrenaline, 10 mg·ml-1 + 5 μg·ml-1; AstraZeneca, Södertälje, Sweden).

The first muscle sample from each leg was taken from the vastus lateralis approximately midway between the origin and the insertion, from a location which was distal to the part that was compressed by the tourniquet during training (occluded leg). In the non-occluded leg, the first incision was taken from the corresponding location and this incision was also used for the second biopsy (1 hour post-exercise) but the needle was angled in a different direction so that the sample was taken about 5 cm from the first. The second (24 h) and third incisions (48 h) were placed approximately 3 and 6 cm proximally to the first incision. Care was taken to avoid affected tissue from earlier biopsies.

Originally, biopsies were taken from a further three participants, i.e., ten subjects in total, but unfortunately samples were lost for some of the subjects because of a freezer failure during transport, and because of technical difficulties with the biopsy sampling. Complete sets of biopsies (including both limbs and all time points) were achieved in 7 of the subjects for SC numbers. For protein signaling, N = 7 from pre to 24 hours post-exercise, and N = 6 for 48 hours post-exercise.

Tissue processing for protein signaling

Muscle biopsy specimens were freeze-dried, freed of blood and connective tissue, and then homogenized in ice-cold buffer (80 µl/mg dry weight) containing 2 mM HEPES, pH 7.4, 1 mM EDTA, 5 mM EGTA, 10 mM MgCl2, 50 mM β-glycerophosphate, 1% TritonX-100, 1 mM Na3VO4, 2 mM dithiothreitol, 20 µg/ml leupeptin, 50 µg/ml aprotinin, 1% phosphatase inhibitor cocktail (Sigma P-2850), 40 µg/µl PMSF. Homogenates were then centrifuged at 10,000 g for 10 min at 4°C in order to remove cellular debris and the resulting supernatant was stored at -80°C. Protein concentrations were determined in aliquots of supernatant diluted 1:10 in distilled water using a bicinchoninic acid assay (Pierce Biotechnology, Rockford, IL, USA). Samples were diluted in Laemmli sample buffer (Bio-Rad Laboratories, Richmond, CA, USA) and homogenizing buffer to obtain a final protein concentration of 1.5 µg/µl. Following dilution, the samples were heated at 95°C for 5 min to denature proteins present in the supernatant. Samples were then kept at -20°C until further analysis.

Immunoblot analysis

Samples containing 30 µg total protein were separated by SDS-PAGE on Criterion cell gradient gels (4-20 % acrylamide; Bio-Rad Laboratories) for separation of all proteins investigated. Electrophoresis was performed on ice at 200 V for 120 min, following which, the gels were equilibrated in transfer buffer (25 mM Tris base, 192 mM glycine, and 10% methanol) for 30 min in order to optimize transfer. The proteins were then transferred to polyvinylidine fluoride membranes (Bio- Rad Laboratories) at a constant current of 300 mA and 100 V for 3 h at 4ºC, after which the membranes were stained with MemCodeTM Reversible Protein Stain Kit (Pierce Biotechnology) (Antharavally et al., 2004) to confirm equal loading of the samples. All samples were run simultaneously on multiple gels, however,

with all samples from each subject loaded on the same gel always beginning with the pre-exercise sample followed by the free-flow leg and BFR leg samples for each time-point.

Membranes were blocked for 1 h at room temperature in Tris-buffered saline (TBS; 20 mM Tris base, 137 mM NaCl, pH 7.6) containing 5% non-fat dry milk Subsequently, the membranes were incubated overnight with commercially available primary polyclonal phosphospecific antibodies (mTOR Ser^{2448}; p70S6k Thr^{389}; eEF2 Thr^{56}; AMPK Thr^{172}; ERK1/2 Thr^{202}/Tyr^{204}; p38 Thr^{180}/Tyr^{182}; Akt Ser^{473}; p90RSK Thr^{573}; Cell Signaling Technology Inc., Danvers, MA, USA).

Antibodies were diluted 1:1,000 (1:500 for p90RSK Thr^{573} and 1:4,000 in the case of eEF2 Thr^{56}) in TBS supplemented with 0.1% Tween-20 containing 2.5% non-fat dry milk (TBS-TM). After incubation with these primary antibodies, the membranes were washed with TBS-TM and then incubated for 1 h at room temperature with secondary anti-rabbit IgG antibodies conjugated with horseradish peroxidase (diluted 1:10,000; Cell Signaling Technology Inc.). Next, the membranes were washed serially (twice for 1 min followed by three times for 15 min) with TBS-TM followed by 4 additional washes with TBS for 5 min each. Finally, membranes with the antibodies bound to the phosphorylated proteins were visualized by chemiluminescent detection on a Molecular Imager ChemiDocTM XRS system and the bands were analysed using Quantity One® version 4.6.3 software (Bio-Rad Laboratories).

