EXERCISE-INDUCED MUSCLE DAMAGE: ROLE OF THE CALPAIN-CALPASTATIN SYSTEM IN SKELETAL MUSCLE MYOFIBRILLAR PROTEIN COMPOSITION

by

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ABSTRACT:

The purpose of this study was to examine the relationship between the activation of the calcium stimulated cysteine protease, calpain, its endogenous inhibitor, calpastatin, and myofibrillar protein composition (troponin I (TnI), troponin T (TnT) and tropomyosin (TM)) in an exercise-induced muscle damage model. It was hypothesized that this protease system initiated skeletal myofibrillar protein loss (and perhaps subsequent peptide release) from the contractile apparatus. In addition, lowering calpain activity (by the use of an exogenous inhibitor) was hypothesized to attenuate the composition of myofibrillar protein substrates for calpain (i.e. TnI, TnT, TM).

To test these hypotheses, male Wistar rats (~315g) were randomly assigned to one of six groups: 1) control sedentary (n=8), 2) control + cysteine protease inhibitor (E64c)(n=8), 3) running (25 meters/minute (-16°) for 45 min.)(n=8), 4) running + E64c (n=8), 5) running + 6 hr. recovery (n=8), or 6) running + 6 hr. recovery + E64c (n=8). Calpain I and II isoforms were isolated from rat hind-limb skeletal muscles, purified via phenyl-sepharose chromatography and their activities quantified using a casein-release assay. Calpastatin was isolated by a heat-release procedure and assayed based on its ability to inhibit calpain. Finally, myofibrillar proteins were resolved from a muscle homogenate via SDS-PAGE and their concentrations quantified using computer densitometry.

Calpain I and II activities increased by 36.1% and 37.5% respectively, immediately following exercise, and at 6 hours of recovery were 16.4% and 15.9% compared to controls ($p<0.05$). With E64c administration, the run-induced activation of calpain I

(3.7%) and II (8.2%) following exercise was much less, which was maintained into the recovery (5.3% and 9.7% respectively)($p>0.05$). Calpastatin activity did not change with exercise, however at the 6 hour recovery, activity was elevated by 74.8% ($p<0.05$). With E64c injection, this activity increase remained elevated over control levels (97.4%)($p<0.05$).

The proportion of bands corresponding to myofibrillar sTnI, sTnT and TM decreased to 75.1%, 75.6% and 90.1% of control values respectively, immediately following exercise ($p>0.05$) and at 6 hours of recovery were 73.2%, 75.4% and 80.3% ($p>0.05$). With E64c injection, any exercise-induced loss of sTnI (108.0% of control), sTnT (105.5% of control) or TM (110.7% of control) following running was arrested ($p>0.05$). This trend was maintained into the recovery (104.2%, 101.8% and 113.8% respectively)($p>0.05$). The proportion of cytosolic protein bands corresponding to sTnI, sTnT and TM increased to 363.8%, 343.5% and 430.1% of control respectively, immediately after exercise ($p<0.05$) and at 6 hours of recovery were 386.4%, 372.7% and 473.2% ($p<0.05$). With E64c administration, the exercise-induced increase in sTnI (532.3% of control), sTnT (509.0% of control) and TM (478.6% of control) was enhanced ($p<0.05$). This change remained into the recovery (537.4%, 530.7% and 514.9% respectively)($p<0.05$).

It is concluded, based on E64c's ability to attenuate both the exercise-induced activation of calpain and myofibrillar protein loss/breakdown, that calpain is a causative factor for protein composition changes *in vivo*. Calpastatin also dominates the protease system during recovery, shifting the environment to one of protein maintenance.

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CHAPTER 1: INTRODUCTION:

It is now well established that intense eccentric exercise (i.e. exercise above 70% Vo_2max. where the active muscle fiber is lengthened) results in significant damage to the skeletal muscle myofiber and the ability of the contractile apparatus to function normally. These temporary changes are evident on both macro and micro scales, in the form of mitochondrial swelling (Armstrong et al., 1983), sarcoplasmic reticular vacuolization (Armstrong et al., 1983; Friden et al., 1989) and most notably, significant sarcomeric z-line alterations (z-line streaming)(Belcastro et al., 1988). They are also very similar to manifestations caused as a result of the *in vitro* action of the calcium activated neutral protease, calpain (Belcastro et al., 1994; Goll et al., 1992). When these microscopic observations are taken in conjunction with increased calpain activity during eccentric exercise (18% and 22% for calpain I and II respectively) (Belcastro et al., 1993), calpain's proposed involvement in the exercise-induced muscle injury process seems plausible.

Typically associated with intense physical exercise and subsequent "transient and specific z-line removal" (Belcastro et al., 1994) is a myofibrillar disruption that results in the dissociation and release of numerous enzymes (creatine kinase (CK) and lactate dehydrogenate (LDH)(Komulanen et al., 1994; Komulanen et al., 1995; Green et al., 1992; Kayashima et al., 1995)) and structurally bound myofibrillar proteins (sTnI, sTnT, TM). Calpain's role in this exercise-induced muscle damage mechanism, is hypothesized to be an initiator for metabolic protein turnover by releasing these peptides from their filamentous structures during periods of increased contractile activity (Belcastro et al.,

1988; Goll et al., 1991). Skeletal troponin I (sTnI) in particular, appears to be an excellent candidate for exercise-induced calpain degradation. Not only is troponin I (cTnI) a very good *in vitro* substrate for calpain (DiLisa et al., 1995), but Dong Gao et al. (1997) recently disclosed that calpain reproduces both partial and selective degradation of cTnI reminiscent of the muscle injury caused by myocardial stunning. Furthermore, these changes can be prevented with the application of calpastatin, an endogenous calpain inhibitor. Sorichter et al. (1996) have also shown that with intense eccentric exercise, there is an increase in sTnI breakdown and hence its blood plasma concentration. Although the evidence for targeted and specific myofibrillar degradation by calpain is significant, the unique mechanism(s) behind its action, as well as its role in releasing peptides into the cytoplasm and eventually the blood plasma, are still unclear. Elucidating the interactive roles of different calpain isoforms, and their endogenous inhibitor calpastatin, in an exercise-induced muscle injury state is crucial to further understanding of the biological role of the calpain-calpastatin system.

Since skeletal muscle protease activity and consequential protein breakdown are thought to occur early in the muscle injury process, the first objective of this study was to quantify the activity of calpain isoforms (I and II), and calpastatin, immediately following exercise, as well as after a 6 hour recovery period. To obtain this data, an eccentric exercise model was employed in which calpain and calpastatin were isolated from sedentary, exercised and recovered rat muscle via phenyl-sepharose chromatography. Their activities were quantified using a casein-release assay. This procedure also allowed us to investigate the synergistic actions/relationship between calpain isoform and

calpastatin activities. For example, it is presently unknown whether this interaction, and hence calpain's cumulative degradative ability, is controlled primarily by a net breakdown potential (i.e. by calpain activity) or a net maintenance potential (i.e. by calpastatin activity). The delineation between calpain I and calpain II activities, and their relative contributions to the system as a whole is also unclear.

The second aim of this study was to investigate fluctuations in the myofibrillar and cytoplasmic protein composites (sTnI, sTnT, TM) associated with increased contractile activity. To investigate myofibrillar and cytoplasmic proteins, sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was employed. Finally, to further each of these objectives, as well as elucidate calpain's role in myofibrillar peptide loss/degradation, E64c, a synthetic cysteine protease inhibitor, was utilised.

The overall theory of this investigation proposes that very early in the muscle injury process, calpain is autolyzed and subsequently activated via increased intracellular calcium levels within the muscle cell (Belcastro, 1993). Following calpain's activation, and its inevitable interaction with calpastatin (Fig. 1a), it acts to selectively proteolyse troponin I, as well as various other contractile and cytoskeletal proteins (DiLisa et al., 1995; Belcastro et al., 1994). These myofibrillar proteins and/or peptide fragments move from a structurally bound state to a more mobile cytoplasmic position, and then eventually into the blood plasma (i.e. 3 compartment model)(Fig. 1a,b). This leakage out of the muscle cell is made possible by the exercise-induced disorganisation of the plasma membrane proteins, in part by calpain (Fig. 1b).

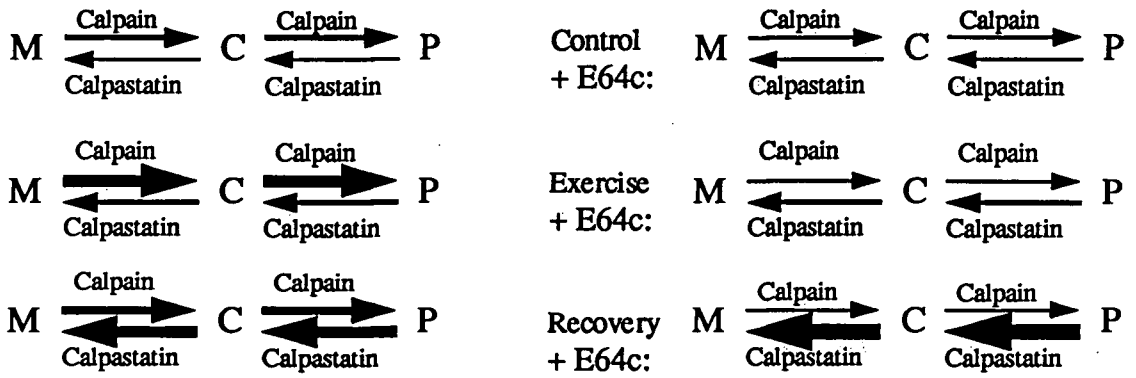

Figure 1a.

Proposed relationship between the calpain-calpastatin system and changes in protein composition of the 3 compartment muscle model. "M" denotes the myofibril component; "C" denotes the cytosolic component; "P" denotes the plasma component and the arrows denote the relative activities of calpain and calpastatin (which affect the flux of protein from one compartment to another) in each of the above groups (Control, Run, Recovery, with and without E64c treatment).

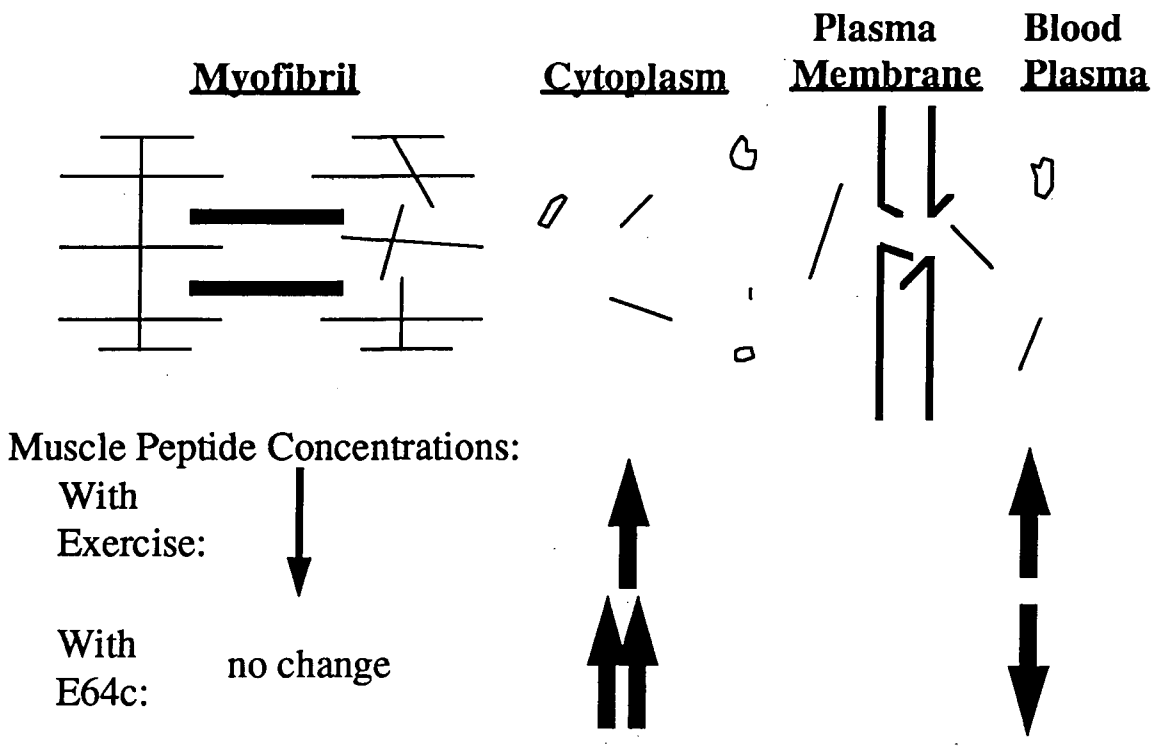

Figure 1b.

Proposed sequence of myofibrillar protein degradation and release.

It was hypothesized that the activities of both calpain I and II would be elevated, post eccentric exercise. Additionally, this increase was hypothesized to occur in close association with a decrease in the proportion of myofibrillar protein bands corresponding to TnI, TnT and TM, and an increase in their cytoplasmic proportions. A more direct implication of calpain as a degrading protease for myofibrillar proteins was expected upon the application of E64c. This cysteine protease inhibitor was hypothesized to prevent increases in calpain isoform activity, as well as myofibrillar decreases and cytoplasmic increases in the proportion of protein bands corresponding to TnI, TnT and TM.

By investigating the calpain-calpastatin relationship, as well as their cumulative role in the composition of myofibrillar proteins, possibly through their degradation, release and transport, this study attempted to clarify the role of the calpain-calpastatin system in myofibrillar protein changes during exercise-induced muscle damage. In light of the recent clinical utility of certain myofibrillar proteins (sTnI) as markers for muscle breakdown disorders (myocardial infarction, muscular dystrophies) and injuries (exercise-induced), elucidating the underlying mechanism(s) for these conditions has become even more important.

RESEARCH QUESTIONS:

a) What is the relationship between striated muscle calpain activity (isoforms I and II) and calpastatin, in response to downhill running?

b) Are exercise-induced changes in the ratio(s) of the calpain-calpastatin system associated with changes in myofibrillar-complexed proteins?

RATIONALE:

In view of the myofibrillar degradation caused by intense physical exercise (i.e. of skeletal muscle troponin I (sTnI) and troponin T (TnT)), elucidating the mechanism(s) underlying myofibrillar protein breakdown, and consequential release into the cytoplasm and blood plasma, have become quite important. Based on its early activation during exercise, as well as the observation that many myofibrillar peptides appear to be exceptional *in vitro* substrates, calpain is a particularly strong candidate as a degrading enzyme. The specific role of different calpain isoforms, as well as their endogenous inhibitor calpastatin, must also be distinguished to further differentiate the mechanism(s) of protein breakdown. This investigation may also be clinically important, as recently the utility of sTnI as a potential marker for exercise-induced muscle injury (skeletal muscle damage) has improved significantly.

OBJECTIVES:

- To quantitate immediately following exercise, as well as after a recovery period, calpain isoform (I and II) and calpastatin activity, in the presence and absence of E64c, a cysteine protease inhibitor.
- To investigate the relative effects of calpain isoform (I and II) and calpastatin activity, in the presence and absence of E64c, on the overall proteolytic ability of the calpain-calpastatin system.
- To quantitate immediately following exercise, as well as after a recovery period, the relative amount and composition changes in myofibrillar proteins for both the myofibrillar complex and the myofiber cytoplasm (i.e. the proportion of bands corresponding to sTnI, sTnT, TM).

HYPOTHESES:

- In comparison to calpain activity in sedentary controls, calpain activity (both I and II isoforms) will increase with exercise.
- Calpain activity (both I and II) will be suppressed upon pre-exercise treatment with E64c.
- Associated with an increase in calpain activity, a decrease in myofibrillar protein levels and an increase in cytoplasmic protein levels (i.e. in the proportion of bands corresponding to TnI, TnT, TM) will occur.
- Associated with an inhibition of calpain activity via the protease inhibitor E64c, no change from control values in myofibrillar or cytoplasmic protein levels (i.e. in the proportion of bands corresponding to TnI, TnT, TM) will occur.

CHAPTER 2: LITERATURE REVIEW:

1. *Overview:*

Considering the importance of normal muscular function in basic everyday activities, as well as in athletic performance, the study of muscle injury and the mechanisms behind its manifestation, have become extremely significant. Of particular interest is the temporary and repairable muscle damage caused by intense physical exercise, specifically eccentric exercise (Stauber, 1989; Armstrong et al., 1983). This component of muscular contraction, as opposed to either the concentric or isometric elements, involves the lengthening of the muscle concurrent to its activation (Armstrong et al., 1983). Furthermore, eccentric actions are typically associated with muscle injury both within and among myofibrils, usually functioning as the very initiators of damage. The evidence of this muscle injury ranges from the overt loss of force production (Davies & White, 1981) and the tenderness resultant from delayed onset muscle soreness (DOMS) (Armstrong, 1984) to the less obvious histological damage of the muscle cell (Lieber & Friden, 1988). Additionally, a transient increase in the serum blood concentrations of a number of muscle proteins is also evident with eccentric exercise (i.e. versus concentric or isometric)(Armstrong, 1984). These include both cytosolic proteins such as creatine kinase (CK) and myoglobin (Mb), as well as structurally bound proteins like myosin heavy chain (MHC) and troponin subunits (e.g. TnI, TnT).

In spite of a general lack of verified causative factors or cellular mechanisms behind the pathophysiology of muscle injury, the potential involvement of the non-lysosomal, calcium-dependent neutral protease, calpain (CANP), appears promising. Calpain's early post-exercise activation and extensive list of myofibrillar protein substrates supplant it as a very likely initiator and/or regulator of muscle damage.

Although the loss of force production and the presence of DOMS are important indicators of muscle damage, it is the measurable plasma increases in muscle-associated proteins which have provided the best clinical evidence of injury. Whether a result of extreme physical exertion (i.e. intense exercise), or specific pathologies (i.e. muscular dystrophy, cardiac ischemia and/or hypertrophy), the use of these protein "markers" has become crucial to the early diagnosis of muscle injury and hence to disease intervention, rehabilitation, and the monitoring of exercise training (i.e. overtraining). Although the plasma values of such myofibrillar protein markers have been relatively well characterised, their cellular breakdown patterns, as well as calpain's potential involvement in their composition, is unknown. By elucidating these mechanisms behind the production, and release, of various myofibrillar protein markers, it will improve the scientific understanding of general muscle breakdown conditions and the clinical care of specific pathologies.

While the calpain protease system appears to play a significant role in the muscle injury process, its own regulation is almost entirely uncharacterised. The relative control of overall proteolytic activity exerted by calpain and/or its endogenous inhibitor, calpastatin, is unclear and must be delineated if any complete biological understanding of calpain-induced muscle damage is to be achieved.

2. Eccentric exercise-induced muscle injury:

Eccentric exercise and hence the action of lengthening the muscle while it is active, is the predominant initiator of muscle injury (Armstrong et al., 1983). Not only does negative work result in the generation of a greater force output per muscle fiber than either concentric or isometric contractions (Stauber, 1989; Cannon et al., 1991), but also a significantly higher mechanical stress and/or tension per fiber (Belcastro et al., 1985). In addition, it appears that this damage is predominantly observed in fast-twitched muscle fibers, a manifestation Laughlin & Armstrong (1983) associate with the typical reduction in blood flow to type II (fast-twitched) fibers in favour of more oxidative type I (slow-twitched) fibers during exercise. This shift in blood flow to meet the increased demand for oxygen and metabolite delivery in type I fibers, appears to limit the capacity to remove exercise by-products and muscle damaged proteins, as well as resupply novel amino acids and plasma proteins for tissue repair/regeneration in type II fibers. When the observation of increased tension per active myofiber is coupled with the overall requirement of less metabolic energy (i.e. reduced oxygen consumption, smaller motor recruitment, and lowered substrate/metabolite utilisation), the causative role of eccentric exercise in muscle injury specific to fast-twitched fibers appears justified.

In 1990, Armstrong constructed a model of eccentric exercise-induced muscle injury consisting of 4 main phases. The first phase is most likely mechanical in nature and defined as the precise point, during eccentric work, at which the "mechanical strain produced in the muscle exceeds the ability of that muscle to resist." As a result of this

initial event, the myofibril engaged in negative work is forced to dissipate the newly absorbed energy as either heat or plastic deformations (defined as "permanent changes" in the size or shape of the muscle components). Although the production of heat is definitely involved in a number of fatigue related processes (e.g. it can be correlated with increased protein and lipid degradations (Nadel et al., 1972)), it is the resultant muscle deformations which are most immediately obvious. As a result of the increased tension in both the muscle fibers and associated connective tissue, disruptions in the myofibers as a whole, the contractile apparatus itself, and the adjacent connective tissue is evident (Cannon et al., 1991). Specifically, mitochondrial swelling (Armstrong et al., 1983), sarcoplasmic reticulum (SR) vacuolization (Friden et al., 1989) and sarcomeric z-line alterations (Belcastro et al., 1988) are all resident in eccentrically exercised muscle (Fig. 2). So too are disruptions in the plasma membrane and the basil lamina of the muscle cell. Furthermore, McNeil et al. (1992) comment that these focal membrane deformations, as well as other myofiber disruptions, are not only early events in the muscle injury model, but also very common ones as well.