Immunohistochemistry

Serial cross-sections (8 μm) were incubated with antibodies (ab). In order to visualize SCs/myoblasts, sections were analyzed for immunoreactivity against CD56/NCAM (monoclonal ab, ab9018, Abcam; 1:200). Alexa-488 (FITC) (goat anti-rabbit; Invitrogen-Molecular Probes, Eugene, Oregon, USA) was used as a secondary antibody. The sections

were finally counterstained with DAPI (for nuclear staining) and mounted under coverslips (ProLong Gold Antifade Reagent with DAPI, P36935, Invitrogen- Molecular Probes). Images of the stained cross-sections were captured using an Axiocam camera (Zeiss, Oberkochen, Germany) mounted on a Axioskop-2 light microscope (Zeiss). Multiple images (x10, x20, and x40 objectives) were taken so that the whole muscle biopsy cross-section was captured.
A nuclei in which the DAPI staining was surrounded by a ring of NCAM staining and which was localised to the membrane of the muscle fibre in a position consistent with a SC was considered as positive (Petrella et al., 2006; 2008). Dreyer et al. (2006) reported no incidences where a NCAM positive cell was located outside the basal lamina border, which suggests that NCAM stained cells outside the myofibres are rare. Data are presented as number of positive cells per 100 myofibres.

Statistical analyses

To identify statistically significant changes in protein signaling, SCs and MVC from baseline, one-way repeated-measures ANOVA with Dunnett's post hoc test was performed for both the BFR and the free-flow leg. A two-way ANOVA (with Bonferroni's post hoc test) was used to assess differences between the legs. Unless otherwise indicated, data are presented as means ± SE. The level of significance for all statistical analyses was $p < 0.05$.

Results

Exercise performance

The number of repetitions performed before concentric torque failure in the BFR leg decreased from 28±5 in the 1st set to 6±1 in the 5th set, and the total number of repetitions was 57. MVC was reduced more in the BFR leg than in the free-flow leg at one and two minutes post-exercise (with maintained BFR) (-62% vs. -22%, and -61% vs. -21%, $p<0.01$).

Protein signalling

Phosphorylation (p) of Akt, a kinase upstream of mTOR, and p-mTOR (Ser^{2448}) were not significantly altered in any leg at any post-exercise time points. In contrast, after one hour of recovery, p-p70S6k (Thr^{389}) was 2.8 times higher than baseline in the BFR leg ($p<0.05$), whereas it was unaltered in the free-flow leg. As a result, p-p70S6k (Thr^{389}) was greater in the occluded leg ($p<0.05$) than in the free-flow leg. At 24 hours post-exercise, p-p70S6k (Thr^{389}) was ~3.5 times higher in the occluded leg ($p<0.05$) and ~3.8 times higher in the free-flow leg ($p<0.05$) compared to pre-exercise, with no difference between the conditions. At 48 hours, p-p70S6k (Thr^{389}) was no longer elevated over baseline for either of the legs (Figure 1).

P-ERK1/2 (Thr^{202}/Tyr^{204}) did not change significantly compared to baseline at any time point following exercise regardless of the conditions. P-p90RSK (Thr^{573}) was not significantly changed in any leg at any time point following exercise compared to baseline.

For p-p38 Thr^{180}/Tyr^{182}, two bands were seen, and both increased at the 1 hour time point ($p<0.05$) for the BFR leg, but did not change significantly at any other post-exercise time points regardless of the conditions compared to baseline (Figure 2). For p-AMPK (Thr^{172}) and phosphorylation of the eukaryotic elongation factor 2 (eEF2) at Thr^{56}, no significant changes were observed over time in any condition. AMPK is shown in Figure 3.

Satellite cells

The number of SCs per muscle fibre increased from pre-exercise to after exercise for all post-exercise time points and in both legs (33-53%, Figure 4).

Discussion

There were several notable findings of the present study: firstly, there was a prolonged increase (up to at least 24 hours) in the phosphorylation of p70S6k at Thr^{389} in the BFR leg. Interstingly, p-p70S6k (Thr^{389}) was elevated also in the free-flow leg at 24h, but not at 1h. Secondly, the phosphorylations of Akt, mTOR, ERK1/2 and p90RSK, kinases that have a positive influence on p70S6k, did not change along with the p-p70S6k (Thr^{389}). On the other hand, the phosphorylation of p38, another kinase that can positively impact on the mTOR-p70S6k pathway (Cully et al., 2010), increased at 1h in the BFR leg but not at the later time points. Thirdly, the phosphorylation of AMPK did not increase, if anything it tended to decrease with the BFR protocol. Finally, there was a remarkable increase in the number of visible satellite cells after both the BFR and the free-flow exercise protocols.

Responses of p70S6k

The increase in the p-p70S6k (Thr^{389}) in the occluded leg is in line with the results of previous studies. The novel finding in the current investigation is that the possible duration of the elevated p-p70S6k (Thr^{389}) with BFR training is extended to at least 24 hours, when studied in the fed state. As p-p70S6k (Thr^{389}) is a predictor for MPS (Kumar et al., 2009, Burd et al., 2010a) and exercise-induced hypertrophy (Baar & Esser, 1999, Terzis et al., 2008), our observations may help explain why BFR resistance training at 20-50% of 1RM can be effective for hypertrophy even with only 2 sessions per week (Takarada et al., 2002, 2004). The finding of elevated p-p70S6k (Thr^{389}) at 24h also in the free-flow leg deserves comment.

Burd et al. (2010b, 2011) recently demonstrated that 4 sets of knee extensions to failure at 30% of 1RM caused increased MPS and p-p70S6K (Thr^{389}) at 4 hours post-exercise, as well as longer-lasting (at least 24-27 hours) elevations in MPS and mTOR signaling. Our findings with regard to the free-flow protocol are therefore in line with those of Burd et al. (2010b, 2011). Furthermore, the long-lasting (≥24 hours) elevations in p-p70S6K (Thr^{389}) in our study are consistent with reports of Cuthbertson et al. (2006) and Burd et al. (2010a).

We estimate that during the first two or three sets in the free-flow leg, our subjects had only 3-5 repetitions left before failure, based on their reports and our previous experiments. Thus, we speculate that the faster increase in mTOR-p70S6k signaling with the BFR training protocol in the present study was caused by some factor(s) such as greater peak muscle activity and/or a greater volume of high-effort repetitions, but that the training of the free-flow leg was still sufficiently strenuous to induce an increase in p-p70S6k (Thr^{389}) at 24 hours.

Response of p70S6k versus Akt and mTOR

The phosphorylations of Akt and mTOR were not significantly altered in either leg at any post-exercise time points, and if tendencies were present, they were rather weak and did not seem to track with the changes in p-p70S6k (Thr^{389}). These findings are at least in partial agreement with those of Fujita et al. (2007) who observed similar responses in p-Akt and p-mTOR, but clearly divergent p-p70S6k between BFR training and free flow training bouts. Several other studies using either conventional heavy resistance training (e.g., Terzis et al., 2008, 2010; Apro & Blomstrand, 2010), or eccentric strength training (Tannerstedt et al., 2009) have also reported dissimilar trends in p-Akt and p-mTOR vs p-p70S6k (Thr^{389}) after acute resistance exercise, with or without intake of amino acids.

These observations are consistent with reports demonstrating that early mechanically stimulated mTOR-p70S6k signaling is independent of the PI3-kinase/Akt-pathway activated

by IGF-1 (O´Neill et al., 2009; Miyazaki et al., 2011). On the other hand, it appears that production of phosphatidic acid (PA), possibly via activation of phosholipase D (PLD), is necessary for mTOR activation with growth-inducing stretch and exercise protocols (O´Neill et al., 2009).

Responses of eEF2

Although not significant, p-eEF2 (Thr^{56}) tended to decrease at 1h in the BFR leg (27%). A dephosphorylation of eEF2 activates the enzyme, which in turn can lead to an increased protein synthesis. However, in the paper of Fujita et al. (2007), p-eEF2 was similarly decreased in the BFR and free-flow conditions, while only the BFR bout resulted in elevated p-p70S6k (Thr^{389}) and muscle protein synthesis. This may be explained by the fact that translation initiation is the rate-limiting step in protein synthesis (Shah et al., 2000).

Phospho-ERK1/2 and phospho-p90RSK

Similarly to the present study, Terzis et al. (2010) did not observe a p-ERK1/2 response, in their case 30 minutes after a strength training bout. However, both human studies (Karlsson et al., 2004; Tannerstedt et al, 2009) and stretch models of cultured myocytes (Atherton et al., 2009) indicate that phosphorylation of ERK1/2 is quickly induced by mechanical stimuli and then decreases rapidly back to baseline (or below) within 15-60 minutes. In contrast, Fry et al. (2010) reported elevated p-ERK1/2 (Thr^{202}/Tyr^{204}) at 1 hour after BFR resistance exercise in older men. Moreover, Burd et al. (2010b) reported that p-ERK1/2 (Thr^{202}/Tyr^{204}) was elevated at 4 hours post resistance exercise after 4 sets of 30% at 1RM to failure.