The consequential phase to this multitude of muscle changes involves the loss of calcium (Ca^{2+}) homeostasis. Although the relative contributions of either damage to the sarcolemma or to the SR in this loss of homeostasis are unclear, it is apparent that the intracellular calcium concentration must be maintained within a narrow range. In fact there

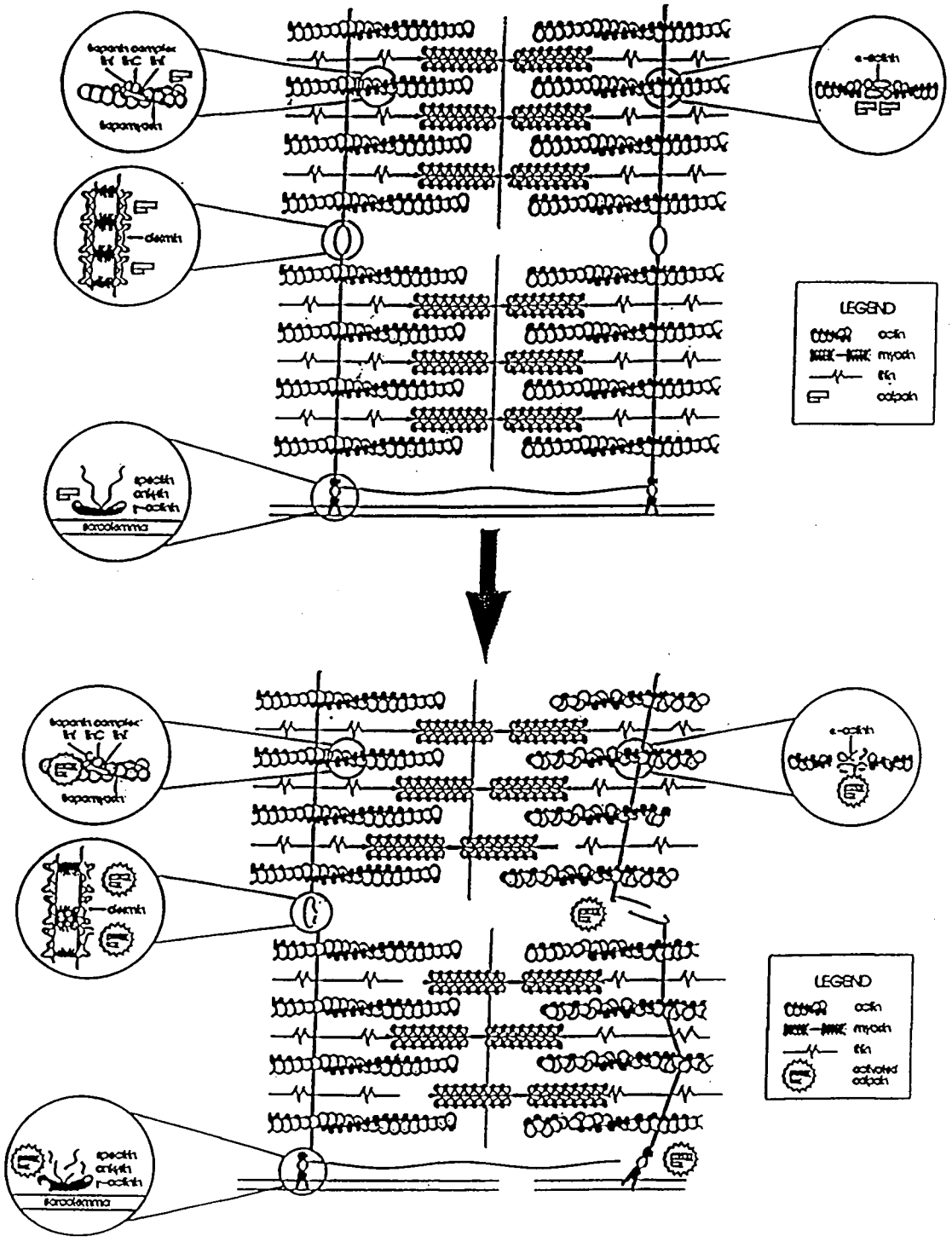

Figure 2. Consequences of muscle degradation via calpain.
(Compliments of Belcastro et al., *in press*)

are a minimum of 7 membrane mechanisms involved in Ca^{2+} control (ATPases, Na^{+}/Ca^{2+} exchangers, Ca^{2+} channels and electrophoretic uniporters). In response to eccentric exercise however, a dramatic increase in intracellular Ca^{2+} is noted at the site of the lesion (Baracos et al., 1984). This is most likely a result of significant damage to both the sarcolemma and the SR, and hence an increase in Ca^{2+} permeability (resulting in a flow of extracellular Ca^{2+} down its concentration gradient and into the cell), and a loss of the cell's ability to sequester intracellular Ca^{2+} respectively (i.e. a decrease of 18% in SR-ATPase activity, post exercise was noted by Belcastro et al., 1981). If the damage is relatively minor (i.e. resulting in a small Ca^{2+} influx), the ATPase pumps can often respond sufficiently to ensure very little change in overall Ca^{2+} homeostasis, and hence a more rapidly reversible form of injury. However, if the intracellular Ca^{2+} concentrations rise too quickly, the pump mechanisms are simply overwhelmed and the reductions in strength and contractility usually associated with muscle damage can not be prevented.

Additionally, the increase in fat oxidation, and hence muscle cell uptake of free fatty acids concurrent with exercise, may also contribute to the loss in Ca^{2+} homeostasis. An unormally elevated concentration of long chain fatty acids perturbs the uptake of Ca^{2+} by the SR (Messineo et al., 1984) and therefore decreases its uptake potential, not to mention acting to increase overall membrane Ca^{2+} permeability as well (Armstrong, 1990; Bindoli et al., 1983). Ca^{2+} influx through stretch-activated channels in the sarcolemma is also a potentially distinct component in the loss of Ca^{2+} homeostasis (Duan et al., 1990).

The third phase, or autogenic component, of Armstrong's muscle injury model is initiated by this increase in intracellular Ca^{2+} concentrations and involves the activation of

indigenous Ca^{2+}-dependent proteolytic and phospholipolytic pathways. These pathways degrade structural and contractile proteins as well as membrane phospholipids respectively. Included are both non-lysosomal enzymes, such as the family of Ca^{2+}-activated neutral proteases (CANPs-- i.e. calpain) and phospholipase A_2, and numerous lysosomal proteases (i.e. cathepsin). Interestingly, based on experiments involving enzyme activity time courses and consequential substrate degradation rates, it appears that it is the non-lysosomal pathways (i.e. CANPs) which are responsible for the immediate changes associated with eccentrically exercised muscle damage (i.e. within hours)(Cottin et al., 1994). The lysosomal enzymes, however, seem to be involved in the more delayed muscle damage processes (i.e. protein turnover in the days following eccentric exercise). The link between eccentric exercise and autogenic activity in muscle fibers is strengthened not only by the observations of increased blood plasma concentrations of numerous structural, contractile and metabolic muscle proteins (i.e. CK, Mb, MHC, TnI, TnT and TM), but also by increased urea and blood amino acid concentrations (Armstrong et al., 1991) post exercise. It has been subsequently agreed upon that increased contractile activity, primarily that which is eccentric in nature, results in increased muscle protein degradation and hence amino acid oxidation.

In addition to the specific autogenic results of increased intracellular calcium concentrations, it should also be noted that other potentially non-autogenic consequences are often resident as well. Observed decreases in mitochondrial energy production capacity, the initiation of apoptosis (Trump & Berezesky, 1995) and/or decreases in overall actin-myosin interaction (Vollestad & Sejerstad, 1988) are all common to

eccentrically-exercised muscle and may not, at least directly, involve any particular autogens.

The final phase in this muscle injury model is the regeneration component and includes processes involving both phagocytes and macrophages (i.e. inflammation). It is also important to note that this phase, and therefore the muscle injury model as a whole, is complete. By conditioning the muscle with repeated exposures, the amount of injury inflicted in the future (i.e. at the same exercise intensity and duration) will be reduced (Armstrong, 1984). Hence muscle damage, at least in the context of exercise, can be defined as a normal and necessary precursor for muscle adaptation to increased use (Armstrong, 1984).

Based on the Armstrong (1990) eccentric muscle injury model, and the sarcomeric deformations, loss of Ca^{2+} homeostasis and activation of Ca^{2+}-dependent proteolytic pathways associated with such activity, this study employed an eccentric exercise model. The utilisation of downhill running was thought to be optimal for promoting calpain isoform and calpastatin activation (Belcastro, 1993), and consequently for causing myofibrillar protein degradation (Sorichter et al., 1997). It is from this basis that the intimate relationships within the calpain-calpastatin system, as well as between calpain proteolysis and myofibrillar breakdown, could be investigated.

3. *Calpain:*

In defining the very early mechanistic events that are responsible for the breakdown and subsequent release of myofibrillar proteins during exercise, the calcium-activated neutral protease, calpain (CANP, EC 3.4.22.17) seems a likely player. Calpain is made up of a family of Ca^{2+}-dependent non-lysosomal cysteine proteases which are fundamentally similar, yet distinct in their distribution. Two isozymes of particular interest are calpain I (requires uM Ca^{2+} concentrations for optimal activation) and calpain II (requires mM Ca^{2+} concentrations for ideal activation); both ubiquitous in mammalian bodies (Saido et al., 1994). They are heterodimers, consisting of a unique catalytic subunit (80 kDa) and an identical regulatory subunit (30 kDa) (Fig. 3)(Saido et al., 1994; Sorimachi et al., 1990). The catalytic subunit includes both the cysteine protease and Ca^{2+}-binding domains whereas the smaller regulatory subunit possesses four distinct Ca^{2+}-binding regions (Sorimachi et al., 1990).

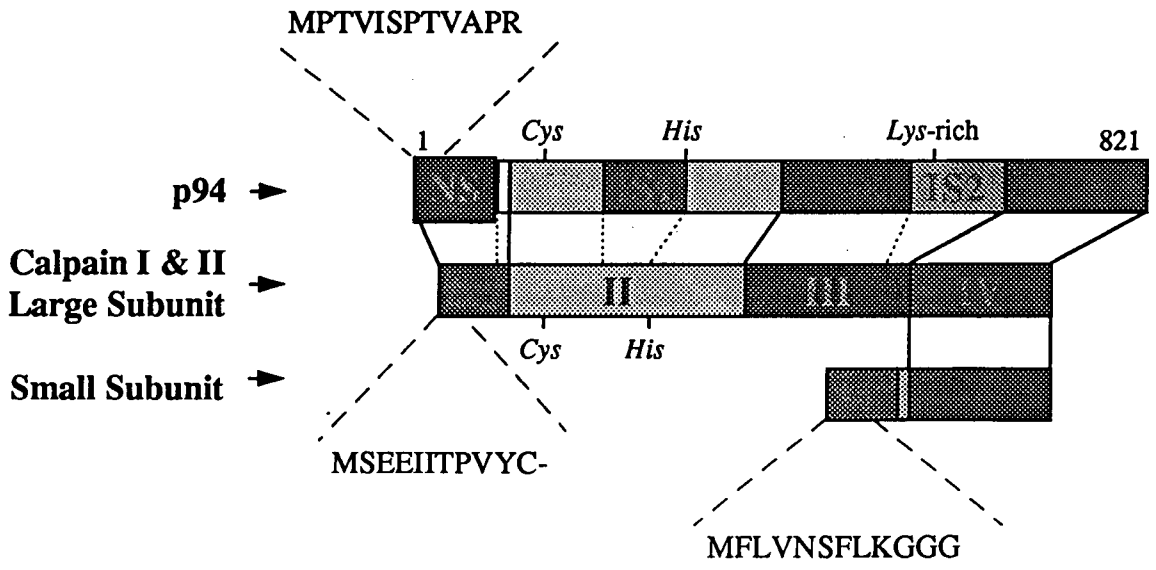

Figure 3.
Schematic representation of calpain

A newly characterised, tissue specific calpain, n-calpain (p94) is also of potential interest (Sorimachi et al., 1989; Sorimachi et al., 1990) (Fig. 3). Both n-calpain and its mRNA are exclusive to skeletal muscles, suggesting a muscle specific role (Sorimachi et al., 1989). Never the less, this protease still retains the same fundamental structure as both type I and II calpains (~ 50 % amino acid sequence homology) (Sorimachi et al., 1990). This includes domain II (the most conserved domain), the enzyme's proteolytic regulator, and hence indicates a potentially similar protease ability to calpain I and II (both in terms of calpain substrates and z-line/cytoskeletal deformations). It does however, contain 3 unique sequences: NS, IS1, and IS2 in domains I, II , and III respectively (Sorimachi et al., 1990)(Fig. 3). Interestingly, n-calpain mRNA is expressed at a rate approximately 10 times greater than the other calpains (Sorimachi et al., 1993), yet the peptide itself is evident in only very limited amounts. This may in part, be due to its autocatalytic regulation (at the nuclear membrane) by the IS2 region immediately after translation (Sorimachi et al., 1989). Because n-calpain possesses a potential nuclear localisation signal, its involvement in muscular development and differentiation also seems plausible (i.e. by modifying specific transcriptional factors in the nucleus) (Gregoriou et al., 1994). n-calpain is particularly applicable to the realm of muscle injury not only because it is muscle specific but also because it is activated (and hence engages in proteolysis) by intracellular Ca^{2+} concentrations in the range of 10 to 1000 nM (i.e. nanomolar range)(Sorimachi et al., 1990). It is therefore much more sensitive to $[Ca^{2+}]$ than calpain I or II. Fluctuations in the concentration of intracellular calcium typically occur at

submicromolar levels, and hence n-calpain (and probably calpain I) appears likely to function under normal physiological conditions.

3.1 Calpain Inhibition:

All isoforms of calpain are inhibited indigenously by the ubiquitous protein, calpastatin (Goll et al., 1992) which is located in the same locale. The primary structure of this peptide, as determined by cDNA cloning, consists of approximately 700 amino acid residues. This includes a larger 73 kDa form as well as a 48 kDa "erythrocyte inhibitor", both of which prevent the activation and expression of the calpain catalytic domain.

More specifically, calpastatin contains 4 repeated mutually homologous domains (140 a.a. residues) composed of subdomains A, B, and C, as well as a consensus sequence (TIPPXYR)(Kawasaki et al., 1989; Maki et al., 1990). Unlike subdomain B (directly inhibits calpain) (Maki et al., 1989) and the consensus sequence, which are essential for calpain inhibition, subdomains A and C are not crucial, but do still potentiate calpastatin function (Ma et al., 1993). Calpastatin also displays molecular polymorphism, likely a result of mRNA splicing (Lee et al., 1992), post-translational modification and/or reversible phosphorylation (Adachi et al., 1991; Takano et al., 1993). This distinct structure-function relationship, when combined with the unique physio-chemical properties inherent to calpastatin (i.e. high resistance to denaturants and lack of secondary structures) present a rather differentiated mechanistic form of proteolytic inhibition; the tripartite structures of calpastatin and calpain provide the basis for a strong and intimate interaction (i.e. subdomains A, B, C and calpain's catalytic and 2 Ca^{2+} binding domains).

In the presence of Ca^{2+}, and possibly upon phosphorylation, calpastatin (Adachi et al., 1991) complexes with calpain (with a high affinity), thereby inhibiting it at Ca^{2+} concentrations lower than those necessary for autolysis or proteolytic processing (Kapprell & Goll, 1989). Recently, Takano et al. (1997) commented that subdomains A and C bind to the large and small subunits of calpain respectively. Although the specifics of this interaction are still unclear, they have proposed that the amphipilic helices, typically formed by subdomains A and C, may interact with the hydrophobic "patches" produced by the calpain molecule (Takano et al., 1997). It was further noted by Dayton et al. (1976) that neither pH nor ionic strength alone were significant in effecting its inhibition of calpain; only when a combined interaction occurred could they modify calpastatin's proteolytic inhibition.

Although the specific avenue of calpastatin's inhibition on calpain is becoming better understood, the relative contributions by calpain isoform and calpastatin activation to overall protease activity is still unknown. Delineating whether a net breakdown potential (calpain activity) or a net maintenance potential (calpastatin activity) is dominant during exercise-induced muscle injury is a goal of this study.

In addition to the endogenous calpastatin inhibitor, a number of synthetic calpain inhibitors, most irreversible, have been designed. Although the majority are active-site targeted peptides, none of them are highly specific for calpain (unlike calpastatin). The most commonly used are leupeptin, calpeptin, calpain inhibitor I, and MDLZ8170, all belonging to the peptide aldehyde class of inhibitors. More applicable to this study however, is the class of epoxysuccinyl peptide inhibitors (i.e. E64 and E64c). The oxirane,

E64c, was isolated from cultures of *Asperigillus japonicus*, and forms irreversible sulphide bonds with the active Cys thiol. Reflecting its limited non-specificity however, E64c not only potentiates inhibition of calpain, but also of cathepsin B and L as well as papain. This makes some *in vivo* conclusions difficult. There is also some question as to its membrane permeation ability, a potential problem which can be rectified by converting E64c into E64d (an ethyl ester), a membrane permeable isoform. In spite of these limitations, E64c is still more appropriate in many *in vitro* applications as it is significantly more active than E64d.

3.2 Calpain Activation:

The dichotomy between the apparent *in vitro* Ca^{2+} concentration requirements of calpain and the actual *in vivo* [Ca^{2+}] is a dilemma in explaining calpain's activation. Although it is unlikely that phospholipids in cell membranes bind directly with calpain to lower its [Ca^{2+}] requirement for activation (as was previously believed), it does appear that its [Ca^{2+}] needs for autolysis, and hence for conversion form an inactive proenzyme into an active protease, can be lowered by Ca^{2+} binding to specific sites (Goll et al., 1992). It seems Ca^{2+} coupling to distinct sites on the calpain protein may induce a series of conformational changes and elicit pre-determined responses associated with each region (i.e. autolysis, proteolytic activity, calpastatin binding) (Goll et al., 1992). The "autolytic phenomenon" likely involves the removal of the amino-terminal propeptide sequence which normally suppresses the protease activity of the peptide (Sorimachi et al., 1990)(Fig. 3). It is also evident that the autolysis and eventual dissociation of the catalytic

80K and regulatory 30K calpain subunits, respectively, lowers the Ca^{2+} requirements for the subsequent proteolysis of a substrate (Saido et al., 1994; Yoshizawa et al., 1995). Although the mechanism(s) by which autolysis and calpain activation occur are unclear, it is obvious that subunit autolysis is an "important early event in the intramolecular activation of calpain" (Saido et al., 1994).

3.3 Calpain Substrates:

Calpain's proteolytic activity is both tightly controlled and somewhat limited. It also exhibits relatively strict substrate specificity, even though an extensive number of substrate classes have been identified (Brooks et al., 1983). *In vitro*, calpain cleaves cytoskeletal proteins such as actin-binding proteins (spectrin, talin, filamin, α-actinin, desmin) and microtubule associated proteins, myofibrillar peptides (troponin, tropomyosin, myosin light chain kinase), adhesion molecules (integrin, cadherin, N-CAM), growth factor receptors (EGF receptors), ion transporters (Ca^{2+}-ATPase), kinases (PKC, PKA, CaMK), phosphatases, phospholipases, cytokines (interleukin-1) and transcription factors (Fos, Jun) (Saido et al., 1994). Calpain's proteolysis of these contractile, metabolic, and structural elements via increased [Ca^{2+}] within the muscle is not surprising upon review of its rather ubiquitous association with most organelles, as well as metabolic enzymes (i.e. glycolytic), signal transduction pathways and cytosolic membrane structures (i.e. phospholipids, structural proteins, transport channels and membrane receptors)(Johnson, 1990).

In vitro experiments have shown that the calpains cleave at a limited number of specific sites in native proteins (myofibrillar and cytoskeletal), producing large protein fragments (which may remain biologically active yet avoid typical control mechanisms) (Belcastro et al., 1997, *in press*). Furthermore, calpain also seems to play a "key role in the disassembly and remodelling of the cytoskeletal matrix, particularly of its filamentous cytoskeletal attachments to the plasma membrane and/or other molecular structures" (i.e. these protein fragments can not re-associate or bind with their previously linked cytoskeletal counterparts)(Belcastro et al., 1997, *in press*).

Of particular interest to this study however, is the close association of calpain with the I (desmin) and T (α-actinin) bands, and in general, the myofiber. Calpain's propensity for the degradation of myofibrillar proteins (and hence potential markers such as TnI and TnT) coupled with observations of "transient and specific z-line removal" from striated muscles following injury, has indirectly linked calpain to muscle damage (Belcastro et al., 1994; Goll et al., 1992). It has therefore been hypothesized that calpain initiates the metabolic turnover of numerous myofibrillar proteins by releasing them from their filamentous structures, especially during periods of increased contractile activity. In fact Belcastro (1993) observed that eccentric exercise increases overall calpain activity (18% and 22% for calpain I and II respectively) as a result of enhanced Ca^{2+} sensitivity (i.e. lowered pCa_{50}) and increased susceptibility of the myofibrillar substrate proteins to calpain proteolysis. In addition, purified myofibrils prepared from exercised muscle also display myofibrillar disruption (i.e. loss of z-line proteins) reminiscent of calpain's action (Belcastro et al., 1988; Goll et al., 1991). Belcastro (1993) also states that the rates of

intracellular protein degradation of selected proteins are "sensitive to the hormonal, ionic and nutritional status of the cell", all of which are optimal for calpain activation during sustained exercise (Fagan et al., 1992). It is this connection between calpain and exercise-induced muscle damage that prompted this study to utilise the eccentric running model.