Therefore, phosphorylation of ERK1/2 may have occurred in the present investigation, but was not detected due to the 1 hour time-point for the first post-exercise biopsies. Alternatively, the state of feeding (fed vs fasted), and/or differences in the training protocols

may have influenced the results. Regardless, it is interesting that the phosphorylations of ERK1/2 and the downstream effector p90RSK tended to be depressed in the BFR-leg not only at 1h but also at 24h, while p-p70S6k (Thr^{389}) was markedly elevated at both time points.

Phospho-p38MAPK

The increased p-p38 at an early post-exercise time-point in response to BFR exercise is a novel finding. Activation of p38 is associated with hypertrophy in myotubes undergoing mechanical stretch (Hanke et al., 2010) and p38 is strongly activated in skeletal muscle by eccentric exercise (Wretman et al., 2001, Tannerstedt et al., 2009, O'Neil et al., 2009). In C2C12 myocytes, the p38alpha/beta inhibitor SB-202190 decreased protein synthesis with 40% (Hong-Brown et al., 2004) and p-p70S6k (Thr^{389}) with ~60% (Deldicque et al., 2007). P38 is also necessary for testosterone-induced fibre hypertrophy in mice (Brown et al., 2009) and for enhanced differentiation by creatine in C2C12 cells (Deldicque et al., 2007).

Notably, p38 has been demonstrated to be both necessary and sufficient for activation of the mTOR pathway; and activation of p38 increases the size of human cells, whereas inhibition of p38 reduces cell size (Cully et al., 2010). However, p38 is also implicated in muscle atrophy induced by cytokines such TNF-alpha, and by reactive oxygen species (ROS), via the induction of MuRF1 and atrogin-1/MAFbx (Norrby & Tågerud, 2010, and references therein). These divergent cellular responses to p38 are currently unexplained, but may be related to differences in cellular localizations of p38 (Norrby & Tågerud, 2010) and/or the degree of ROS formation. It was remarked by Cully et al. (2010) that the relationship between p38 phosphorylation and mTORC1 activation is not linear, and that only low doses of hydrogen peroxide (H_2O_2) elicited mTORC1 activation.

Adding further to the complexity is the existence of four different isoforms (alpha, beta, delta and gamma), some of which sometimes have opposite functions (Lovett et al., 2010)

The p38alpha and p38gamma isoforms are the most abundant isoforms in skeletal muscle (Lovett et al., 2010). A recent study indicates that p38alpha is the isoform responsible for activation of the mTOR-p70S6k pathway (Zheng et al., 2011). It was also reported that p38beta has a negative role in mTOR signaling (Zheng et al., 2011).

The high band observed for p38 in our study is presumably p38gamma (Boppart et al., 2000), which is known to respond to endurance exercise (Boppart et al., 2000; Pogozelski et al., 2009). The lower band is likely to be primarily p38alpha (Boppart et al., 2000). Thus, our BFR resistance exercise protocol seems to activate both the alpha and gamma isoforms of p38. Our 1 hour results for p38 are similar to those reported by Terzis et al. (2010) and by Hulmi et al. (2010) for moderate to high-volume heavy resistance training (3-5 sets of 6-10RM). It could thus be that the p38gamma is activated by moderate-to-high repetition resistance exercise and that this is associated with effects on strength-endurance.

Phospho-AMPK

The phosphorylation of AMPK did not change significantly, but if anything, it tended to be decreased with BFR training at 24 and 48 hours post-exercise (41-44%). Also of note, p-AMPK was not elevated at 1 hour. Phosphorylation of AMPK at Thr^{172} has been reported after high-volume strength training (Dreyer et al., 2006b) and high-intensity interval cycling (Gibala et al., 2009). Because strenuous low-load BFR exercise leads to low intramuscular pH and decreased levels of PCr and ATP (Ingemann-Hansen et al., 1981; Krustrup et al., 2008), we expected that p-AMPK (Thr^{172}) would be elevated shortly after exercise. As with p-ERK1/2, we cannot rule out that p-AMPK (Thr^{172}) may have increased temporarily.

However, Fry et al. (2010) reported no increase in p-AMPK (Thr^{172}) after a bout of BFR resistance exercise before the release of the pressure cuff, as well as 1-3 hours post-exercise, in older men. Therefore, our findings are in line with those of Fry et al. (2010).

Short-term hypoxia can inhibit AMPK activity in some cell types (Brugarolas et al., 2004), which could be a mechanism to counteract an increase in p-AMPK (Thr^{172}) that would otherwise likely be caused by the energetic stresses of BFR training.

Satellite cell numbers

The 33-53% increase in the number of visible SCs per muscle fibre post-exercise is quite remarkable in comparison to some of the previous studies on the SC response with resistance training. For example, Mackey et al. (2010) reported increases of 25% and 18% in SCs per fibre after 12 weeks of heavy strength training and very light resistance exercise, respectively. Petrella et al. (2006) reported a 49% increase the number of SCs per 100 fibres in young men after 16 weeks of heavy strength training.

However, SC numbers can increase within 24-48 hours in response to an acute bout of maximum eccentric exercise (Dreyer et al., 2006a; Mikkelsen et al., 2009, Paulsen et al., 2010) and electrical stimulation (Mackey et al., 2009). In the study of Dreyer et al. (2006a), SC number was elevated by 140% at 24h post-exercise in young men, and in the paper by Paulsen et al. (2010), SCs per fibre were ~73% higher than in the control muscle, averaged over all time points after exercise. Similarly, Mikkelsen et al. (2009) reported a 96% increase in SC number 8 days after eccentric exercise. Thus, it seems that some modes of resistance exercise can indeed induce rapid and large increases in the amount of SCs per fibre.

An intriguing finding in our current study is the ~33-36% elevation in the number of visible SCs already 1 hour after exercise. This took place in both the BFR and the free-flow legs. We believe that this early increase does not reflect an actual elevation in cell number, but is an effect caused by the swelling and the elongation of the activated cells (Figures 5 and 6). In support of this notion, SCs have been shown to become enlarged with activation already within 10 minutes after injury (Anderson, 2000).

In an electron microscopy study on a ischemia-reperfusion in primate skeletal muscle (Gregory & Mars, 2004), it was reported that the quiescent SCs in the pre-ischemia samples had a length of about 6.5 µm, whereas the length of early activated SCs at 6 hours after tourniquet-induced ischemia (3 hours) was about 10-15 µm, (i.e., the length was doubled). A doubling in the cross-sectional area of early activated SCs, and reorientation of some SCs to the long axis of the muscle fibres, were also observed. All these effects would increase the likelihood of detecting SCs in immunohistochemistry sections. In addition, it should be mentioned that NCAM stains a greater part of the satellite cell than Pax7, which is confined to the nucleus. Accordingly, Mackey et al. (2009) reported that NCAM-stained SCs could be followed through four or five 7 µm sections, while Pax7-labelled SCs could not be followed through more than two sections. Regarding the later increases in SC number in the current study, we assume that these were at least in part caused by proliferation.

Limitations

We acknowledge that we did not measure muscle protein synthesis, hence we do not know whether the anabolic signaling translated into elevations of protein synthesis. It also remains to be shown if hypertrophic signaling and SC responses persist with subsequent training bouts. However, preliminary data suggest that a training period with a low-load BFR resistance exercise protocol similar to the one in the present study results in myofibre hypertrophy and strength gains (Nielsen, Wernbom, et al., unpublished).

Perspectives

An acute bout of multiple sets to failure of unilateral low-load resistance exercise with BFR can result in an early enhanced phosphorylation of p70S6k (Thr^{389}) which lasts up to a least 24 hours post-exercise, and in an early phosphorylation of p38 as well as a greater number of

visible satellite cells per muscle fibre. Our findings thus confirm and extend those of previous studies on the signaling responses to BFR exercise. The SC response is of particular note, as it suggests the potential for myonuclear addition with longer-term BFR resistance exercise.

Notably, both the BFR leg and the free-flow leg demonstrated increased p-p70S6K (Thr^{389}) and satellite cells numbers. This indicates the possibility that performing certain exercises such as knee extensions with low loads close to concentric failure can trigger multiple hypertrophic mechanisms even without restriction of the blood flow by a pressure cuff. Therefore, our findings agree with and extend those of Burd et al. (2010b, 2011).

Low-load protocols which are effective even without BFR have numerous potential applications. Like BFR resistance exercise, they may be especially useful with regard to the rehabilitation of various injuries and other conditions where the use of large training loads may be contraindicated or not possible (Wernbom et al., 2009; Burd et al., 2010b).