When observations of cardiac troponin I being an excellent substrate for calpain *in vitro*, (especially for calpain I, which is 10X more active in degrading TnI *in vitro* and *in situ* than calpain II) (DiLisa et al., 1995) are linked with distinct increases in calpain activity and TnI breakdown during exercise, the calpain-calpastatin system has a plausible role as a causative factor in myofibrillar protein degradation and peptide release (into the cytoplasm and blood plasma) during muscle injury. The basis for this study's investigation of such a link is further supported by the immediate increase in sTnI following exercise observed by Sorichter et al. (1996)(i.e. within 6 hours post-exercise). Furthermore, calpain's role in the breakdown of other, non-myofibrillar proteins (and hence the release of potential plasma markers) such as CK and LDH is also a distinct possibility, as it is clearly associated with both on an intracellular basis. Interestingly, it appears as though not all myofibrillar proteins are victims of the calpain system. The much delayed increase in serum MHC values (i.e. 48 hrs. post-exercise) (Sorichter et al., 1996) indicates that perhaps, the heavy chain release is not a result of calpain action but of a distinct mechanism; a result supported by the poor *in vitro* substrate capability of MHC.

3.4 Calpain Targeting:

In light of the variety of calpain substrates, it is somewhat predictable that there is no single criterion which pre-selects a target for calpain proteolysis. The regulation of protein degradation includes both intrinsic and extrinsic factors (Bond & Butler, 1987). PEST sequences (i.e. hydrophilic sequences rich in proline, glutamic acid, serine, and threonine) are located near cleavage sites in a number of calpain substrates (Wang et al., 1989), yet are not always prerequisites for proteolysis. When PEST domains on the Ca^{2+}-ATPase are point mutated for example, there is no change in susceptibility of the peptide to calpain processing (Molinari et al., 1995). Another possible contributor to the pre-markation process is phosphorylation. This is especially evident with troponin, as its phosphorylation by PKA and PKC causes a conformational change and alters its sensitivity to calpain (DiLisa et al., 1995). In fact an inverse relationship arises between the Ca^{2+} sensitivity of the myofibrillar Mg-ATPase and troponin's susceptibility to proteolytic attack (DiLisa et al., 1995). Oxidation status ([$NADH/NAD^{+}$]) also seems to be a targeting mechanism, as does the oxidation of thiol groups and the construction of mixed disulfides (Rivett, 1986). The sulfhydrl content of a peptide is also a possibility; complexed myofibrils for example, exemplify a low SH content and a consequential increase in their susceptibility to proteolysis (Belcastro et al., 1994). Even altered carbohydrate metabolism appears to play a crucial role in calpain targeting, as diabetics show increased troponin and tropomyosin proteolysis by calpain (Belcastro et al., 1994).

4. Troponin I and muscle injury:

While Calpain's role in myofibrillar breakdown is extremely complex, one protein which appears particularly susceptible is troponin I (sTnI). Troponin I is a subunit of the relatively well characterised ternary troponin complex. It is a regulatory component of the skeletal muscle thin filament in striated muscle, specifically a basic globular protein consisting of approximately 180 amino acids (molecular weight of 21 kDa) which binds to actin, tropomyosin, TnT, and TnC (Ohtsuki et al., 1986; Zot & Potter, 1987). Its main function in muscular contraction is to inhibit the interaction between F-actin and myosin at rest, primarily by impeding the actomyosin Mg^{2+}-ATPase in a Ca^{2+}-dependent manner (i.e. inhibition at submicromolar Ca^{2+} concentrations and activation at micromolar concentrations)(Greaser & Gergely, 1971) (Fig. 4).

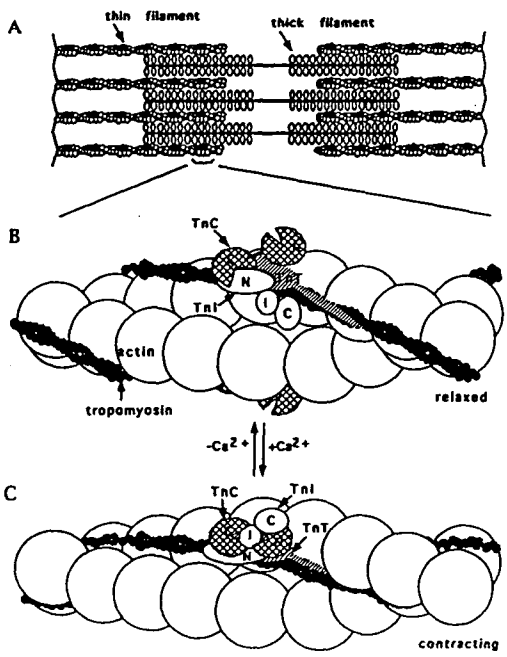

Figure 4. Model of the muscle contraction process.
(Compliments of Farah & Reinach, 1995)

This inhibitory action and actin binding is determined primarily by the minimum central dodecapeptide sequence corresponding to residues 104-115 (Ip), which possesses approximately 50% of the inhibitory activity of whole TnI on a molar basis. For full TnI inhibition however, the COOH-terminal region (i.e. carboxyl end at least up to residue 156) is required. This is supported by reconstitution experiments and the sequence homology between residues 108-115 and 137-144 (Talbot & Hodges, 1981). In effect, the muscle contraction model consists of 2 distinct, but related, states. In the relaxed condition, and hence the relative absence of Ca^{2+}, the Ca^{2+}/Mg^{2+} sites in the COOH terminal domain of TnC (i.e. the Ca^{2+} binding protein responsible for stimulation of actin and tropomyosin interaction at micromolar Ca^{2+} concentrations) are filled by Mg^{2+}, though the NH_2-terminal Ca^{2+}-specific sites are empty (Ohtsuki et al., 1986; Ingraham & Swenson, 1984). Concurrently, the NH_2-terminal region is bound to the COOH-terminal region of TnC as well as the TnT COOH terminus, whereas the inhibitory and COOH terminal regions of TnI make contact with actin and tropomyosin (thereby inhibiting actin-myosin interaction) (Fig. 5).

In the contracting state, where the Ca^{2+}-specific NH_2-terminal TnC sites are bound with Ca^{2+}, this domain's affinity for the inhibitory and COOH-terminal regions of TnI increase and therefore it is released from actin and tropomyosin. It is then hypothesized that this act may allow tropomyosin to change positions on the actin filament and liberate the actomyosin interaction that results in contraction. Farach & Reinarch (1995) also state,

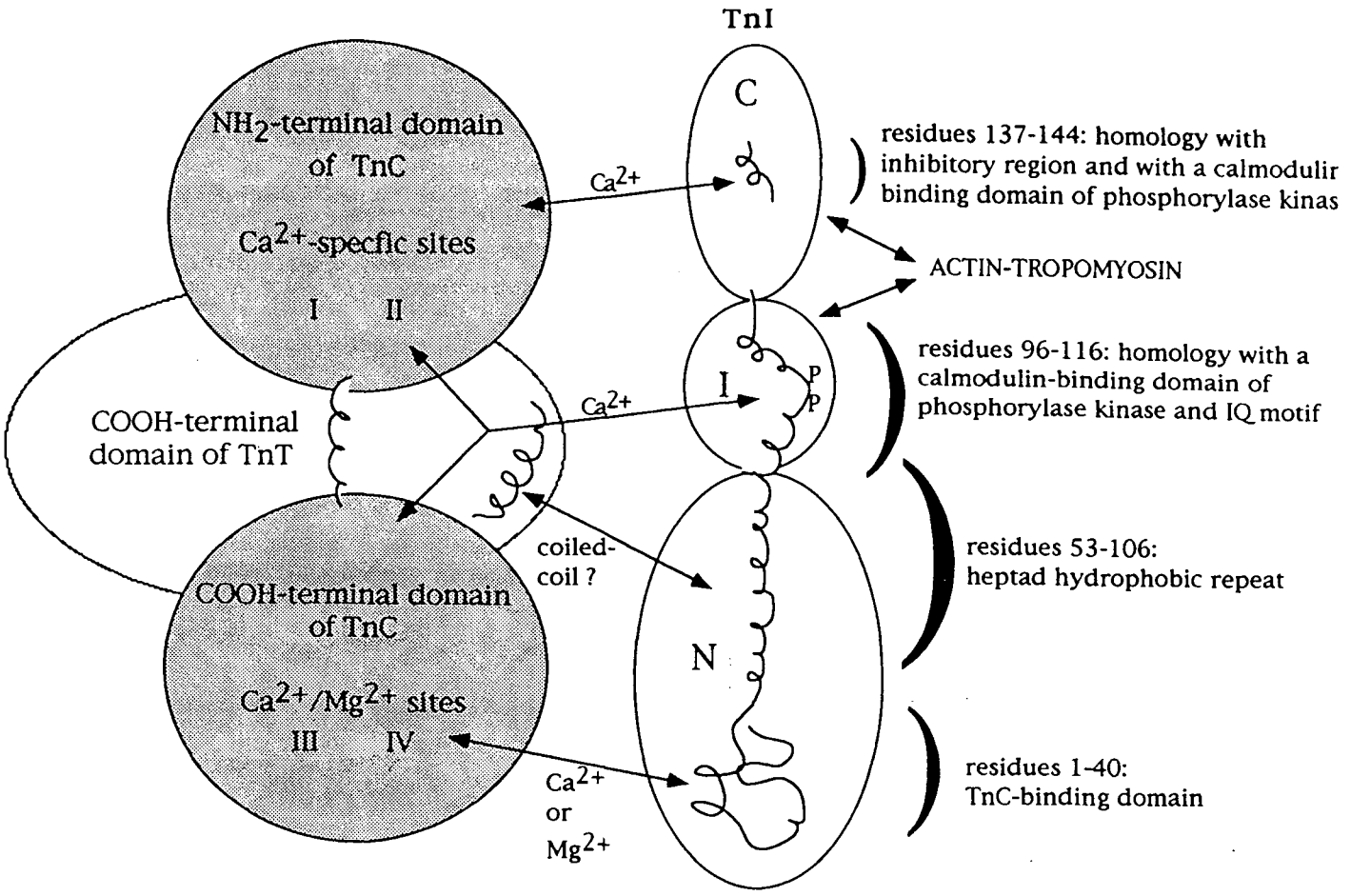

Figure 5. Molecular model of troponin interaction in skeletal muscle.
(Compliments of Farah & Reinach, 1995)

it is important to consider that "troponin's mechanism of control involves the inhibition of an otherwise strong and favourable interaction between actin and myosin, a characteristic common to systems where a quick, concerted and highly sensitive response to a stimulus is necessary".

Based on the very high affinity between TnI and TnC in both the presence ($Ka=1.7\text{-}7.0 \times 10^{9}\ M^{-1}$) and absence of Ca^{2+} ($Ka=0.6\text{-}1.2 \times 10^{8}\ M^{-1}$), it appears as though whether relaxing or contracting conditions are resident, both units remain associated (even

though there is a 1 or 2 reduction in magnitude). It follows that it is a "subset of Ca^{2+}-dependent interactions" which must be responsible for the regulatory mechanism of the troponin complex, not simple association/dissociation of TnI and TnC as previously hypothesized. Furthermore, the Ca^{2+}-independent interactions are also crucial, as they are required to maintain the structural integrity of the complex (Ohtsuki et al., 1986). Upon further study, it appears TnI and TnC interact with one another in an anti-parallel arrangement (Figs. 4,5). The Ca^{2+}-independent interactions occur between residues 1-40 of the NH_2-terminal region of TnI and the COOH-terminal domain of TnC, whereas Ca^{2+}-dependent interactions are found between the inhibitory and COOH-terminal domains of TnI and the NH_2 terminal domain of TnC, as well as the inhibitory region of TnI and the COOH-terminal domain of TnC. It also appears that the TnI-TnT interaction, and hence the mediator of tropomyosin cooperativity, occurs between residues 40-98 of the TnI NH_2-terminal region and the globular COOH-terminal domain of TnT.

Because TnI is a structurally bound protein, unlike CK or Mb for example, it requires both significant membrane disruption and specific degradation of the subcellular contractile apparatus to ensure leakage into the cytoplasm and subsequently the blood. Plasma TnI concentrations are therefore indicators of quite significant muscle fiber damage. In regards to the relative distribution of bound myofibrillar sTnI versus free cytosolic sTnI (and eventual blood plasma sTnI), before or after exercise-induced muscle damage, very little is known. Sorichter et al. (1997) however, have quantified the normal, resting sTnI cytoplasmic precursor pool as approximately 3.4% of total muscle sTnI. The

exercise-induced changes in myofibrillar protein distribution and composition are a major focus of this study.

5. Troponin T, Tropomyosin and muscle injury:

In addition to skeletal muscle troponin I, another member of the myofibrillar troponin complex which appears to be a good substrate for calpain isoform degradation, is troponin T (TnT)(Saido et al., 1994). TnT is a structurally asymmetric protein of approximately 31 kDa which is responsible for troponin-tropomyosin binding. TnT's amino-terminal domain (residues: 1-158) seems to be involved in achieving a cooperative association between Ca^{2+} binding to the thin filament and tropomyosin binding to the actin molecule (Figs. 4,5) (Ohtsuki et al., 1986; Zot & Potter, 1987). Specifically, it appears to be residues 70-158 of the NH_2-terminus which are directly involved in this interaction along the carboxy-terminal third of the tropomyosin coil (i.e. from Cys190 to the COOH-terminal where it overlaps with the NH_2-terminal region of the adjacent tropomyosin). TnT's carboxy-terminal domain (residues 159-259) however, is responsible for binding TnI and TnC, as well as tropomyosin (i.e. near residue Cys190), and hence is crucial to the organisation of the entire complex (note: this binding is reduced by TnC-Ca^{2+} interaction).

Another myofibrillar protein which is an excellent *in vitro* calpain substrate is tropomyosin (Saido et al., 1994). It is a threadlike peptide of approximately 20,000 Daltons. Tropomyosin proteins are arranged end to end as a continuous filament along the groove of each actin coil/spiral (double helix), acting to cover the cross-bridge binding site and therefore aid in the regulation of the contraction cycle. It has been proposed for over

20 years that in the absence of Ca^{2+}, tropomyosin is positioned such that actomyosin-ATPase is inhibited. In the presence of Ca^{2+} however, tropomyosin shifts via troponin control to remove the inhibition on contraction (Fig. 4). It is still unclear though if this inhibition is "resident at the actin-binding step and/or subsequent to actin binding" (Chalovich, 1992).

Because of the structurally bound nature of sTnT and tropomyosin, much like sTnI, the breakdown and consequential release of these proteins into the cytoplasm and blood is reliant on specific degradation of the myofibrillar complex by a protease system (as well as significant plasma membrane damage). While very little is known of sTnI distribution within the myofiber, cytoplasm or blood plasma, (pre or post-muscle injury), there is even less known about TnT and TM. Elucidating the flux in myofibrillar and cytoplasmic peptide levels, as well as the influence of the calpain-calpastatin protease system on their composition, is crucial to understanding the mechanism(s) behind general muscle injury.

6. *Markers for exercise-induced muscle injury:*

Based on the exercise-induced degradation of numerous contractile proteins, their release into myofiber cytoplasm and subsequent transient increases in blood plasma, the use of these proteins as markers for muscle injury has become a very useful clinical tool. A good marker incorporates a number of crucial qualities, including: sensitivity (i.e. an immediate increase post-damage), specificity (i.e. for the particular type of damage one is measuring), precision (i.e. reproducibility), and accuracy (i.e. a solid reflection of the

actual cellular muscle damage). Traditionally, the two predominantly cytosolic proteins, creatine kinase (CK) and myoglobin (Mb), have been the preferred indicators. With both proteins, a significant increase in their plasma concentrations is evident relatively early post-eccentric exercise (i.e. during muscle damage) (Clarkson et al., 1986). For reasons which are unclear, the typically biphasic Mb release (and therefore increased blood plasma Mb concentrations) precedes a somewhat delayed increase in serum CK concentrations (Sorichter et al., 1995); this is perhaps a reflection of their distinct half lives in the general circulation, as both proteins are transported from the muscle interstitium to the blood via the lymph because of the extremely low capillary permeability in muscle (i.e. continuous capillaries)(Lindena et al., 1984). In both cases however, their release is likely due to temporary muscle fiber damage and accompanying membrane leakage (i.e. increased permeability) involved with exercise (Friden et al., 1988, 1981). Typically exercise is associated with only sub-lethal injuries and therefore any release via final muscle cell death can virtually be ruled out (Friden et al., 1988; Newham et al., 1983). Because these cytosolic proteins (CK and Mb) require only a small window in the plasma membrane to leak from the cytoplasm into the blood plasma (i.e. before the still viable muscle fiber reseals), they are considered indicative of minor muscle damage.

At some point in time virtually every cytosolic muscle protein has been used as an injury marker. Lactate dehydrogenase (LDH), an NAD^+ dependent enzyme, α-glyceraldehyde-3-phosphate dehydrogenase, and other glycolytic enzymes are no exception. Few, if any, have had much success.

In 1992 however, Mair et al. documented the use of a newer marker, myosin-heavy chain (MHC). There are 4 distinct isoforms of MHC characterised thus far: three fast (type IIb, IIa, IIx) and one slow (I). All have been reported in adult mammalian locomotory muscles and retain a common nucleotide binding site located at the interface between the 25 kDa N-terminal (i.e. motor driven) and the central 50-kDa tryptic fragments (Pette & Staron, 1996). Unlike CK or Mb, MHC is a structurally bound protein of the thick filaments and hence indicative of more significant muscle cell damage. Mair et al. (1992) postulated that the significantly slower increase in plasma MHC concentrations following exercise (i.e. versus CK or Mb) is due to the requirement not only for plasma membrane disruption, but also for the specific degradation of the contractile apparatus by various proteases (peak plasma concentrations of MHC were noted approximately 48 hours after exercise). Furthermore, the rise in serum MHC concentrations also proved to be a far superior indicator for myocardial infarction than either CK or Mb (Adams et al., 1994).

In response to the success of MHC release as an indicator for muscle damage, other structurally bound contractile proteins have also recently been enlisted as possible markers (including desmin, α-actinin, spectrin, ankyrin, and TnT). Particularly successful however, is cTnI which both Adams et al. (1993) and Mair et al. (1994) have concluded is not only a more specific marker for the diagnosis of myocardial injury than CK, Mb or MHC, but a more accurate one as well.

With the evidence that there are 3 distinct isoforms of TnI (slow-twitched, fast-twitched, and cardiac) (Wilkinson & Grand, 1978), one of which is specific for fast-

twitched muscle and hence eccentric exercise associated damage, it is not difficult to conceptualise the use of plasma skeletal TnI (sTnI) as a marker for muscle injury. Since complete muscle fiber disruption appears to occur within 1 day of cyclic eccentric exercise (Lieber et al., 1994) there is a definite need for a plasma marker more indicative of this early muscle damage (i.e. versus MHC). To this end, Sorichter et al. (1997) support sTnI as a possible candidate for this role, as peak values post-exercise occur within a 24 hour time period. Unlike CK or Mb, which are found in all muscle types, or MHC whose skeletal isoform cross-reacts with the antibody for its cardiac form, sTnI could potentially be a marker specific to skeletal muscle damage (i.e. first, because it differs considerably form cardiac TnI and does not cross-react with the same antibodies (Bodor et al., 1992) and second, because studies in rats have indicated that cTnI is not expressed in skeletal muscle after muscle damage (Saggin et al., 1990)).

The use of TnI in the diagnosis of either exercise-induced muscle injury or serious degenerative diseases, such as the family of muscular dystrophies is a distinct possibility. Currently CK (measured via a CK assay) is the preferred indicator for both the diagnosis of neo-natal Duchenne muscular dystrophy and the monitoring of athletic training status because of its ease of use (versus a CK gel, which although it would enable the distinction between the CK isoforms, is too tedious and time consuming for a practical diagnosis). The handicap in using CK assays, however is two-fold: first, CK assays possess an extremely high variability, both on an inter-subject as well as intra-subject basis. Second, after being compared to histologically quantified muscle damage, changes in plasma CK levels correlate with the appropriate magnitude of muscle injury only 70 % of the time

(Komulainen et al., 1995). In fact in some individuals, significant muscle damage may not induce changes in CK levels at all. Upon reflection of the specificity, sensitivity and expedience of the 22 minute assay for cTnI now being used in the diagnosis of myocardial infarction however, a similar sTnI assay for skeletal muscle injury would prove extremely useful.

For patients with the potential for concurrent myocardial and skeletal muscle injuries this marker could prove especially important. Definitive delineation of their relative contributions to an overall disease-state could aid patients with renal failure, seizures, skeletal muscle myopathies, trauma injury and/or post operative complications. Such precision would drastically simplify care (something the current use of CK is unable to do, as its causative source is often impossible to distinguish).

Although the utility of these myofibrillar markers as a diagnostic for muscle injury is undeniable, the precise mechanism(s) underlying the breakdown, release and transport of these proteins are not available in the literature.

Additionally, it should be noted that the potential exists for the detection of not only full length sTnI peptides exists, but also for sTnI fragments as well. It has very recently been proposed that plasma cTnI fragment detection may provide an even superior marker for myocardial infarction. These fragments, generated by proteolysis, are hypothesized to proceed through the injured cell and into the associated plasma in a more rapid fashion (i.e. vs. full length peptides). The same concept can also be applied to skeletal muscle injury and hence to the sTnI fragments produced by exercise and/or muscular pathologies (Fig. 1b).