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Figure legends

Fig. 1: Increase in phosphorylation of p70S6K at Thr^{389} in the BFR resistance trained leg at 1h, and in both the BFR and the free-flow leg at 24h. * = $p<0.05$ vs. baseline. # = $p<0.05$ vs. free-flow leg. Examples of western blots from two subjects are shown.

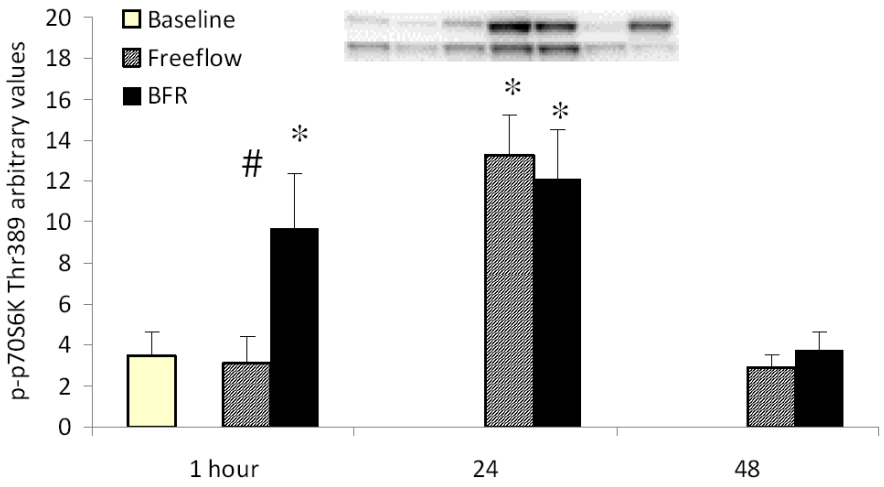
Fig. 2: Levels of phosphorylated p38 at Thr^{180}/Tyr^{182} in the BFR resistance exercised leg and the free-flow leg at 1h, 24 hrs and 48 hrs after exercise. Upper panel; upper band, presumably p38gamma, lower panel; lower band, presumably p38alpha. .* = significantly different from baseline levels.

Fig. 3: Phosphorylation of AMPK at Thr172 at baseline, and at 1h, 24h and 48h post-exercise. Examples of western blots from two subjects are shown.

Fig. 4: Changes in satellite cells per fibre. * = $p<0.05$ vs. baseline.

Fig. 5: Satellite cell marked with NCAM (fluorescent green) and Dapi (blue). Note the long cytoplasmic extension, a sign of activation.

Fig. 6: Two satellite cells marked with NCAM (fluorescent green) and Dapi (blue). Note the long extensions.


Baseline
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p-p70S6K Thr389 arbitrary values
20
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*
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*
1 hour
24
48

Figure 1.