SYNOPSIS:

In view of the strong link between exercise-induced myofibrillar protein degradation (i.e. sTnI, sTnT, TM), their consequential release into the cytoplasm and blood plasma, and the calcium dependent cysteine protease, calpain, the objectives of the current study and their corresponding hypothesis are:

To quantitate immediately following exercise, as well as after a recovery period (6 hours), calpain isoform (I and II) and calpastatin activity, in the presence and absence of E64c, a cysteine protease inhibitor. It was hypothesized that as compared to sedentary controls, calpain activity (both I and II isoforms) would increase with exercise (25 m/min at -16° for 45 minutes) and be suppressed upon pre-exercise treatment with E64c (subcutaneous 1 mg/kg injection).

It was also an objective to investigate the relative effects of calpain isoform (I and II) and calpastatin activity, in the presence and absence of E64c, on the overall function of the calpain proteolytic system. No distinct hypotheses were prepared for this objective because no current data was available on exercise-induced calpastatin activity changes.

Finally, we also aimed to quantitate immediately following exercise, as well as after a recovery period, the relative composition changes of myofibrillar proteins in both the myofibrillar complex and the myofiber cytoplasm (i.e. sTnI, sTnT, TM). It was hypothesized that associated with an increase in calpain activity, a decrease in myofibrillar protein bands (i.e. corresponding to sTnI, sTnT, TM) and an increase in the proportion of cytoplasmic protein bands would be evident. Furthermore, no changes in myofibrillar or cytoplasmic protein bands were expected upon E64c inhibition of calpain activity.

CHAPTER 3: RESEARCH METHODOLOGY:

Animal care:

Approval for the use of male Wistar-firth rats in this study was granted by the Animal Care Committee of the University of British Columbia (ref. A97-0213). Experiments were performed in accordance with the Canada Council on Animal Care requirements (1980-1984). All animals were separated into groups of four and placed in cages (1'x 1' x 2') in a temperature controlled environment (22-24°C). They were allowed "Purina rat chow" (commercial rat food) and acidified water *ad libitum*; their time line was divided into 12 hours of light followed by 12 hours of dark.

Exercise protocol:

Male Wistar-firth rats (~ 315 g) were randomly assigned to one of six groups:

1) sedentary control group (n=8)

2) control group with sub-epidermal E64c injections (n=8)

3) downhill running group sacrificed immediately post exercise (n=8)

4) downhill running group + E64c, sacrificed immediately post exercise (n=8)

5) downhill running group sacrificed 6 hrs. post exercise (n=8)

6) downhill running group + E64c, sacrificed 6 hrs. post exercise (n=8)

It should be noted that the E64c-treated animals were injected sub-epidermally with an E64c concentration of 1 mg/kg (E64c in ddH_20) 1 hour prior to exercise (note: animals which did not receive E64c injections were given a pin prick stimulus to simulate

the injection 1 hour prior to exercise). All animals were familiarised on a motor driven treadmill for 5 minutes at 15 m/min., 2 days prior to the test day. At this point they were considered accustomed to the exercise environment (Laughlin & Armstrong, 1982; Laughlin & Armstrong, 1983).

The experimental treatment consisted of downhill running (16° decline) at 25 m/min for 45 minutes (Belcastro et al., 1981). It is important to note that downhill running has been linked to more significant muscle damage, more eccentric muscle action, and a lower metabolic cost than either level or uphill running (Clarkson & Tremblay, 1988). It is therefore the preferred model for eccentric exercise-induced muscle damage. The animals were encouraged to run by noise and/or air stimulation, according to the guidelines of the Canada Council on Animal Care. The rats provided a recovery were returned to their cages and allowed to resume normal activity for the 6 hour time period. As before, the animals were kept in a temperature controlled environment (22-24°C) and provided food and water *ad libitum*. Also, information as to the running quality and weight of each rat was recorded.

Sample collections:

In all groups, rats were given a dose, intra-peritoneal injection of sodium pentobarbital (~O.8 mL Euthanol per animal). Blood samples obtained by cardiac puncture were collected in EDTA-treated tubes (K_3-treated 5 mL Vacutainer sterile interior tubes (Franklin Labs, N.J., U.S.A.))(note: the needle tip was removed upon filling the tube to ensure smooth blood transfer and a reduction in lysing any blood cells). The

plasma was then immediately separated by centrifugation (3000 rpm for 10 minutes) (using a Hermle centrifuge, model z360k-rotor 27072V) and frozen at -80°C until analysed (i.e. for CK, sTnI, sTnT, TM).

Following blood sampling, the vastus lateralis muscle was excised from the right hindlimb and immediately frozen in liquid nitrogen. Similar to the plasma, all tissue samples were stored at -80°C until analysed. The remaining hindlimb muscles (soleus, plantarus, gastrocnemius and the quadriceps complex) from the right and left leg were then removed and again frozen in liquid nitrogen prior to analysis (approx. 5 grams of tissue was required for the calpain isoform and calpastatin isolation and subsequent assay). All tissue samples were excised in less than 5 minutes to minimise any post-mortem artefact (i.e. rigor mortis associated metabolic or proteolytic changes). Wistar rats were chosen as the particular animal model not only because they can be easily trained to run on a declining treadmill (Laughlin & Armstrong, 1982), but also because rat reaction to treadmill-induced exercise muscle damage has been well characterised in the past (Belcastro et al., 1983; Belcastro, 1993, Belcastro et al., 1997).

Isolation and quantification of Calpain I, II and Calpastatin:

This method is a modification of Gopalakrishna & Barsky (1985). Skeletal muscle samples (5g) were homogenised in 5 volumes of ice-cold homogenisation buffer (50 nM Tris/HCl, 1mM EDTA, 150 nM pepstatin A, and 0.01 M DTT) using an Ultra-Turrax polytron (T25 piston)(IKA-Labortecnik, U.S.A.) at half-maximal setting for 3x15 seconds (while immersing the tube in ice). All separation and chromatography was performed at 4°C. The homogenate was then centrifuged at 13,000 g for 30 minutes (using a Hermle centrifuge, model z360k-rotor 27072V) and the supernatant decanted through glass wool (normal grade) to separate any lipids. The supernatant (20 mL for calpain isoform isolation) was mixed with 40 uL of 1 mM-leupeptin and 4 mL of Phenyl-Sepharose beads (which had been previously washed with Buffer A + 0.25M NaCl. Buffer A consists of 20mM Tris/HCl, 0.1 mM $CaCl_2$, 10 mM DTT and 20 uM leupeptin). To this mixture, 1.2 mL of 5M NaCl was added. The solution was then shaken for 5 minutes (while immersed in ice), stopped, 0.1 M $CaCl_2$ added, and shaken for another 10 minutes. The end product was packed into a 10 mL plastic column (Econo-pac chromatography columns, 1.5 x 12 cm polypropylene columns)(Bio-Rad Labs., CA, U.S.A.) and successively eluted with 8 mL of: 1) buffer A with 0.2 M NaCl, 2) without NaCl, and 3) without leupeptin (this series of washes acts to rid the column of abstract muscle proteins). Calpain II was eluted with 16 mL of buffer B (consists of 20mM Tris/HCl, 1mM EGTA, and 10 mM DTT) with 0.1M NaCl. Calpain I was eluted with 8 mL of buffer B alone (i.e. no NaCl) (note: all DTT was added to the above solutions fresh every day).

For storage, subsequent protein quantification and gel electrophoresis, the calpain I and II solutions were concentrated using an Ultrafree-15 Centrifugal filter device, with Biomax-10K NMWL membranes (Millipore, MA, U.S.A.). A 2 to 15 mL sample was poured into the filter device without touching the membrane surface. The filter unit was then placed in a 50 mL centrifuge tube, balanced and centrifuged at 2,000g for 6 minutes (calpain I) and 10 minutes (calpain II) (using a Hermle centrifuge, model z360k-rotor 27072V). The remaining 200 uL - 500 uL solution was quantified by the appropriate protein assay (note: these filter units were good for one use only).

To isolate calpastatin, 1 mL of the supernatant was boiled for 5 minutes, followed by a 10 minute centrifugation at 3000 rpm (using a Hermle centrifuge, model z360k-rotor 27072V) to precipitate heat-denatured proteins. The calpastatin supernatant was then collected using a Pasteur pipette and stored at -80°C until analysed.

Calpain assay:

Ca^{2+}-dependent proteolytic activity was determined by measuring the release of trichloroacetic acid-soluble peptides from Hammersten casein, absorbing at 280 nm (Yoshimura et al., 1984). 3 mL samples of eluant were combined with 1.5 mL of Ca^{2+} assay medium (0.1 M Tris/OH (pH=7.5), 1mM NaN_3, 0.002 M DTT, 0.05 mM $CaCl_2$ (for calpain I) or 1 mM $CaCl_2$ (for calpain II), and 5 mg of caesin/mL) and incubated at 25°C for 30 minutes. The DTT was again, made fresh every day. The reaction was stopped by adding 0.25 mL of ice-cold 50% trichloroacetic acid. The solution was then centrifuged at 3000 rpm for 10 minutes (using a Hermle centrifuge, model z360k-rotor 27072V) and the

supernatant (i.e. soluble digestion products) measured spectrophotmetrically at 280 nm. All activities were measured in triplicate. One unit of calpain activity was defined as the amount of enzyme which produces an increase of 1 absorbance unit at 280 nm after 30 minutes of incubation at 25°C, corrected by subtracting the activity of 1mM EDTA.

Calpastatin assay:

Calpastatin activity was assayed based on its inhibition of calpain II activity. Inhibitor activity was measured by adding 150 uL of boiled fractions of isolated calpastatin to 0.2 enzyme units of calpain II. This solution was then pre-incubated at 25°C for 10 minutes. Following the pre-incubation, the remaining proteinase activity was determined using the protein assay described above (i.e. calpain assay). 1 unit of calpastatin activity was defined as inhibiting 1 unit of calpain II completely.

Glycogen assay:

Approximately 35-50 mg samples of the rat vastus lateralis were kept on ice throughout the addition of 0.5 mL of 30% KOH saturated with $NaSO_4$ (being sure the tissue was totally immersed in solution). The tubes were then boiled in a water bath for 20-30 minutes (until a homogenous solution was obtained), removed and cooled in ice. 1.1-1.2 volumes of 95% ethanol was added to precipitate the glycogen from the alkaline digestate. The samples stood in ice for 30 minutes and were subsequently centrifuged at 810g for 20-30 minutes (using a Hermle centrifuge, model z360k-rotor 27072V). Next, the supernatants were carefully aspirated. The glycogen precipitates were dissolved in 3.0

mL of distilled H_2O, of which a small aliquot was pipetted into a 150x20 mm test tube and brought to a sample volume of 1 mL by the addition of distilled water. 1.0 mL of 5% phenol solution was added, followed by 5.0 mL of 96-98% H_2SO_4 (rapidly, in 10-20 seconds), the stream of acid being directed against the side of the test tube (mixed well). The tubes were allowed to stand for 10 minutes, shaken, and placed in a water bath at 25-30°C before readings were taken. Blanks were prepared by using 1.0 mL distilled water and the absorbance for each sample was read at 490 um. All tests were carried out in triplicate. To convert the absorbance reading to grams of glycogen per 100 grams of tissue, the formula: $(ABS/K)x(V/v)x(10^{-4}/W)$ was used, where V = total volume of glycogen solution, v = volume of aliquot used in the colour reaction, ABS = absorbance at 490 um, W = weight of tissue sample in grams, K = slope of the standard curve, units = 1 per microgram of glycogen. This method was published by Lo et al. (1970).

Creatine Kinase assay:

This assay was commercially available from Sigma (Sigma Diagnostics CK assay, Procedure No. 520, MO. U.S.A.) Into each test tube, 0.1 mL of a 10-fold dilution of blood plasma in water (1 part plasma, 9 parts water) was added, followed by 0.5 mL of phosphocreatine solution (Pcr) (mixed by tapping). Into a blank test tube, 0.1 mL of water and 0.5 mL Pcr solution was pipetted. The tubes were then placed in a 37°C water bath for 5 minutes to warm up. 0.2 mL of ADP-Glutathione solution was added to start the reaction (mixed by tapping) and incubated for 30 minutes. Following the incubation period, 0.2 mL of p-hydroxymercuribenzoate solution was added to stop the reaction.

Then, 1.0 mL α-napthol solution, 1.0 mL diacetyl solution, and 7.0 mL of distilled water were added (the contents of all tubes were mixed after each addition). Subsequently, the tubes were placed in a 37°C water bath for 15-20 minutes to promote colour development. Finally, the tubes were centrifuged at 3000 rpm for 5 minutes (using a Hermle centrifuge, model z360k-rotor 27072V), transferred to cuvets and the absorbances of the test solution versus the blank solution were read (i.e. at 520 nm). These readings were completed within 10 minutes of the final centrifugation. The standard calibration curve was prepared (in advance) using 6 tubes (0 mL Pcr solution + 1.0 mL water; 0.2 mL Pcr sol. + 0.8 mL water; 0.4 mL Pcr sol. + 0.6 water...1.0 mL Pcr sol. + 0 mL water). To each "standard" tube, 1 mL of α-napthol, 1 mL of diacetyl solution and 7 mL of distilled water was added. Each of the contents were mixed after their addition. After 15 minutes the absorbance was read using the first tube as a reference (again, within 10 minutes of colour development).

Myofibril purification:

The myofibril preparation involved weighing the sample, followed by its immersion in 20 volumes of homogenisation buffer (39 mM sodium borate, 25 mM KCl and 5 mM EGTA, pH=7.1). The tissue sample was then homogenised in an Ultra-Turrax polytron with a T25 piston (IKA-Laborternik, U.S.A.) at a setting of 6.5 for 15 seconds (until a homogeneous solution resulted). A small portion of the homogenisation buffer was saved and used to rinse the piston and tube to ensure all remnants of protein were in solution. The samples were centrifuged at 2900 rpm for 12 minutes (all centrifugations used a Hermle model Z 360K-rotor 22072V). Following centrifugation, the supernatant was

retained and marked "cytosolic-1", while the pellet was resuspended with buffer II (100 nM KCl, 50 nM Tris, pH=7.1). The new solution was then centrifuged again, as above. Following this centrifugation, the supernatant was retained (marked "cytosolic-2") and the pellet resuspended in wash buffer (100 nM KCl, 50 nM Tris, 1.0% w/v Triton X-100, pH=7.1) and centrifuged (as above). The supernatant was discarded. This was done twice. Following the last wash buffer, the myofibrils were resuspended in suspension medium (100 nM KCl, 20 nM Tris, pH=7.1). A quantitative protein assay (Lowry et al., 1951) was completed to quantify the yield of protein for each phase (i.e. homogenate, cytosolic-1, cytosolic-2 and myofibril)(see below). If these robust myofibrils were stored, centrifugation was repeated and they were resuspended in a low salt buffer (100 nM KCl, 2 mM $MgCl_2$, 2 mM EGTA, 10 mM Tris, pH=7.1). The solution was stored at -80°C.

Quantitative Protein assay:

This protein assay was adapted from Lowry et al. (1951). First, standard solutions were prepared (0 mL protein stock (5mg/mL BSA in distilled water)) + 0.5 mL LSB buffer (100 nM KCl, 2 mM Mg Cl_2, 2 mM EGTA, 10 mM Tris, pH=7.1), 0.1 mL protein stock + 0.4 mL LSB, 0.2 mL protein stock + 0.3 mL LSB buffer,...,0.5 mL protein stock + LSB buffer). Subsequently, 0.1 mL from all standards and samples was diluted with 0.2 mL of 0.3 M KOH. This is a copper-alkaline solution which was prepared fresh when needed. To 48 mL of reagent A (0.1 M NaOH, 2% w/v sodium carbonate, pH=12.5), 1 mL of 0.5% w/v copper sulphate and 1% w/v sodium potassium tartrate was added. 0.1 mL of all standards and samples were mixed with 2.5 mL of copper-alkaline solution and

stood at room temp. for 10 minutes (in duplicate). Next, 0.25 mL of diluted F-C reagent was added (dilute Folin-Ciocalteu reagent 1:1 with distilled water) to each tube while mixing with a vortex (setting "3" on a Vortex Genie 2, Scientific Industries, U.S.A.). It then stood for 30 minutes. Absorbances were measured against a blank tube (distilled water) at 750 nm. Finally a printed standard curve of OD_{750} versus Protein Concentration (mg/mL) was obtained to determine the unknown concentrations.

Dye-Binding Protein assay:

In the case of purified calpain isoforms I and II, the concentrations were too low for quantification via the Lowry et al. (1951) protein assay, hence the standard assay (Bio-Rad, CA., U.S.A.) was used to quantify the protein amounts. The dye reagent was prepared by diluting 1 part dye reagent concentrate with 4 parts distilled, deionized water, which was then filtered through Whatman #1 filter paper to remove any particles. This reagent had a shelf life of approximately 2 weeks. Subsequently, 3 to 5 dilutions of a protein standard (BSA-1 mg/mL) were tested. The linear range of the assay was 0.2 mg/mL to 0.9 mg/mL. 100 uL of each of the standards and samples were pipetted into a dry test tube (in duplicate) and 5.0 mL of diluted dye reagent added to each tube. They were then vortexed (setting "3" on a Vortex Genie-2, Scientific Industries, U.S.A.) and incubated at room temperature for at least 5 minutes. The final absorbance was measured at 595 nm within 1 hour.

Poly Acrylamide Gel Electrophoresis (SDS-PAGE) analysis:

Proteins were resolved on 1-dimensional sodium dodecyl sulphate-poly acrylamide gel electrophoresis (SDS-PAGE) unit with a discontinuous buffering system (Lammeli, 1970) (Fig. 6). Concentrations of protein in the homogenate were adjusted to 50 mg/ 80 uL well. Of that 80 microliters, the SDS-PAGE sample buffer always composed a minimum of 5 uL (62.5 mM Tris-HCL, pH=6.8, 20% glycerol, 2% SDS, 5% β-mercatoethanol, and 0.5% (w/v) bromophenol blue (in water)). All buffers were also degassed for 15 minutes prior to use.

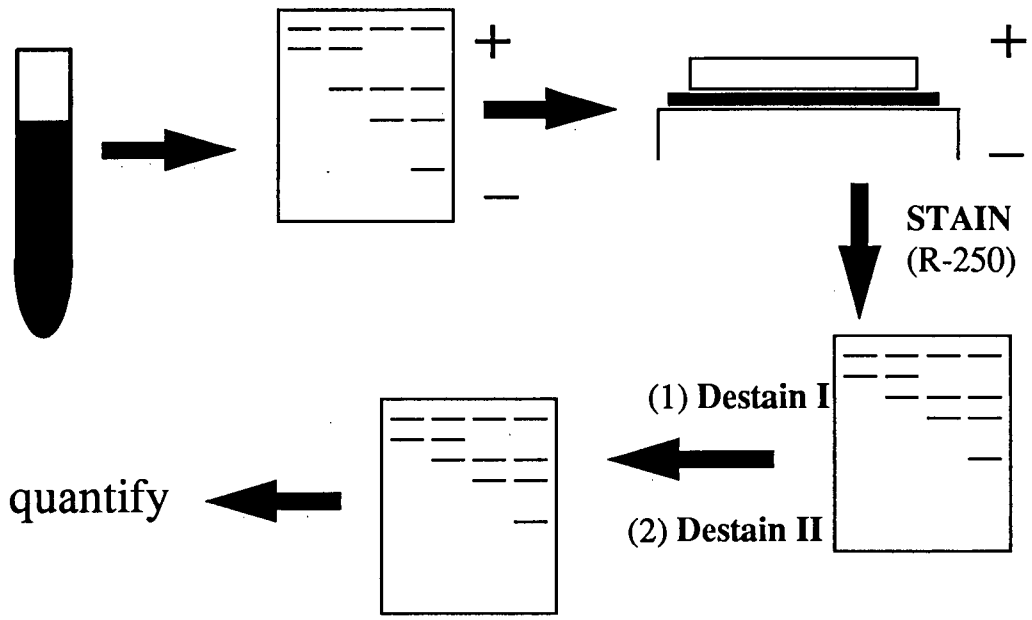

Figure 6.
Schematic representation of an SDS-PAGE

The 5-15% SDS PAGE gels were prepared from stock solutions and used to synthesize 1.5 mm thick gradient gels. The 5% gel solution (approx. 23 mL) was made of 3.75 mL 30% acrylamide, 5.63 mL Tris-OH (pH=8.8), 225 uL 10% SDS, 12.83 mL distilled water, 67.5 uL of 10% ammonium persulphate, and 7.5 uL TEMED. The 15% gel solution (approx. 23 mL) was composed of 11.25 mL 30% acrylamide, 5.63 mL Tris-OH (pH=8.8), 225 uL 10% SDS, 5.39 mL glycerol, 13.5 uL 10% ammonium persulphate, and 7.5 uL TEMED, (note: gel polymerisation was initiated by the addition of ammonium persulphate and TEMED and hence the gels were poured immediately after their addition). These two solutions were then mixed in a mixing chamber (linear gradient maker) and cast between two glass plates in a BIO-Rad (CA, U.S.A.) gel assembly. Approximately 1.5 hours was allowed for polymerization. Subsequently, the 4% stacking gel (2.6 mL 30% acrylamide, 5.0 mL Tris (pH=6.8), 200 uL SDS, 12.2 mL distilled water, 100 uL 10% ammonium persulphate, and 200 uL TEMED) was poured immediately after polymerisation was initiated (i.e. into the area below and surrounding the well comb). The stacking gel was allowed to set for 1 hour. Prior to loading the samples into the wells, the protein samples were heated to 45°C for 20 minutes, with the sample buffer, to solubilize the proteins (this included a sample of the recombinant molecular weight standard marker (e.g. Sigma-M-0671)). During this time the polymerised gels were mounted on the central core of the SDS-PAGE unit. Once the upper chamber was filled with Tank buffer (25 mM Tris-HCL, pH=6.8, 20% glycerol, 2& SDS, 5% β-mercaptoethanol) the samples were injected into each lane using a small pipette (note: the electric wire in both the upper and lower chambers must be immersed in tank buffer to prevent overheating). Protein

concentrations in each lane were equated and loaded accordingly (i.e. to avoid concentration errors). It should also be noted that all components in contact with the acrylamide were first washed with 95% ethanol to ensure all impurities were removed. Finally, a stir bar was in continuous motion throughout the gel run (to ensure tank buffer circulation) and the cooling water hoses were connected and in full flow (to cool the central core unit).