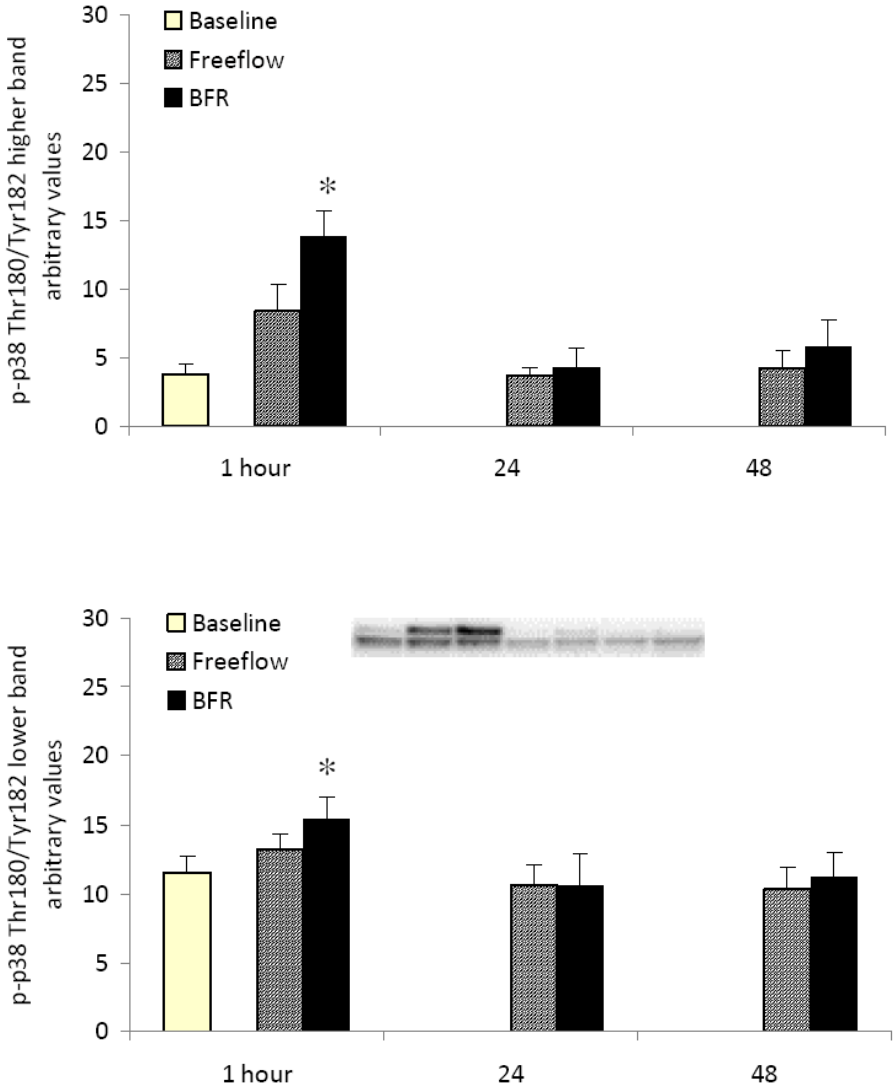

Baseline
Freeflow
BFR
p-p38 Thr180/Tyr182 higher band arbitrary values
30
25
20
15
10
5
0
*
1 hour
24
48
Baseline
Freeflow
BFR
p-p38 Thr180/Tyr182 lower band arbitrary values
30
25
20
15
10
5
0
*
1 hour
24
48

Figure 2.

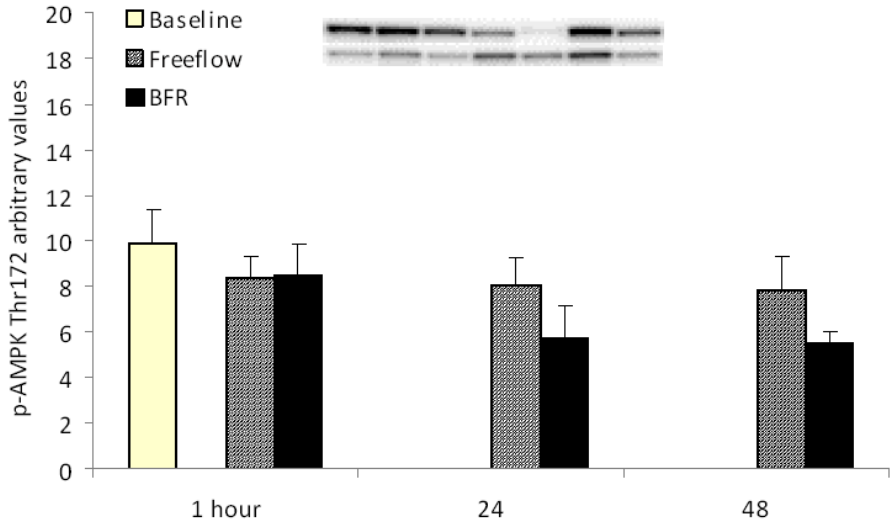
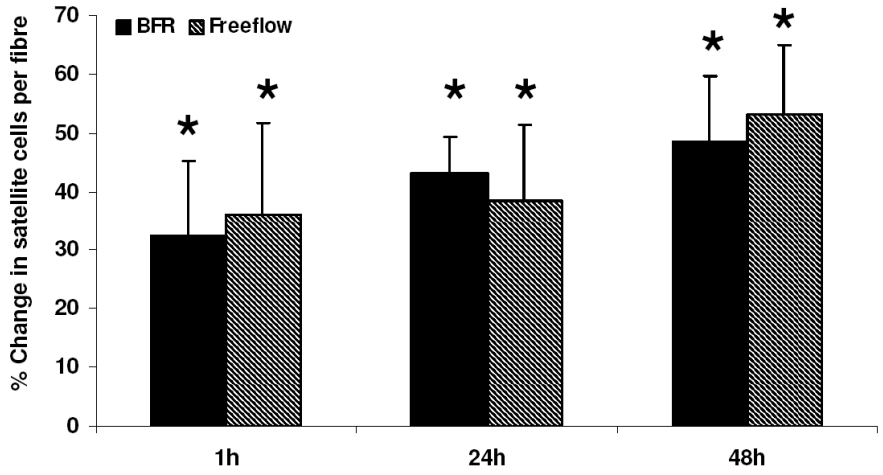

Baseline
Freeflow
BFR
p-AMPK Thr172 arbitrary values
20
18
16
14
12
10
8
6
4
2
0
1 hour
24
48

Figure 3.