The electrophoresis was carried out at a constant amperage of 40 mA for 16 hours (i.e. for 4 concurrently running gels: therefore 10 mA per gel) and the gels stained with 0.025% Coomassie Blue (R-250) stain (45% methanol, 45% distilled water, 10% glacial acetic acid and 2.75g/1100 mL R-250) for 45 minutes (Morrisey, 1981). Following staining, the gels were destained in 2 washes of destain I (45% methanol, 45% distilled water, 10% glacial acetic acid) overnight. Finally, the gel was destained in destain II (20% methanol, 5% acetic acid, and 75% distilled water) for approximately 3 hours. The completed gel was then dried in 70-100% methanol for 5 minutes, mounted between two sheets of cellophane paper (Bio-Rad, CA, U.S.A.) and clamped between 2 perspex frames.

Data (gel) analysis:

The relative protein composition of each SDS-PAGE preparation (i.e. band intensity) was determined by a MacIntosh II PC utililizing a Hewlett Packard Scanner and IP Lab Gel Image Analysis software version 1.5 (Signal Analytics Corporation). By applying this software, the picture elements were stored as pixels (1 byte per pixel), which the program then assigns values between 0 and 900 (higher values signifying higher densities/intensities). The density of various segments was calculated as the volume of pixel values within each previously selected segment. The following formula was used to compute density quantities:

$$\text{Volume} = \Sigma (I_{xy}) - N*B$$

N= number of pixels

I_{xy}=pixel value

B=background value

In effect, the program acts to provide a specific quantitative value for each gel band (i.e. TnI, TnT, TM) which has been corrected for the background intensity. The intensity value for each band was also quantified relative to a preselected band (i.e. actin band at ~ 40 kDa) to account for potential loading error during sample injection. Actin was our internal standard as it is easily identifiable, significant in density, and close in proximity to the bands of interest.

STATISTICAL ANALYSIS:

All variables were tested for normal distribution and statistically equal variances. Statistical analysis (using SPSS for MS Windows, version 6.1) was based on a 2 by 3 (inhibition by group) factorial design for randomised groups ANOVA with a level of significance set at $p<0.05$. The main effects were defined as the "group" (Control vs. Run vs. Recovery groups) and "inhibition" (E64c vs. no E64c). Where analysis of variance proved significant, differences across inhibition treatments (E64c vs. no E64c) as well as across groups (Control vs. Run vs. Recovery) were evaluated by contrast methods which delineated each contributing factor (i.e. Tukey's post-hoc test for honestly significant differences). In essence, the dependent variables were the calpain isoform activities, calpastatin activities, plasma CK levels, muscle glycogen amounts and the gel band intensities corresponding to myofibrillar and cytosolic sTnI, sTnT and TM. The independent variables were the eccentric exercise condition (i.e. exercised versus control), the time of recovery post exercise (i.e. 0 or 6 hours) and the discretionary application of E64c. The ANOVA table employed is shown as table 1:

	GROUP		
	Control	Exercise (Run)	Recovery
w/o E64c	n = 8	n = 8	n = 8
w E64c	n = 8	n = 8	n = 8

Table 1.
Summary of 2 by 3 (inhibition by group) way ANOVA table

CHAPTER 4: RESULTS:

Creatine Kinase

All rats in the exercise (treadmill running at 25 m/min. (-16°) for 45 minutes) group ran for 45 ± 4 minutes (mean ± S.D.). To confirm the occurrence of an eccentric exercise response in this study, as well as investigate the potential interaction between calpain and creatine kinase (CK), blood CK values were quantified (Fig. 7).

Although there were no significant differences in CK plasma levels across various groups, with or without E64c treatment, the observed trends were consistent with exercise-induced muscle damage (Fig. 7). A large increase in CK concentrations was noted immediately post exercise (control: 991.87 ± 409 I/U vs. exercise: 2411.8 ± 1972 I/U)($p>0.05$). The lack of statistical significance is most likely a product of the immense variances resident in CK measurements. As previously indicated, the intra and inter subject variability in CK values is always large. The exercise-induced increase in plasma CK values was also evident into the recovery period (1569.3 ± 621 I/U)($p>0.05$). Interestingly, with the addition of E64c, there was a slight increase in the plasma CK values of each group (control + E64c: 1204 ± 584 I/U; exercise + E64c: 3061 ± 1140 I/U; recovery + E64c: 2380 ± 1114 I/U)($p>0.05$).

Glycogen

In addition to CK, muscle glycogen values were also quantified to verify the exercise response. Glycogen concentrations for the control group (1039.3 ± 283.6 ug/g) decreased by 50.9% immediately after exercise (510.5 ± 151.2 ug/g)($p<0.05$)(Fig. 8).

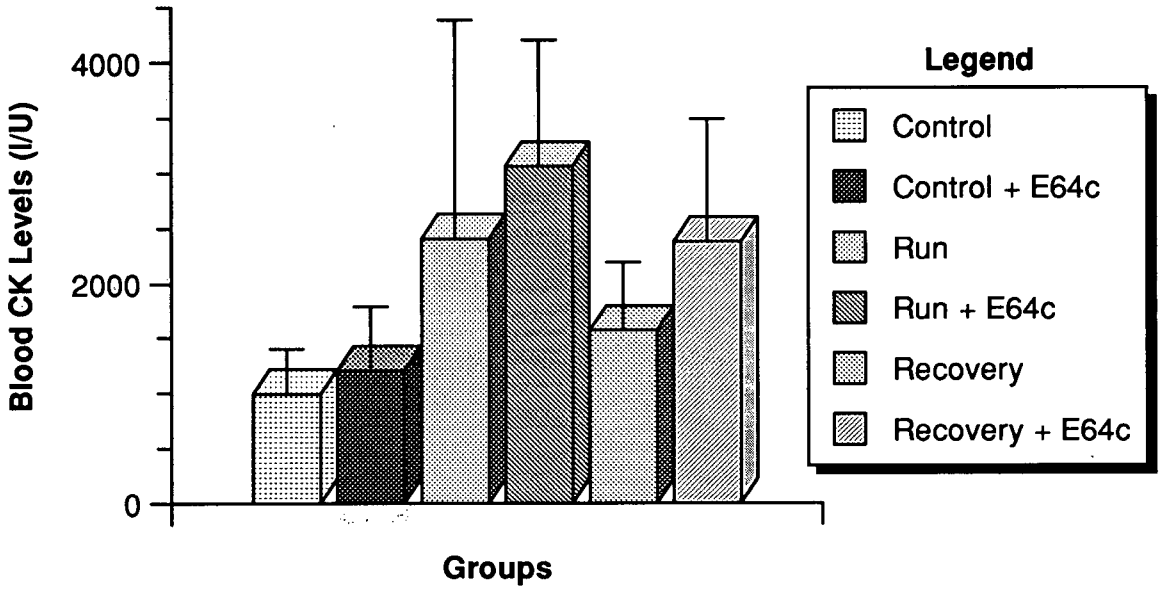

Figure 7.

Bar-plot representing blood plasma CK concentrations (I/U)(quantified via a commercially available heat-release assay). Results are presented as means ± S.D. No statistically significant differences were evident between any of the groups (Control, Run or Recovery, with or without E64c).

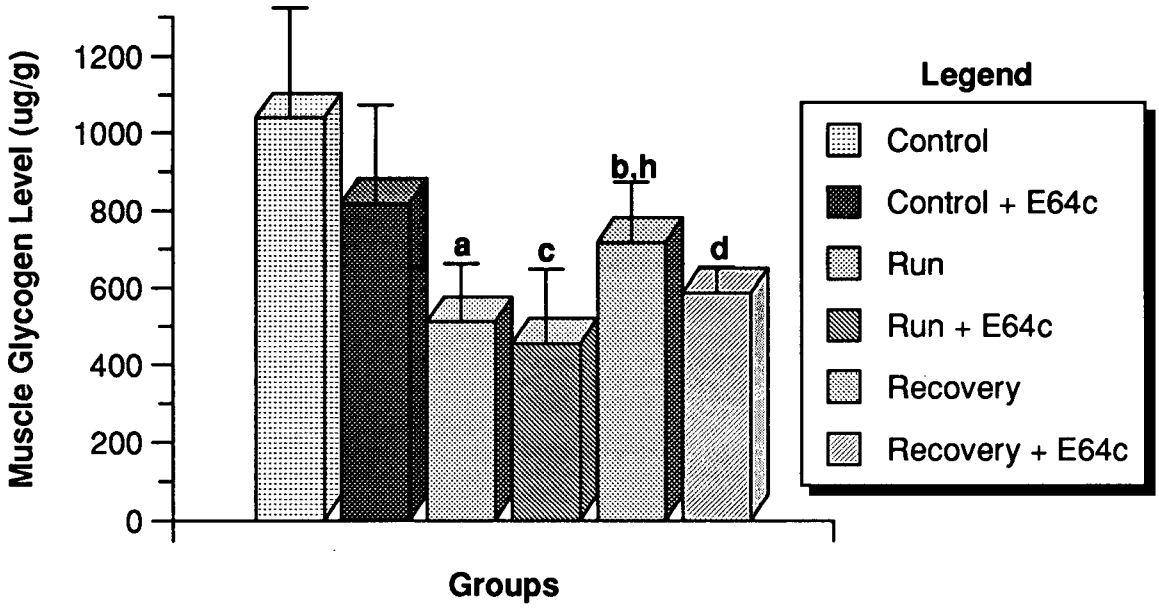

Figure 8.

Bar-plot representing muscle glycogen concentrations (ug/g)(quantified via a commercially available precipitation assay). Results are presented as means $\pm$ S.D.; "a" denotes a significant difference between Run and Control groups ($p<0.05$)(the running protocol consisted of 25 m/min downhill treadmill running (- 16°) for 45 minutes); "b" denotes a significant difference between Recovery and Control groups ($p<0.05$); "c" denotes a significant difference between Run and Control groups treated with E64c ($p<0.05$)(E64c treatment consisted of a subcutaneous 1 mg/kg injection); "d" denotes a significant difference between Recovery and Control groups treated with E64c ($p<0.05$); "e" denotes a significant difference between Run and Run + E64c treated groups ($p<0.05$); "f" denotes a significant difference between Recovery and Recovery + E64c treated groups ($p<0.05$); "g" denotes a significant difference between Control and Control + E64c treated groups ($p<0.05$); "h" denotes a significant difference between Run and Recovery groups ($p<0.05$); "i" denotes a significant difference between Run + E64c and Recovery + E64c treated groups ($p<0.05$).

They remained deflated after the 6 hour recovery (715.7 ± 156.9 ug/g)($p<0.05$). With E64c treatment, the control group (816.9 ± 255 ug/g) decreased 44.3% following exercise (454.9 ± 191 ug/g) and 28.4% after the 6 hour recovery (584.6 ± 70 ug/g)($p<0.05$). The differences between the run and recovery groups were also significant ($p<0.05$), indicating a recovery in muscle glycogen content with time. There were however, no differences in glycogen concentration between E64c-treated and corresponding un-treated groups ($p>0.05$).

The combined increase in blood plasma CK levels and decrease in muscle glycogen concentrations with the downhill exercise protocol utilized in this study, indicates exercise-induced stress of the active subjects (and the conditions required to investigate the objectives of this study).

Calpain Isoform Activity

Regardless of the calpain isoform (I and II), its activity (U/g) was increased immediately post exercise (Figs. 9,10). Calpain I activity for the control group (0.665 ± 0.112 U/g) increased by 36.1% immediately after exercise (0.905 ± 0.113 U/g)($p<0.05$). Likewise, calpain II activity for the control group (0.755 ± 0.105 U/g) increased by 37.5% immediately post exercise (1.038 ± 0.094 U/g)($p<0.05$). These findings suggest that at rest, there appears to be a small but consistent basal calpain isoform activity. As predicted, this isoform activity increases dramatically with intense exercise and the muscle-induced damage that accompanies it.

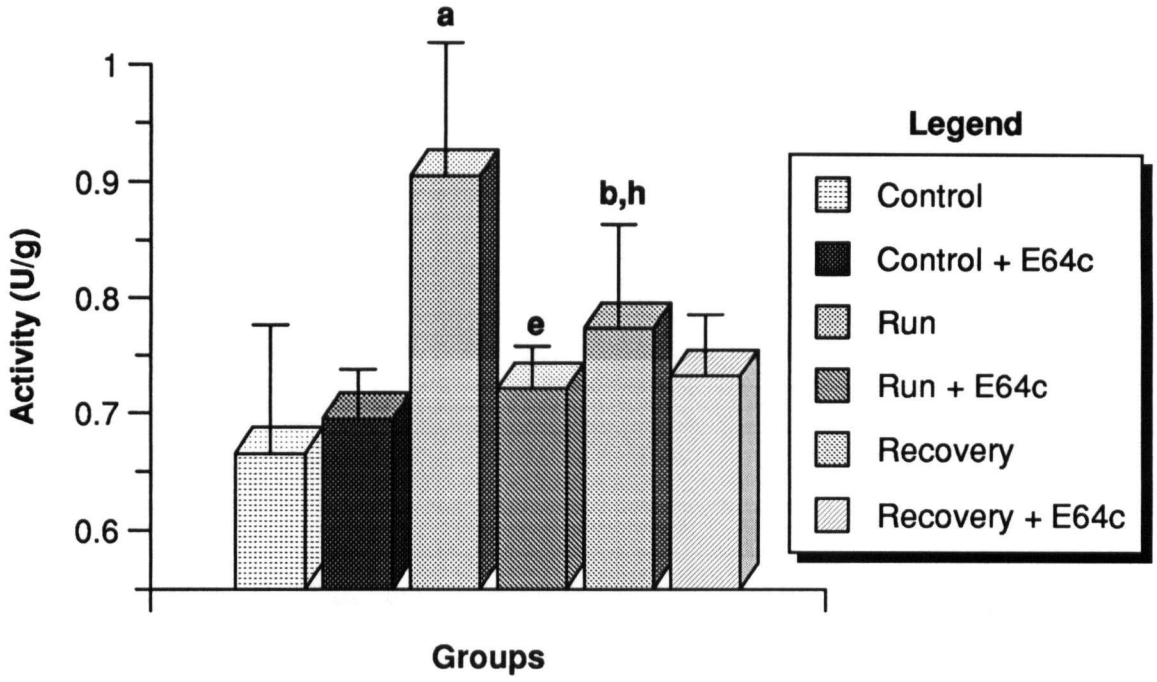

Figure 9.

Bar-plot representing calpain I isoform activity (U/g)(isolated via phenyl-sepharose chromatography and quantified by a casein substrate-release assay). Results are presented as means ± S.D.; "a" denotes a significant difference between Run and Control groups ($p<0.05$)(the running protocol consisted of 25 m/min downhill treadmill running (- 16°) for 45 minutes); "b" denotes a significant difference between Recovery and Control groups ($p<0.05$); "c" denotes a significant difference between Run and Control groups treated with E64c ($p<0.05$)(E64c treatment consisted of a subcutaneous 1 mg/kg injection); "d" denotes a significant difference between Recovery and Control groups treated with E64c ($p<0.05$); "e" denotes a significant difference between Run and Run + E64c treated groups ($p<0.05$); "f" denotes a significant difference between Recovery and Recovery + E64c treated groups ($p<0.05$); "g" denotes a significant difference between Control and Control + E64c treated groups ($p<0.05$); "h" denotes a significant difference between Run and Recovery groups ($p<0.05$); "i" denotes a significant difference between Run + E64c and Recovery + E64c treated groups ($p<0.05$).

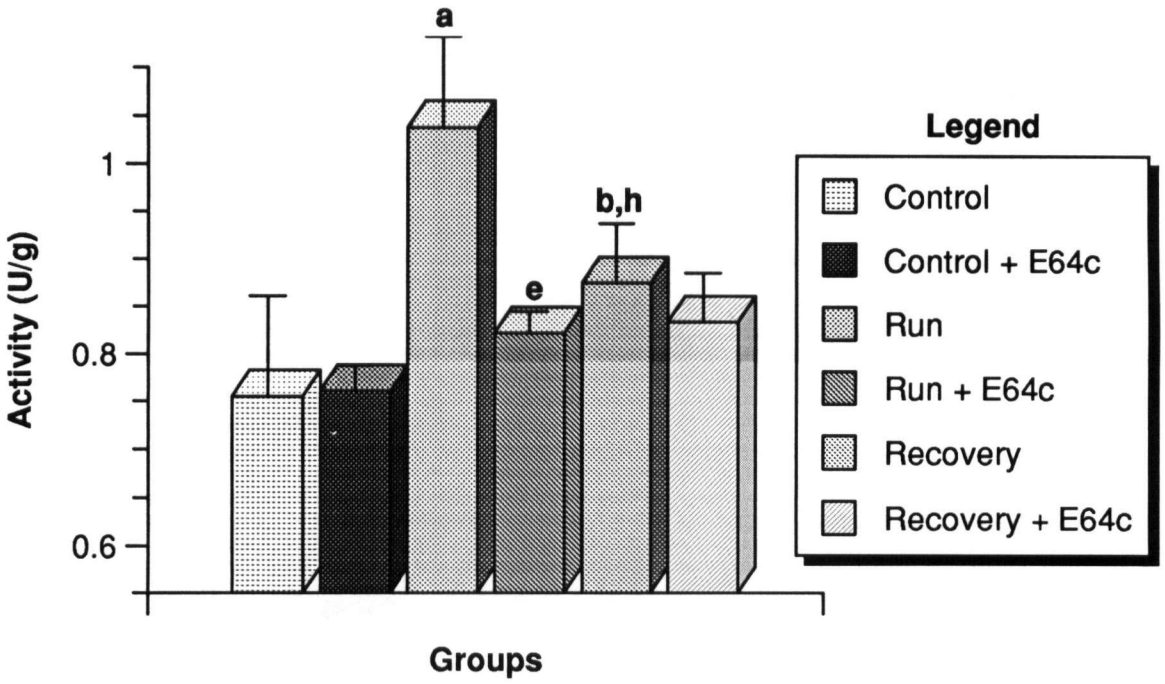

Figure 10.

Bar-plot representing calpain II isoform activity (U/g)(isolated via phenyl-sepharose chromatography and quantified by a casein substrate release-assay). Results are presented as means $\pm$ S.D.; "a" denotes a significant difference between Run and Control groups ($p<0.05$)(the running protocol consisted of 25 m/min downhill treadmill running (- 16°) for 45 minutes); "b" denotes a significant difference between Recovery and Control groups ($p<0.05$); "c" denotes a significant difference between Run and Control groups treated with E64c ($p<0.05$)(E64c treatment consisted of a subcutaneous 1 mg/kg injection); "d" denotes a significant difference between Recovery and Control groups treated with E64c ($p<0.05$); "e" denotes a significant difference between Run and Run + E64c treated groups ($p<0.05$); "f" denotes a significant difference between Recovery and Recovery + E64c treated groups ($p<0.05$); "g" denotes a significant difference between Control and Control + E64c treated groups ($p<0.05$); "h" denotes a significant difference between Run and Recovery groups ($p<0.05$); "i" denotes a significant difference between Run + E64c and Recovery + E64c treated groups ($p<0.05$).

Upon a 6 hour recovery after exercise, calpain activities for both isoforms (I and II) remained elevated over baseline control levels (Figs. 9,10). Calpain I activity remained 16.4% (0.774 $\pm$ 0.089 U/g) above the sedentary control group ($p<0.05$). Calpain II activity was still increased by 15.9% (0.875 $\pm$ 0.062 U/g) over controls ($p<0.05$). It is also important to note that both calpain isoform activities were different in the exercise (run) group versus the recovery (run + 6 hr. recovery) groups ($p<0.05$).

When E64c was administered, the control animals showed no differences in either calpain I (0.695 $\pm$ 0.040 U/g) or calpain II (0.761 $\pm$ 0.026 U/g) activities compared to the controls without E64c ($p>0.05$). It was apparent however, that in both the exercise and recovery groups, E64c had an inhibiting effect (Figs. 9,10). Calpain I activity for the control + E64c group increased only 3.7% immediately after exercise (0.721 $\pm$ 0.036 U/g) when E64c was utilised ($p>0.05$). In a similar fashion, calpain II activity increased only 8.2% post exercise (0.822 $\pm$ 0.022 U/g) with E64c treatment ($p>0.05$). Likewise, when rats were allowed a 6 hour recovery, their calpain activities also remained suppressed with E64c. Calpain I activity increased 5.31% (0.731 $\pm$ 0.054 U/g) over the control + E64c group, whereas calpain II activity increased 9.7% (0.834 $\pm$ 0.051 U/g)($p>0.05$).

Calpastatin Activity

Calpastatin activity from the hindlimb muscles demonstrated a markedly different response to exercise in comparison to either calpain I or calpain II (Fig. 11). Unlike the calpain isoform activity increase immediately after exercise, calpastatin activity showed no

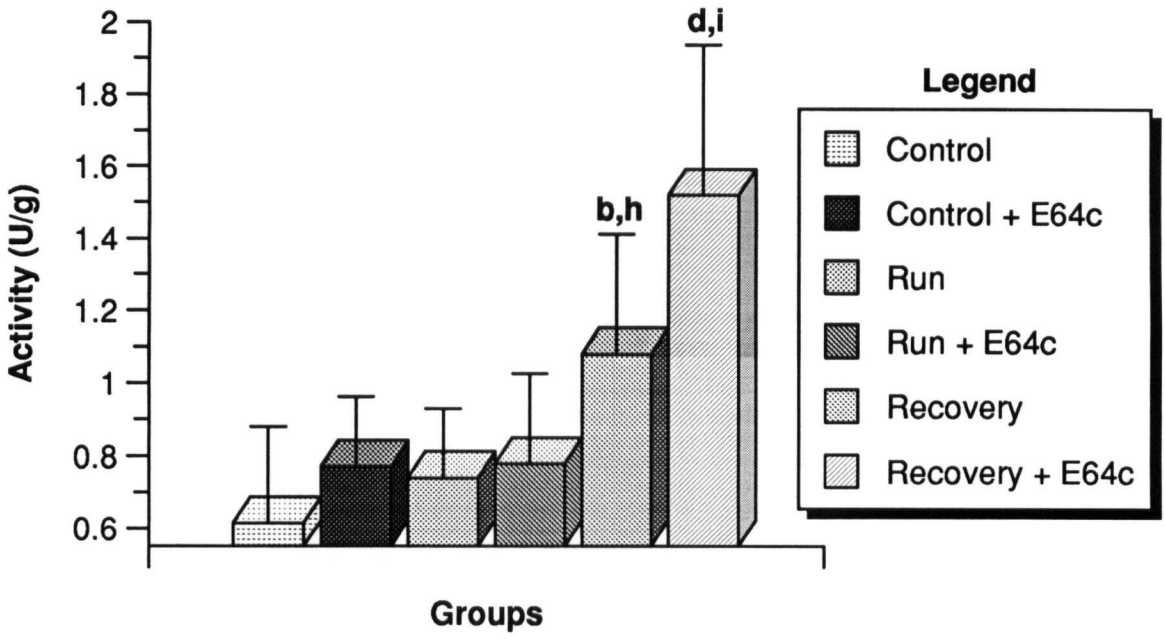

Figure 11.

Bar-plot representing calpastatin activity (U/g)(isolated via heat separation and quantified based on its inhibition of calpain II). Results are presented as means $\pm$ S.D.; "a" denotes a significant difference between Run and Control groups ($p<0.05$)(the running protocol consisted of 25 m/min downhill treadmill running (- 16°) for 45 minutes); "b" denotes a significant difference between Recovery and Control groups ($p<0.05$); "c" denotes a significant difference between Run and Control groups treated with E64c ($p<0.05$)(E64c treatment consisted of a subcutaneous 1 mg/kg injection); "d" denotes a significant difference between Recovery and Control groups treated with E64c ($p<0.05$); "e" denotes a significant difference between Run and Run + E64c treated groups ($p<0.05$); "f" denotes a significant difference between Recovery and Recovery + E64c treated groups ($p<0.05$); "g" denotes a significant difference between Control and Control + E64c treated groups ($p<0.05$); "h" denotes a significant difference between Run and Recovery groups ($p<0.05$); "i" denotes a significant difference between Run + E64c and Recovery + E64c treated groups ($p<0.05$).

change. Calpastatin activity for the control group (0.615 $\pm$ 0.264 U/g) increased 20.3% (0.740 $\pm$ 0.188 U/g) immediately post exercise ($p>0.05$). Only when the animals were provided the 6 hour recovery did calpastatin activity show a significant increase of 74.8% (1.079 $\pm$ 0.332 U/g) over baseline ($p<0.05$).

As might have been predicted, the difference in calpastatin activities between the group sacrificed immediately post-exercise and those after the recovery, was significant as well (increase of 45.8%)($p<0.05$).

Again, unlike the trends observed with calpain isoform activities, there were no significant differences in groups treated with E64c versus their counterparts without E64c injection ($p>0.05$)(Fig. 11). The absence of a difference in calpastatin activity between the control and exercise group sacrificed immediately post-running was also echoed when E64c was applied. There was no increase over baseline treated controls (0.771 $\pm$ 0.190 U/g) with running (0.779 $\pm$ 0.246 U/g)($p>0.05$). There was however, a significant increase in calpastatin activity of 97.4% (1.52 $\pm$ 0.417 U/g) over the control + E64c group ($p<0.05$) when the 6 hour recovery was provided (i.e. Recovery + E64c).

Net Potential Ratios

In addition to examining the absolute changes in both calpain and calpastatin activities, it is also useful to construct net potential ratios to elucidate their relationship within the overall system. These ratios (calpain I/calpastatin and calpain II/calpastatin activity) are an indication of the net potential for protein breakdown and/or maintenance in a particular protease system. For example, an increase in this ratio would indicate an

overall increased breakdown or degradation potential, whereas if it is reduced, an overall decreased breakdown (or potentially an increased protein maintenance) potential is evident. If there is no change in the ratio, one can assume that the protease system is not swayed in either direction.

It is evident from the data that this net potential ratio for calpain I (calpain I/calpastatin) is not altered from the control group (1.31 ± 0.713) immediately after exercise (1.298 ± 0.415)($p>0.05$)(Fig. 12). Likewise, there is no change in the calpain II net potential ratio over the control group immediately post exercise (1.48 ± 0.784 vs. 1.493 ± 0.382 respectively)($p>0.05$)(Fig. 13). The consistency in these ratios indicates there were no changes in the net potential prompting the system in either direction.

During the recovery however, the calpain I net potential ratio for the control group (calpain I/calpastatin) is reduced by 38.5% (0.798 ± 0.339) versus control ($p<0.05$). Similarly, the net potential ratio for calpain II/calpastatin activity decreases by 39.5% (0.904 ± 0.363)($p<0.05$). This indicates that changes in either the calpain protease or calpain inhibitor (calpastatin) did not occur in concert with the other. In reference to this data, it appears as though there is a shift towards an environment of decreasing breakdown (or perhaps increasing protein maintenance) at the 6 hour recovery time point.

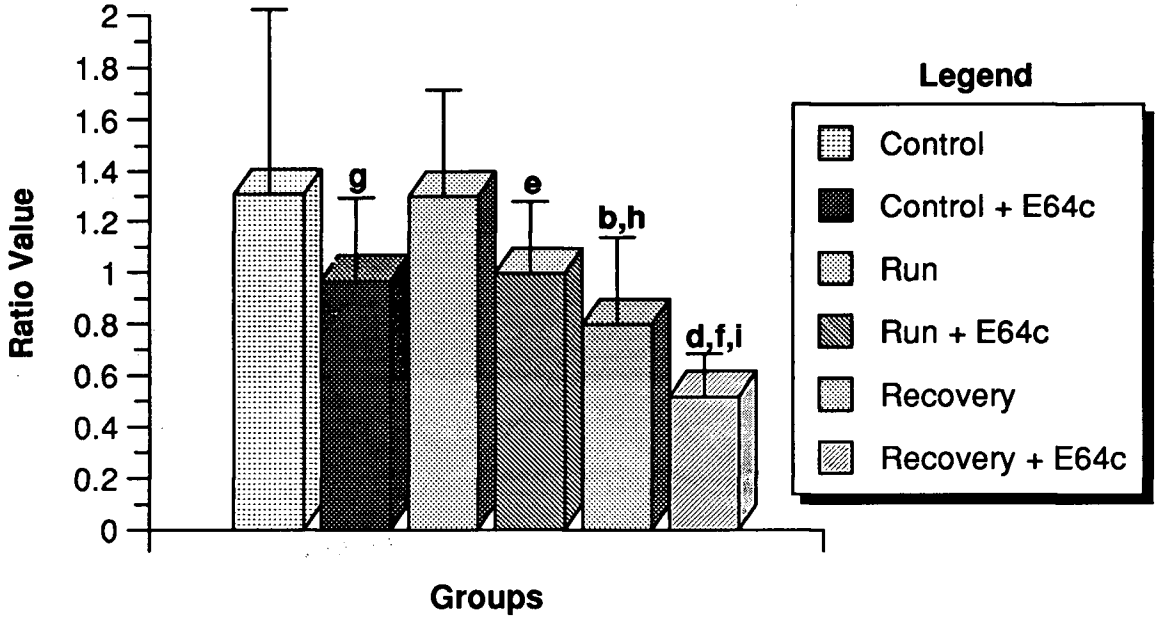

Figure 12.

Bar-plot representing calpain I / calpastatin activity ratios. Results are presented as means ± S.D.; "a" denotes a significant difference between Run and Control groups ($p<0.05$)(the running protocol consisted of 25 m/min downhill treadmill running (- 16°) for 45 minutes); "b" denotes a significant difference between Recovery and Control groups ($p<0.05$); "c" denotes a significant difference between Run and Control groups treated with E64c ($p<0.05$)(E64c treatment consisted of a subcutaneous 1 mg/kg injection); "d" denotes a significant difference between Recovery and Control groups treated with E64c ($p<0.05$); "e" denotes a significant difference between Run and Run + E64c treated groups ($p<0.05$); "f" denotes a significant difference between Recovery and Recovery + E64c treated groups ($p<0.05$); "g" denotes a significant difference between Control and Control + E64c treated groups ($p<0.05$); "h" denotes a significant difference between Run and Recovery groups ($p<0.05$); "i" denotes a significant difference between Run + E64c and Recovery + E64c treated groups ($p<0.05$).

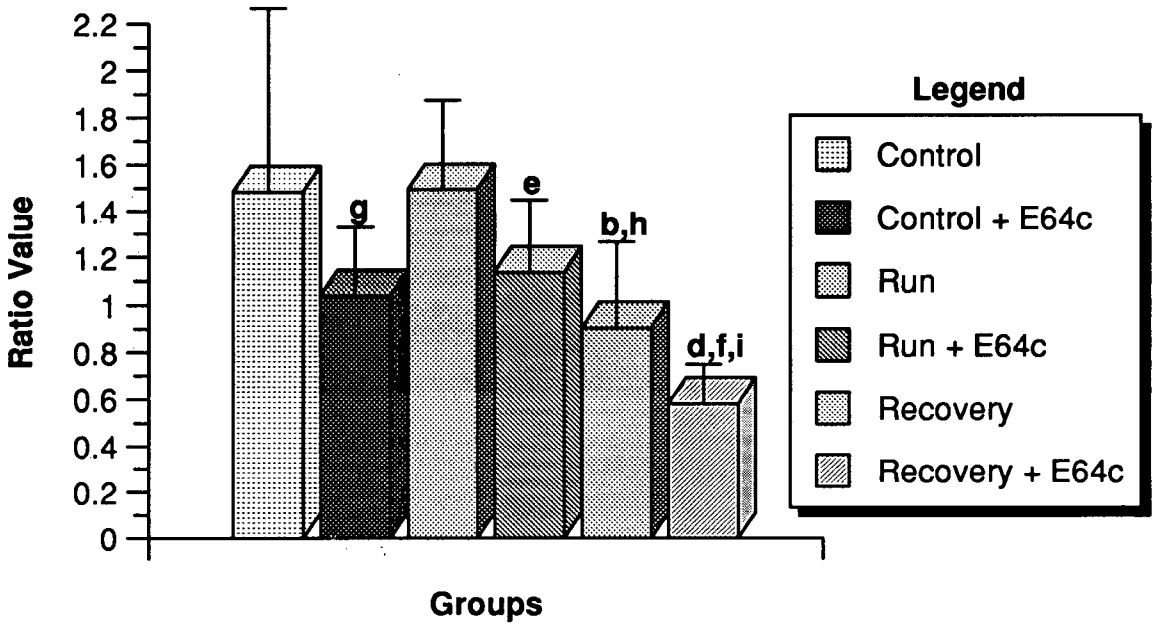

Figure 13.

Bar-plot representing calpain II / calpastatin activity ratios. Results are presented as means ± S.D.; "a" denotes a significant difference between Run and Control groups ($p<0.05$)(the running protocol consisted of 25 m/min downhill treadmill running (- 16°) for 45 minutes); "b" denotes a significant difference between Recovery and Control groups ($p<0.05$); "c" denotes a significant difference between Run and Control groups treated with E64c ($p<0.05$)(E64c treatment consisted of a subcutaneous 1 mg/kg injection); "d" denotes a significant difference between Recovery and Control groups treated with E64c ($p<0.05$); "e" denotes a significant difference between Run and Run + E64c treated groups ($p<0.05$); "f" denotes a significant difference between Recovery and Recovery + E64c treated groups ($p<0.05$); "g" denotes a significant difference between Control and Control + E64c treated groups ($p<0.05$); "h" denotes a significant difference between Run and Recovery groups ($p<0.05$); "i" denotes a significant difference between Run + E64c and Recovery + E64c treated groups ($p<0.05$).

In regards to the E64c-treated groups, the trends associated with the untreated animals hold consistent (Figs. 12,13). There were no changes in either the calpain I/calpastatin net potential ratio from the E64c-treated control group (0.967 $\pm$ 0.328) immediately after exercise (0.995 $\pm$ 0.284) or the calpain II/calpastatin ratio from the treated controls (1.038 $\pm$ 0.294) following exercise (1.14 $\pm$ 0.311)($p>0.05$). The E64c-treated recovery subjects however, showed decreases of 48.2% and 49.0% from baseline controls for both calpain I/calpastatin (0.515 $\pm$ 0.139) and calpain II/calpastatin (0.580 $\pm$ 0.168) ratios respectively ($p<0.05$). As before, this indicates a shift in the system to an environment of decreasing degradation capacity.

It is also interesting to note that in general, the ratio values for the E64c-treated groups decreased from their non-treated equivalents for both the calpain I/calpastatin ratios (control: decreased 26.1%; Exercise: decreased 23.3%; Recovery: decreased 35.8%) and calpain II/calpastatin ratios (control: decreased 30.1%; Exercise: decreased 23.8%; Recovery: decreased 35.8%)($p<0.05$). These reductions in ratio elude to the fact that E64c is acting to decrease the net potential for breakdown and hence enhance the environment towards protein maintenance.

The differences between various calpain isoforms were also investigated by completing a ratio of their activities (calpain I/calpain II activity). By examining this value, it becomes clear if one particular isoform responds differently to the stress of exercise, the treatment of E64c, or any potential activating factors.

It is apparent from the data, that there are no significant changes in the calpain I / II activity ratio across groups ($p>0.05$)(Fig. 14). The ratios for the control group (0.885 $\pm$

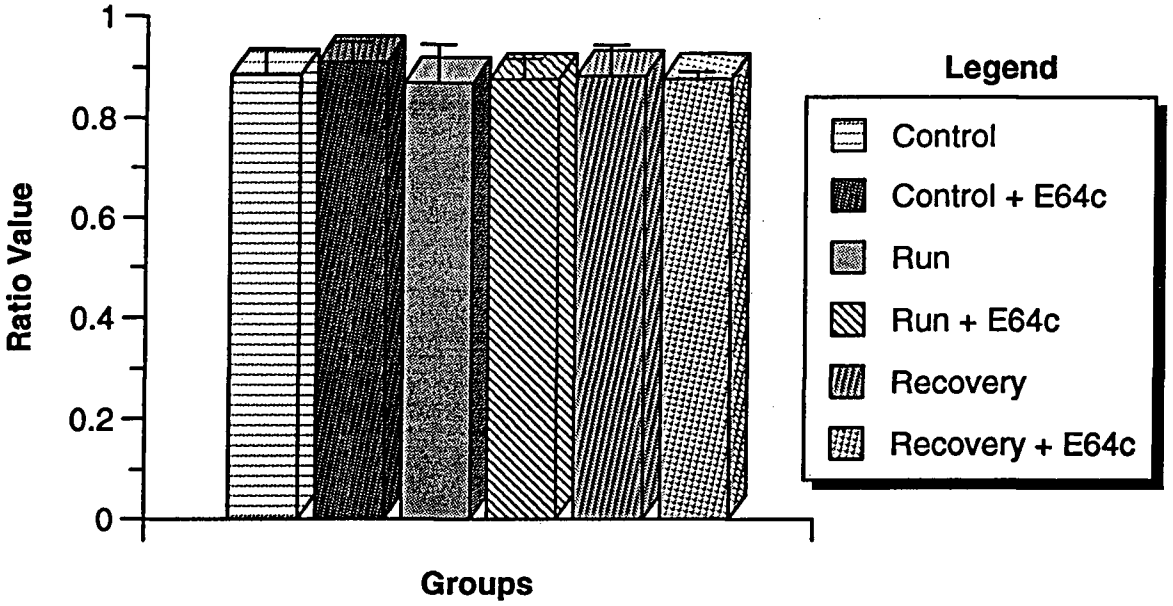

Figure 14.

Bar-plot representing calpain I / II activity ratios. Results are presented as means ± S.D.; "a" denotes a significant difference between Run and Control groups ($p<0.05$)(the running protocol consisted of 25 m/min downhill treadmill running (- 16°) for 45 minutes); "b" denotes a significant difference between Recovery and Control groups ($p<0.05$); "c" denotes a significant difference between Run and Control groups treated with E64c ($p<0.05$)(E64c treatment consisted of a subcutaneous 1 mg/kg injection); "d" denotes a significant difference between Recovery and Control groups treated with E64c ($p<0.05$); "e" denotes a significant difference between Run and Run + E64c treated groups ($p<0.05$); "f" denotes a significant difference between Recovery and Recovery + E64c treated groups ($p<0.05$); "g" denotes a significant difference between Control and Control + E64c treated groups ($p<0.05$); "h" denotes a significant difference between Run and Recovery groups ($p<0.05$); "i" denotes a significant difference between Run + E64c and Recovery + E64c treated groups ($p<0.05$).

0.050), running group (0.869 ± 0.076), and the 6 hour recovery group (0.884 ± 0.060) were all similar ($p>0.05$). This provides evidence for comparable control mechanisms for both isoforms.

Upon examination across the E64c-treated groups, it is apparent that no differences exist ($p>0.05$)(control: 0.911 ± 0.038; exercise: 0.877 ± 0.041; recovery: 0.877 ± 0.014). Furthermore, there are no changes in the E64c-treated groups versus their non-treated equivalents ($p>0.05$), indicating no preferential selectivity of one isoform over the other by E64c.

Calpain Subunit Quantification

Both isoforms of calpain (I and II) consist of heterodimers composed of a unique 80 kDa catalytic subunit as well as an identical 30 kDa regulatory subunit (Saido et al., 1994; Sorimachi et al., 1990). It was therefore helpful to investigate and identify these calpain isoform subunits from the "purified" isolation procedure used to quantify calpain activities under various conditions.

As a result, three distinct bands were identified, specifically at 80 kDa, 76 kDa and 17 kDa (Fig. 15). While antibodies specific for each of these calpain subunits were not utilised, the locale of the bands and the purified nature of the preparation suggests they correspond to the unautolyzed catalytic subunit, autolyzed catalytic subunit and the autolyzed regulatory subunit respectively. In the originally published assay, Ilian & Forsberg (1992) verified the presence of purified calpain subunits via Western blot analysis.

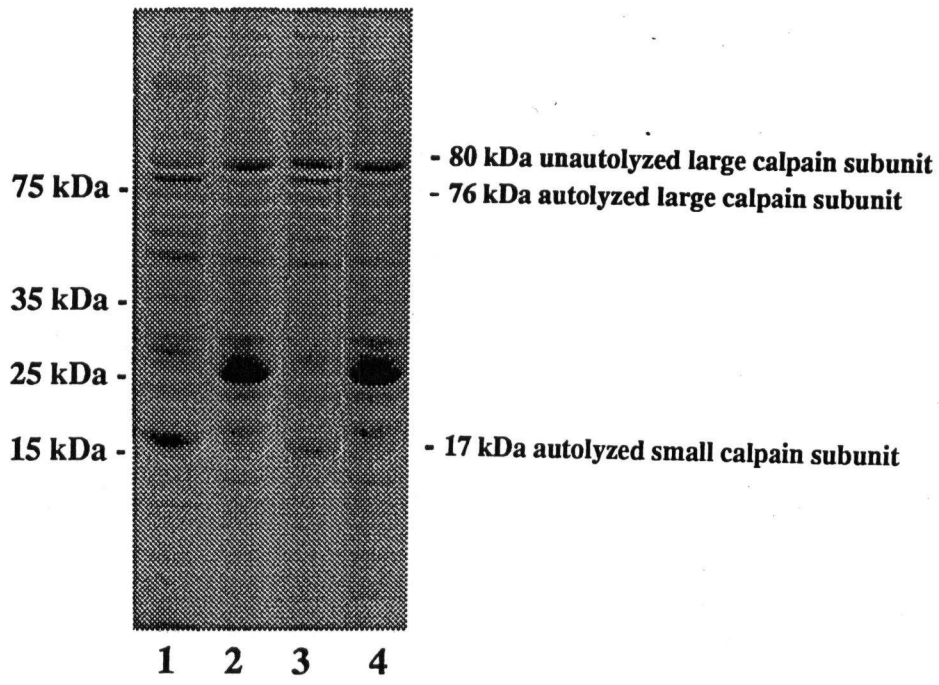

Figure 15.