	Baseline	1 h post		24 h post		48 h post	
Kinase		Free flow	BFR	Free flow	BFR	Free flow	BFR
p-Akt (S473)	7.1±1.3	4.7±1.1	6.6±1.3	6.9±1.5	5.8±0.7	6.0±1.5	5.6±1.2
p-mTOR (S2448)	16.5±1.5	17.9±1.6	18.6±1.4	18.2±1.8	15.2±1.9	17.0±1.2	16.9±1.7
p-p90S6K (T573)	8.8±2.0	6.7±1.8	5.9±2.0	11.1±2.7	5.9±1.6	6.6±2.1	8.7±3.1
p-eEF2 (T56)	11.9±1.4	9.9±0.5	8.7±1.3	11.8±1.5	10.2±2.0	11.3±1.1	13.1±1.6
p-ERK1/2 (T202/T204)	11.2±2.5	7.8±1.8	6.7±1.8	9.5±3.1	6.6±1.9	9.2±2.8	11.9±3.7

Table 1. Phosphorylation (in arbitrary units) of selected kinases at different time points, (means ± standard error).


% Change in satellite cells per fibre
70
60
50
40
30
20
10
0
BFR
Freeflow
*
*
*
*
*
*
1h
24h
48h
Figure 4.

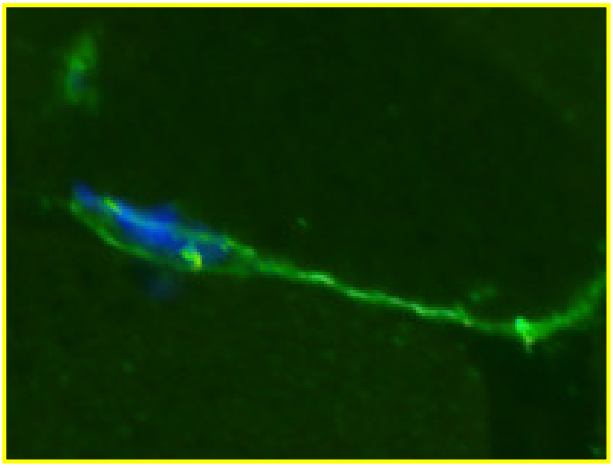


Figure 5.

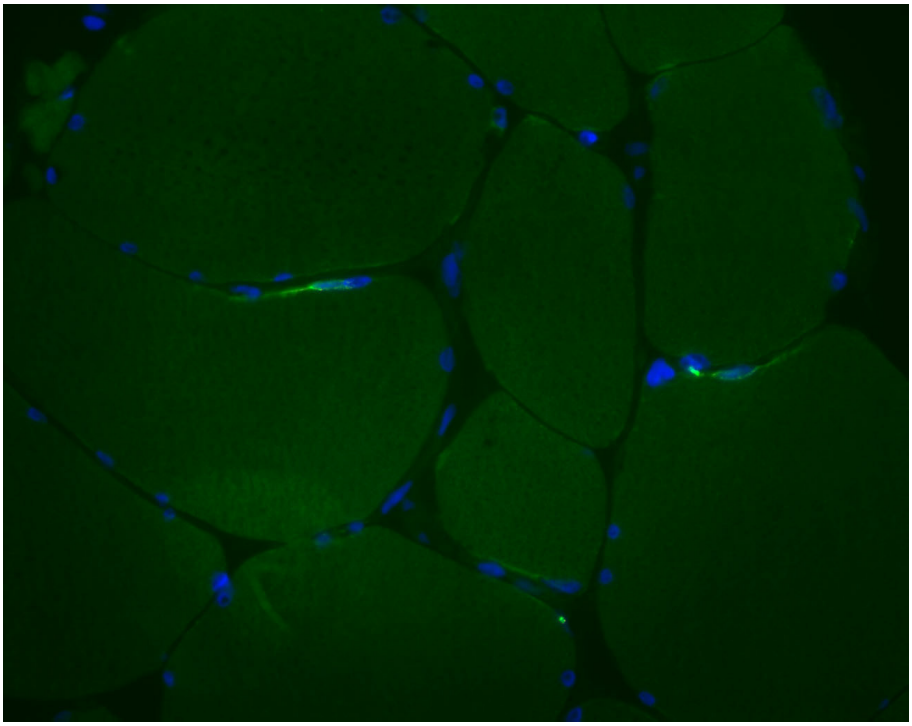


Figure 6.