Example of a representative 5-15% SDS-PAGE gel (run at 40 mA for 16 hours and stained with Coomassie Blue) expressing 80 kDa, 76 kDa and 17 kDa bands. Lane 1 corresponds to a calpain II isolation from the Run group (the running protocol consisted of 25 m/min downhill treadmill running (-16°) for 45 minutes). Lane 2 corresponds to a calpain II isolation from the sedentary Control group. Lane 3 corresponds to a calpain II isolation from the Recovery group (6 hours). Lane 4 corresponds to a calpain II isolation from the Run + E64c group (E64c treatment consisted of a subcutaneous 1 mg/kg injection).

The 80 kDa band for the control group (164.3 ± 3.5 units) showed a decrease of 22.5% immediately after exercise (127.3 ± 8.34 units) for the calpain I isolation ($p<0.05$)(Fig. 16). Likewise, there was a decrease of 22.6% in the 80 kDa subunit for the calpain II isolation with exercise (125.75 ± 6.29 units) from baseline (162.5 ± 2.89 units)($p<0.05$)(Fig. 17). Upon recovery, the 80 kDa subunit was still reduced versus the control group by 13.2% (142.5 ± 4.65 units) for calpain I isolation and 13.1% (141.3 ± 8.02 units) for the calpain II isolation ($p<0.05$). The differences between the run and recovery groups were not significant however ($p>0.05$).

The E64c-treated groups for both the calpain I specific isolation (control: 163.0 ± 5.15 units; exercise: 160.8 ± 6.18 units; recovery: 161.8 ± 10.7 units) and the calpain II purification (control: 163.2 ± 8.35 units; exercise: 159.5 ± 9.33 units; recovery: 160.5 ± 3.70 units) displayed no differences ($p>0.05$). There were however, significant differences between the E64c-treated exercise and recovery groups, and the untreated exercise and recovery groups ($p<0.05$), indicating that E64c inhibited the reductions in the 80 kDa gel band intensity.

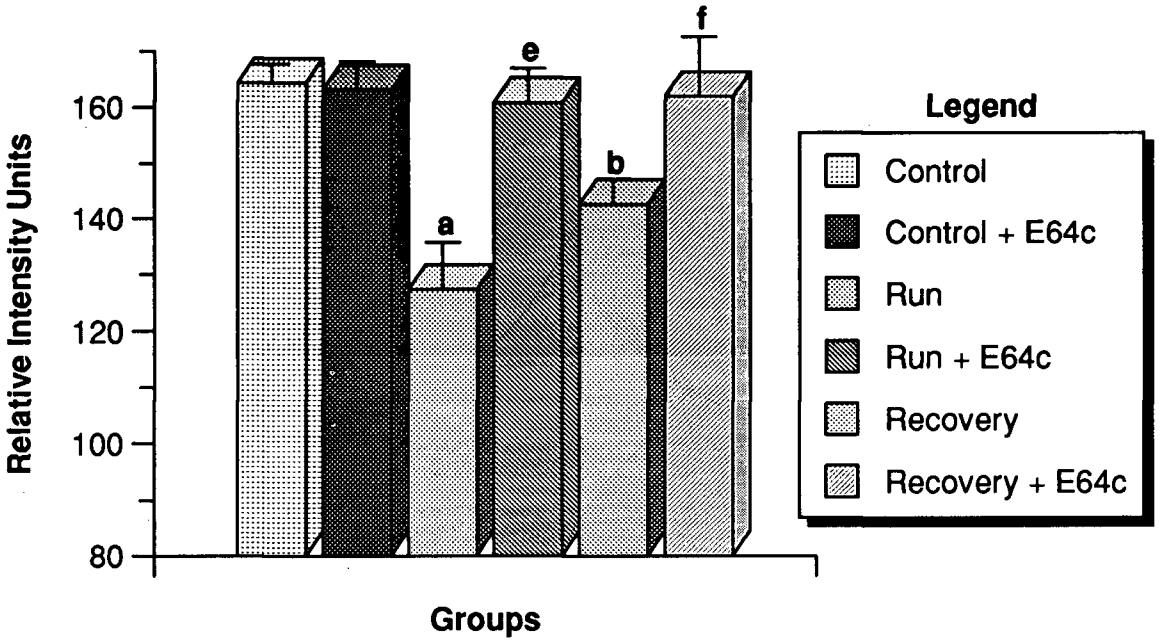

Figure 16.

Bar-plot representing the relative 80 kDa band intensities for the calpain I isolation (these values were obtained via SDS-PAGE and quantified using the IP Lab Gel program). Results are presented as means ± S.D.; "a" denotes a significant difference between Run and Control groups ($p<0.05$)(the running protocol consisted of 25 m/min downhill treadmill running (- 16°) for 45 minutes); "b" denotes a significant difference between Recovery and Control groups ($p<0.05$); "c" denotes a significant difference between Run and Control groups treated with E64c ($p<0.05$)(E64c treatment consisted of a subcutaneous 1 mg/kg injection); "d" denotes a significant difference between Recovery and Control groups treated with E64c ($p<0.05$); "e" denotes a significant difference between Run and Run + E64c treated groups ($p<0.05$); "f" denotes a significant difference between Recovery and Recovery + E64c treated groups ($p<0.05$); "g" denotes a significant difference between Control and Control + E64c treated groups ($p<0.05$); "h" denotes a significant difference between Run and Recovery groups ($p<0.05$); "i" denotes a significant difference between Run + E64c and Recovery + E64c treated groups ($p<0.05$).

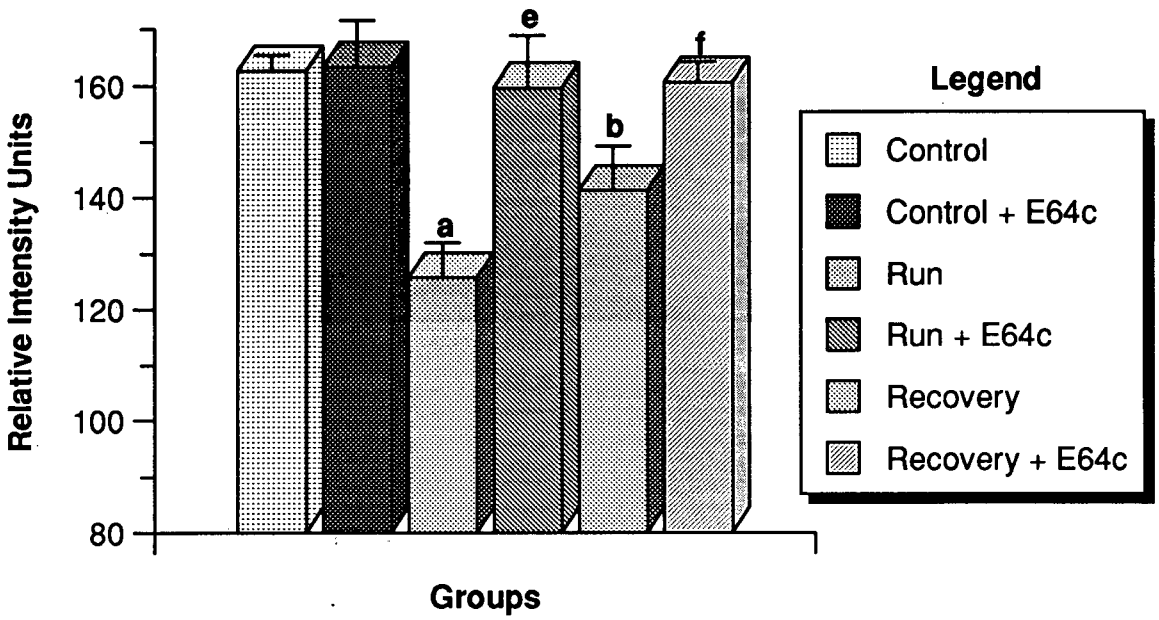

Figure 17.

Bar-plot representing the relative 80 kDa band intensities using the calpain II isolation (these values were obtained via SDS-PAGE and quantified using the IP Lab Gel program). Results are presented as means ± S.D.; "a" denotes a significant difference between Run and Control groups ($p<0.05$)(the running protocol consisted of 25 m/min downhill treadmill running (- 16°) for 45 minutes); "b" denotes a significant difference between Recovery and Control groups ($p<0.05$); "c" denotes a significant difference between Run and Control groups treated with E64c ($p<0.05$)(E64c treatment consisted of a subcutaneous 1 mg/kg injection); "d" denotes a significant difference between Recovery and Control groups treated with E64c ($p<0.05$); "e" denotes a significant difference between Run and Run + E64c treated groups ($p<0.05$); "f" denotes a significant difference between Recovery and Recovery + E64c treated groups ($p<0.05$); "g" denotes a significant difference between Control and Control + E64c treated groups ($p<0.05$); "h" denotes a significant difference between Run and Recovery groups ($p<0.05$); "i" denotes a significant difference between Run + E64c and Recovery + E64c treated groups ($p<0.05$).

The 76 kDa SDS-PAGE band increased 1562.5% over the control group (4.0 ± 6.93 units) with exercise (66.5 ± 3.51 units)($p<0.05$) for the calpain I isolation procedure (Fig. 18). There was also an increase of 2449.7% over the calpain II isolated control group (2.67 ± 4.62 units) with running (68.0 ± 5.29 units)($p<0.05$)(Fig. 19). Following the 6 hour recovery, there was still a significant increase in the 76 kDa band intensity over controls for both the calpain I (31.0 ± 4.55 units) and calpain II (26.0 ± 3.83 units) isolations ($p<0.05$). These were also decreased in comparison to the exercise (running) group (i.e. without the recovery) ($p<0.05$).

When treated with E64c, there was no change in the intensity of the 76 kDa band across groups for either the calpain I isolation (control: 2.33 ± 2.08 units; exercise: 0.4 ± 0.547 units; recovery: 4.0 ± 3.16 units) or the calpain II purification (control: 0.5 ± 1.0 units; exercise: 0.2 ± 0.45 units; recovery: 0 ± 0.0 units)($p>0.05$)(Figs. 18,19). It appears that in the normal sedentary control animals, the intensity of the 76 kDa band is virtually nil. In comparing the E64c-treated groups and those which are not treated, the evidence indicates that E64c inhibits the exercise-induced increases in 76 kDa band intensity values. Because the 80 kDa protein is disappearing from a relatively high concentration to a more moderate level with intense exercise, it fits with the predicted behaviour of an unautolyzed calpain subunit (80 kDa). The rapid and large appearance of the 76 kDa band, and hence the autolyzed form of the large calpain subunit, also seems intuitively reasonable.

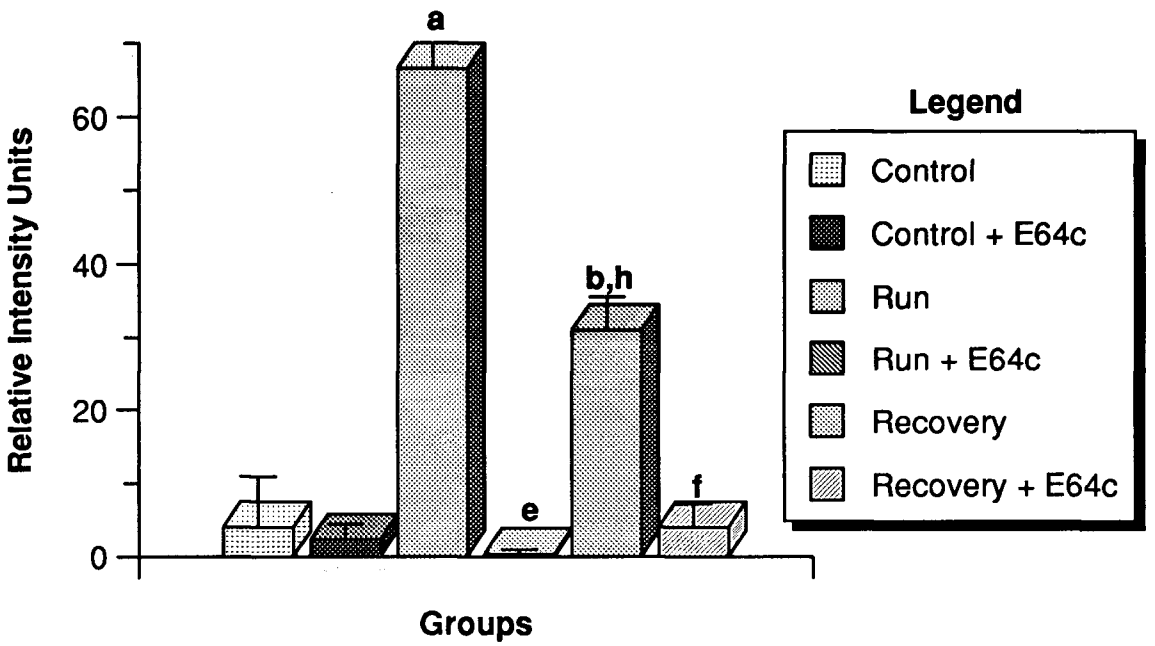

Figure 18.

Bar-plot representing the relative 76 kDa band intensities using the calpain I isolation (these values were obtained via SDS-PAGE and quantified using the IP Lab Gel program). Results are presented as means $\pm$ S.D.; "a" denotes a significant difference between Run and Control groups ($p<0.05$)(the running protocol consisted of 25 m/min downhill treadmill running (- 16°) for 45 minutes); "b" denotes a significant difference between Recovery and Control groups ($p<0.05$); "c" denotes a significant difference between Run and Control groups treated with E64c ($p<0.05$)(E64c treatment consisted of a subcutaneous 1 mg/kg injection); "d" denotes a significant difference between Recovery and Control groups treated with E64c ($p<0.05$); "e" denotes a significant difference between Run and Run + E64c treated groups ($p<0.05$); "f" denotes a significant difference between Recovery and Recovery + E64c treated groups ($p<0.05$); "g" denotes a significant difference between Control and Control + E64c treated groups ($p<0.05$); "h" denotes a significant difference between Run and Recovery groups ($p<0.05$); "i" denotes a significant difference between Run + E64c and Recovery + E64c treated groups ($p<0.05$).

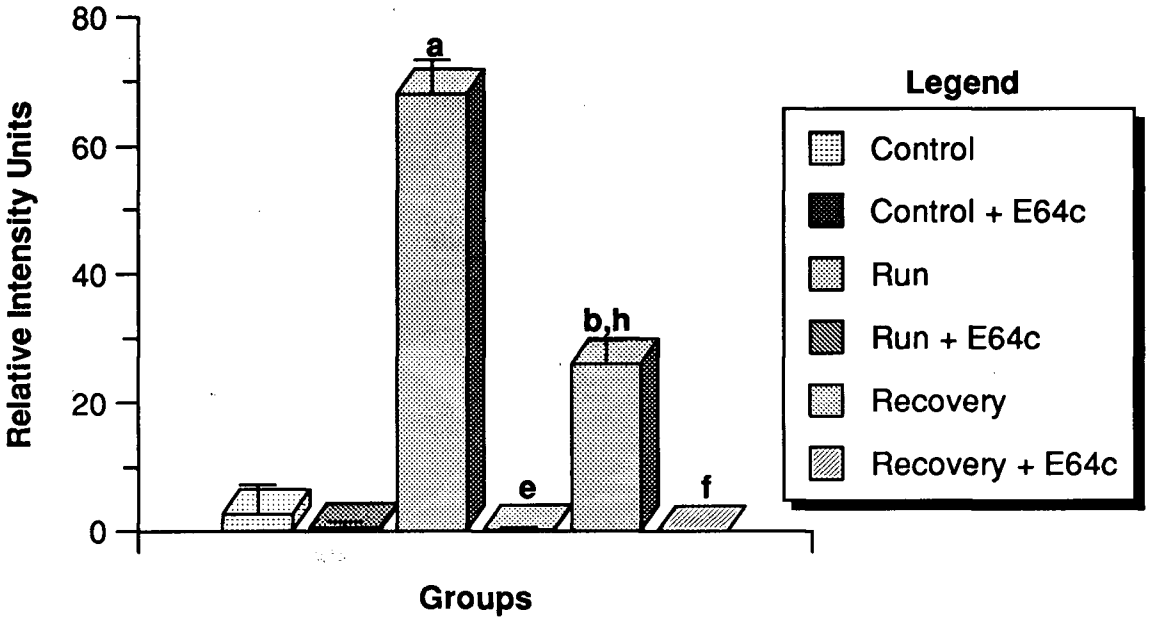

Figure 19.

Bar-plot representing the relative 76 kDa band intensities using the calpain II isolation (these values were obtained via SDS-PAGE and quantified using the IP Lab Gel program). Results are presented as means ± S.D.; "a" denotes a significant difference between Run and Control groups ($p<0.05$)(the running protocol consisted of 25 m/min downhill treadmill running (- 16°) for 45 minutes); "b" denotes a significant difference between Recovery and Control groups ($p<0.05$); "c" denotes a significant difference between Run and Control groups treated with E64c ($p<0.05$)(E64c treatment consisted of a subcutaneous 1 mg/kg injection); "d" denotes a significant difference between Recovery and Control groups treated with E64c ($p<0.05$); "e" denotes a significant difference between Run and Run + E64c treated groups ($p<0.05$); "f" denotes a significant difference between Recovery and Recovery + E64c treated groups ($p<0.05$); "g" denotes a significant difference between Control and Control + E64c treated groups ($p<0.05$); "h" denotes a significant difference between Run and Recovery groups ($p<0.05$); "i" denotes a significant difference between Run + E64c and Recovery + E64c treated groups ($p<0.05$).

Like its larger 76 kDa autolyzed counterpart, the potential autolyzed small 17 kDa calpain subunit is increased from an intensity of virtually zero (Figs. 20,21). In the calpain I isolation, an increase of 1340.9% in band intensity is noted with eccentric running (2.20 $\pm$ 3.19 units vs. 31.3 $\pm$ 4.57 units respectively)($p<0.05$). Calpain II purified solutions showed nearly a 4000% increase from a baseline control intensity of 0.8 $\pm$ 1.10 units to an exercised value of 32.0 $\pm$ 2.16 units ($p<0.05$). Again, as with the 76 kDa band, this increase in band intensity remained during the recovery, however only for the calpain II isolation (12.67 $\pm$ 11.4 units)($p<0.05$)(Fig. 21). There was no measurable change in the intensity of the calpain I purification solution with recovery (4.0 $\pm$ 4.0 units)($p>0.05$)(Fig. 20).

Whether calpain I (control: 0 $\pm$ 0 units; exercise: 5.75 $\pm$ 11.5 units; recovery: 2.75 $\pm$ 5.5 units) or calpain II (control: 0.6 $\pm$ 0.89 units; exercise: 3.0 $\pm$ 3.56 units; recovery: 2.48 $\pm$ 4.95 units) isolation, the 17 kDa band intensity showed no increases when treated with E64c among any of the groups ($p>0.05$). (note: a 30 kDa band representing an unautolyzed regulatory calpain subunit was impossible to distinguish). The data provides evidence for the appearance of a 17 kDa autolyzed calpain subunit with exercise, which may be inhibited by E64c.

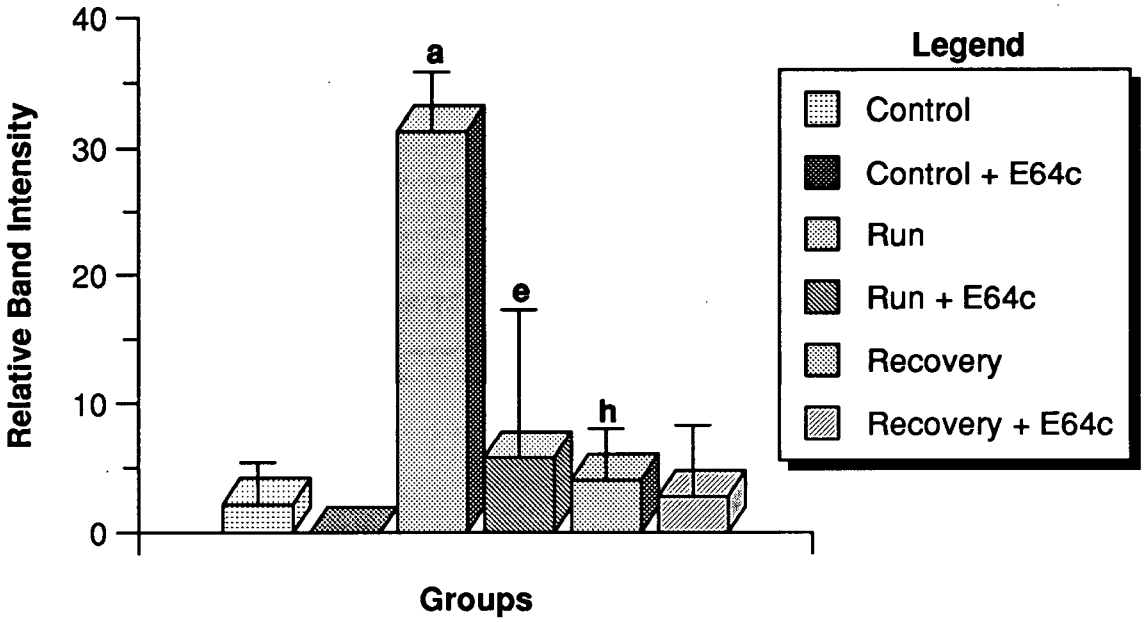

Figure 20.

Bar-plot representing the relative 17 kDa band intensities using the calpain I isolation (these values were obtained via SDS-PAGE and quantified using the IP Lab Gel program). Results are presented as means ± S.D.; "a" denotes a significant difference between Run and Control groups ($p<0.05$)(the running protocol consisted of 25 m/min downhill treadmill running (- 16°) for 45 minutes); "b" denotes a significant difference between Recovery and Control groups ($p<0.05$); "c" denotes a significant difference between Run and Control groups treated with E64c ($p<0.05$)(E64c treatment consisted of a subcutaneous 1 mg/kg injection); "d" denotes a significant difference between Recovery and Control groups treated with E64c ($p<0.05$); "e" denotes a significant difference between Run and Run + E64c treated groups ($p<0.05$); "f" denotes a significant difference between Recovery and Recovery + E64c treated groups ($p<0.05$); "g" denotes a significant difference between Control and Control + E64c treated groups ($p<0.05$); "h" denotes a significant difference between Run and Recovery groups ($p<0.05$); "i" denotes a significant difference between Run + E64c and Recovery + E64c treated groups ($p<0.05$).

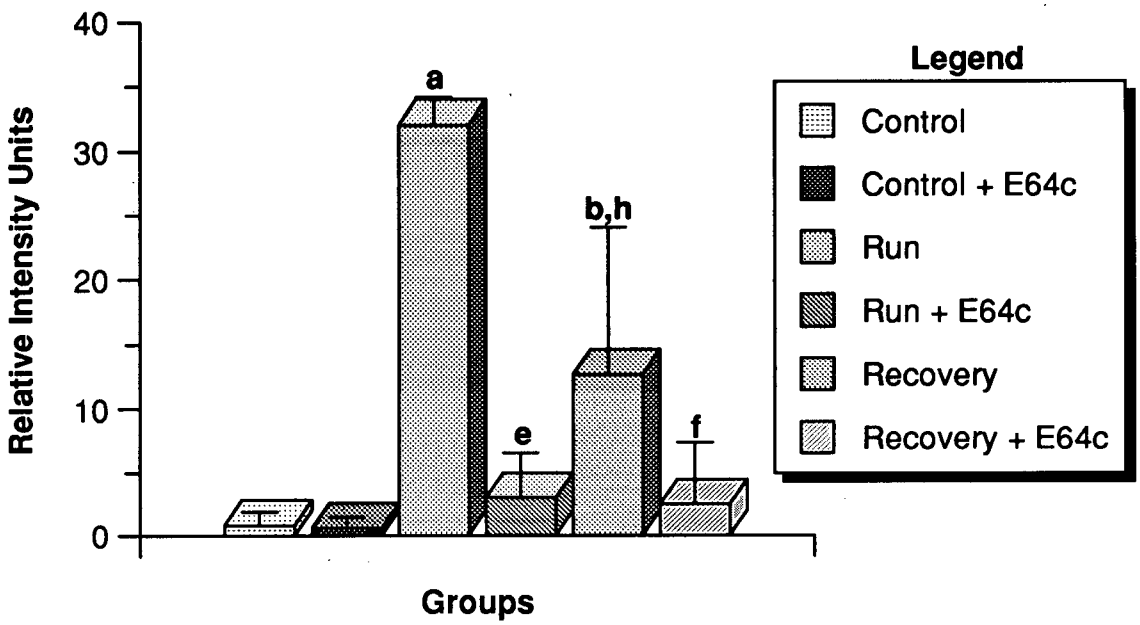

Figure 21.

Bar-plot representing the relative 17 kDa band intensities using the calpain II isolation (these values were obtained via SDS-PAGE and quantified using the IP Lab Gel program). Results are presented as means $\pm$ S.D.; "a" denotes a significant difference between Run and Control groups ($p<0.05$)(the running protocol consisted of 25 m/min downhill treadmill running (- 16°) for 45 minutes); "b" denotes a significant difference between Recovery and Control groups ($p<0.05$); "c" denotes a significant difference between Run and Control groups treated with E64c ($p<0.05$)(E64c treatment consisted of a subcutaneous 1 mg/kg injection); "d" denotes a significant difference between Recovery and Control groups treated with E64c ($p<0.05$); "e" denotes a significant difference between Run and Run + E64c treated groups ($p<0.05$); "f" denotes a significant difference between Recovery and Recovery + E64c treated groups ($p<0.05$); "g" denotes a significant difference between Control and Control + E64c treated groups ($p<0.05$); "h" denotes a significant difference between Run and Recovery groups ($p<0.05$); "i" denotes a significant difference between Run + E64c and Recovery + E64c treated groups ($p<0.05$).

Skeletal Troponin I (sTnI) Distributional Changes

Although not statistically significant once adjusted for relative protein concentrations (Table 17), a decrease in myofibrillar sTnI to 75.1% of control levels was evident immediately following exercise (control: 21.3 $\pm$ 10.6 vs. exercise: 16.0 $\pm$ 15.0 intensity units)($p>0.05$)(Figs. 22,23). Upon recovery, the decrease remained at 73.2% of control values (15.6 $\pm$ 16.3 intensity units)($p>0.05$). With E64c-treatment, again no change among any of the groups was noted ($p>0.05$). The E64c-injected exercise and recovery groups remained at 108.0% (23 $\pm$ 10.2 intensity units) and 104.2% (22.2 $\pm$ 19.8 intensity units) respectively, relative to normal controls. The treated control group showed a slight decrease to 95.8% of control (20.4 $\pm$ 9.3 intensity units)($p>0.05$).

The cytoplasmic sTnI band values showed a unique trend (Fig. 24). Cytoplasmic sTnI band intensity for the control group (119.5 $\pm$ 4.6 units) increased to 363.8% of control immediately after exercise (434.7 $\pm$ 43.9 units)($p<0.05$). With the 6 hour recovery (461.7 $\pm$ 68.4 units), cytoplasmic sTnI values maintained at 386.4% of sedentary control values ($p<0.05$). With E64c treatment, the increases remained intact ($p<0.05$). The treated exercise and recovery groups showed increases to 532.3% (636.1 $\pm$ 116.8 intensity units) and 537.4% (642.3 $\pm$ 38.5 intensity units) of control values respectively ($p<0.05$). The E64c-treated controls increased to 212.7% of the control groups (254.2 $\pm$ 7.4 intensity units)($p<0.05$). Also with the E64c treatment, increases in each group over their respective non-treated counterparts were noted ($p<0.05$).

It is evident from this data that with exercise, a decrease in myofibrillar sTnI band intensity (not statistically significant) is accompanied by a significant increase in cytoplasmic sTnI intensity. With E64c treatment, the myofibrillar sTnI decrease is halted.

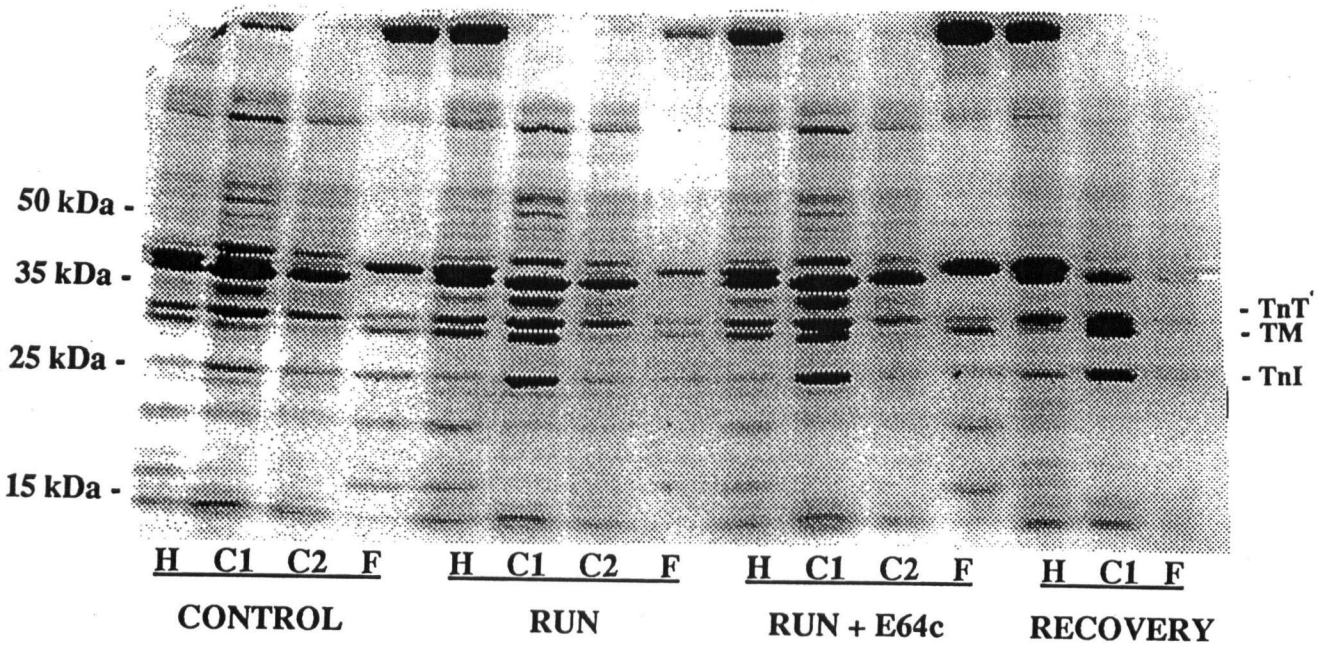

Figure 22.

Example of a representative SDS-PAGE gel (5-15% gel run at 40 mA for 16 hours and stained with Coomassie Blue) expressing myofibrillar and cytoplasmic sTnI, sTnT, and TM. Lanes 1-4 correspond to homogenate, cytosolic (1), cytosolic (2) and fibril samples, respectively, from the Control group. Lanes 5-8 correspond to homogenate, cytosolic (1), cytosolic (2) and fibril samples, respectively, from the Run group (the running protocol consisted of 25 m/min downhill treadmill running (-16°) for 45 minutes). Lanes 9-12 correspond to homogenate, cytosolic (1), cytosolic (2) and fibril samples, respectively, from the Run + E64c group (E64c treatment consisted of a subcutaneous 1 mg/kg injection). Lanes 13-15 correspond to homogenate, cytosolic (1) and fibril samples, respectively, from the Recovery group (6 hours).

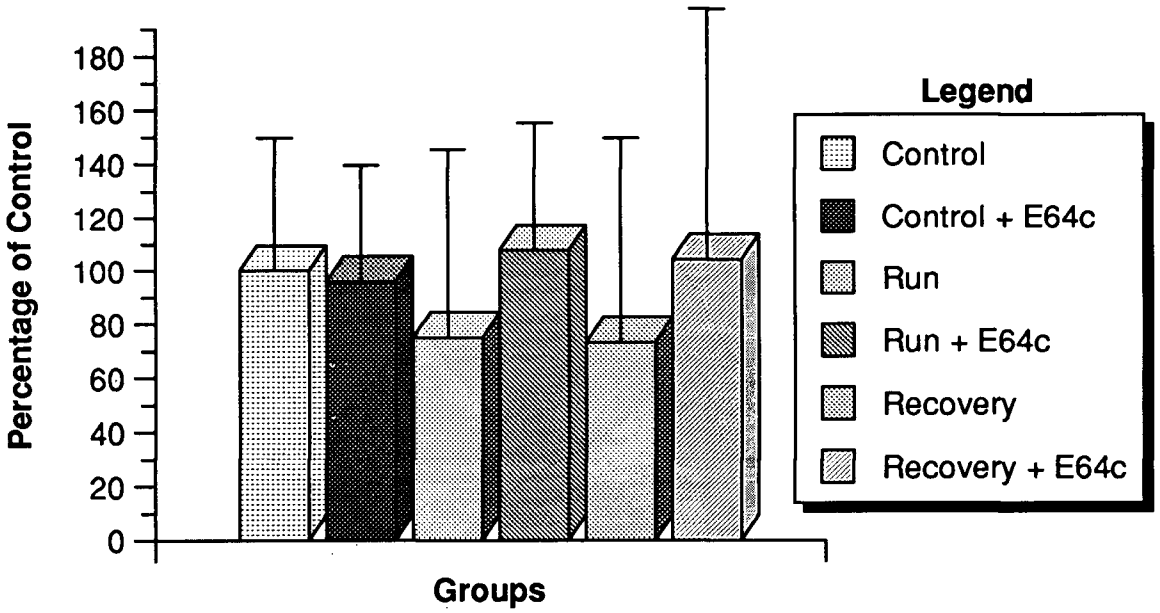

Figure 23.

Bar-plot representing myofibrillar sTnI band intensities (these values were obtained via SDS-PAGE and quantified using the IP Lab Gel program). Results are presented as means ± S.D. No statistically significant differences were evident between any of the groups (Control, Run or Recovery, with or without E64c).

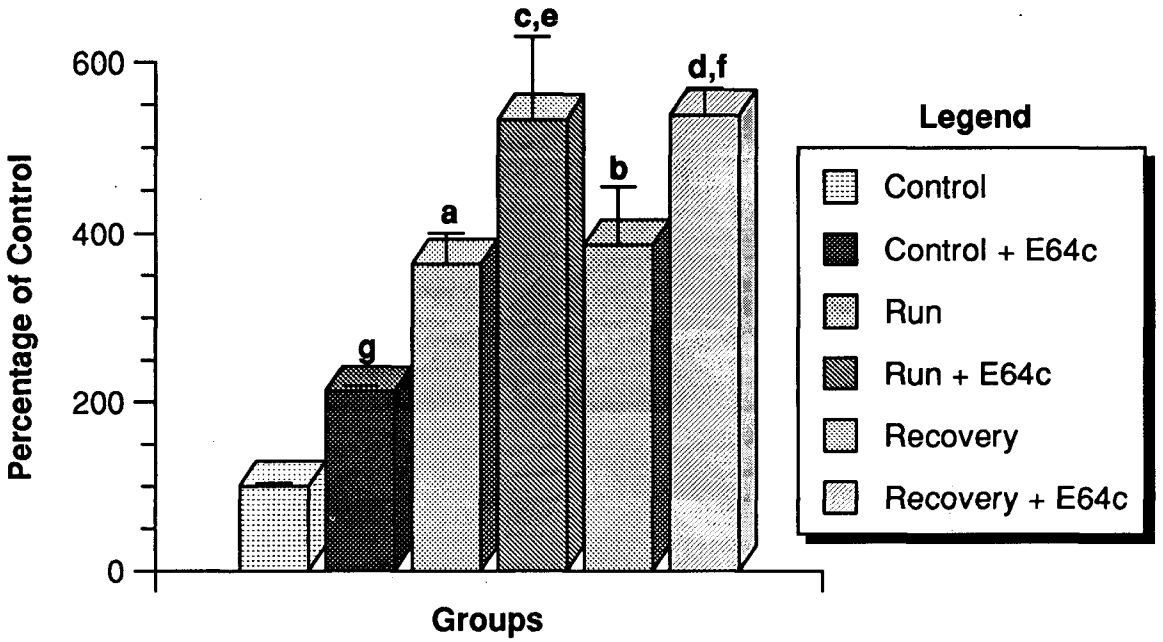

Figure 24.

Bar-plot representing cytoplasmic sTnI band intensities (these values were obtained via SDS-PAGE and quantified using the IP Lab Gel program). Results are presented as means ± S.D.; "a" denotes a significant difference between Run and Control groups ($p<0.05$)(the running protocol consisted of 25 m/min downhill treadmill running (- 16°) for 45 minutes); "b" denotes a significant difference between Recovery and Control groups ($p<0.05$); "c" denotes a significant difference between Run and Control groups treated with E64c ($p<0.05$)(E64c treatment consisted of a subcutaneous 1 mg/kg injection); "d" denotes a significant difference between Recovery and Control groups treated with E64c ($p<0.05$); "e" denotes a significant difference between Run and Run + E64c treated groups ($p<0.05$); "f" denotes a significant difference between Recovery and Recovery + E64c treated groups ($p<0.05$); "g" denotes a significant difference between Control and Control + E64c treated groups ($p<0.05$); "h" denotes a significant difference between Run and Recovery groups ($p<0.05$); "i" denotes a significant difference between Run + E64c and Recovery + E64c treated groups ($p<0.05$).

Skeletal Troponin T (sTnT) Distributional Changes

Like sTnI, a statistically insignificant decrease of 24.4% in myofibrillar sTnT was observed immediately following exercise (control: 23.87 $\pm$ 4.04 units vs. exercise: 18.05 $\pm$ 10.11 units)($p>0.05$)(Fig. 25). After recovery, the decrease remained intact at 24.6% from controls (18.0 $\pm$ 14.90 units)($p>0.05$). With E64c treatment, no differences across any of the groups were evident ($p>0.05$). The E64c-administered exercise and recovery groups showed slight increases to 105.5% (25.19 $\pm$ 12.8 intensity units) and 101.8% (24.30 $\pm$ 15.4 intensity units) of the control group respectively. The E64c-treated control group was also unchanged in relation to the normal control (increased to 102.8% of control)(24.53 $\pm$ 5.06 intensity units)($p>0.05$).

The cytoplasmic sTnT band intensities followed the same patterns as those for sTnI (Fig. 26). Cytoplasmic sTnI density for the control group (134.65 $\pm$ 14.6 units) increased to 343.5% of its value immediately after exercise (462.57 $\pm$ 53.5 units)($p<0.05$). With the recovery (501.88 $\pm$ 71.3 units), sTnT values in the cytoplasm remained at 372.7% of the control group ($p<0.05$). When E64c was administered, all groups displayed an increase in absolute cytoplasmic sTnI values over their untreated counterparts ($p<0.05$). E64c-treated exercise and recovery groups increased to 509.0% (685.43 $\pm$ 33.5 intensity units) and 530.7% (714.53 $\pm$ 9.76 intensity units) respectively of the controls ($p<0.05$). The control + E64c group also increased to 210.6% of normal control values (283.53 $\pm$ 18.4 intensity units)($p<0.05$).

This data indicates that after exercise, a small decrease in myofibrillar sTnT band intensity and a significant increase in cytoplasmic sTnT band density occurs. With the

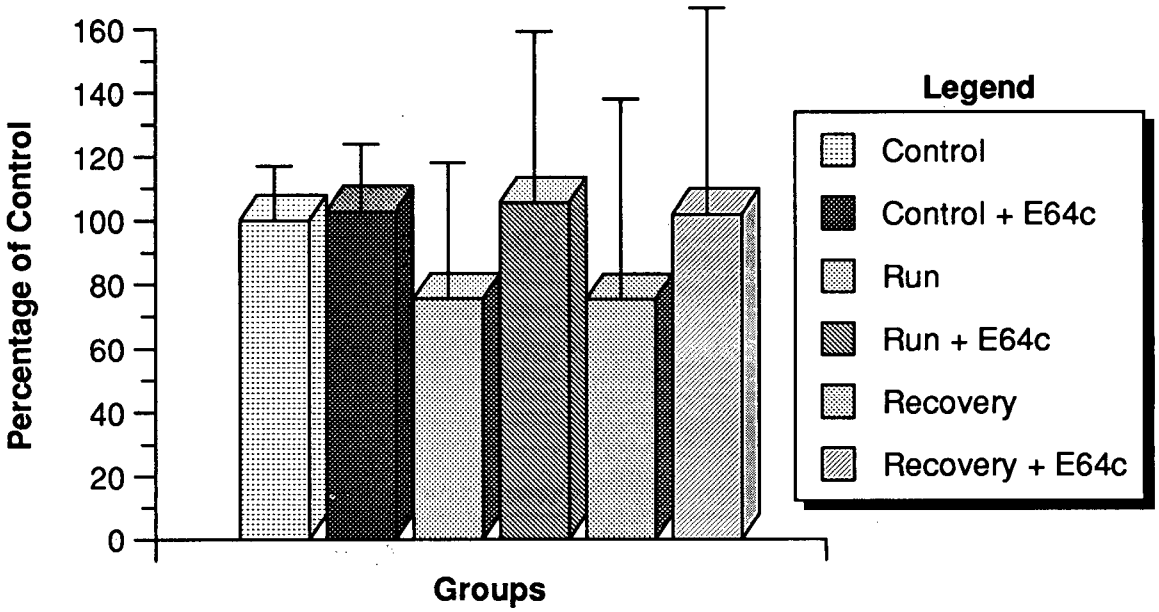

Figure 25.

Bar-plot representing myofibrillar sTnT band intensities (these values were obtained via SDS-PAGE and quantified using the IP Lab Gel program). Results are presented as means ± S.D. No statistically significant differences were evident between any of the groups (Control, Run or Recovery, with or without E64c).

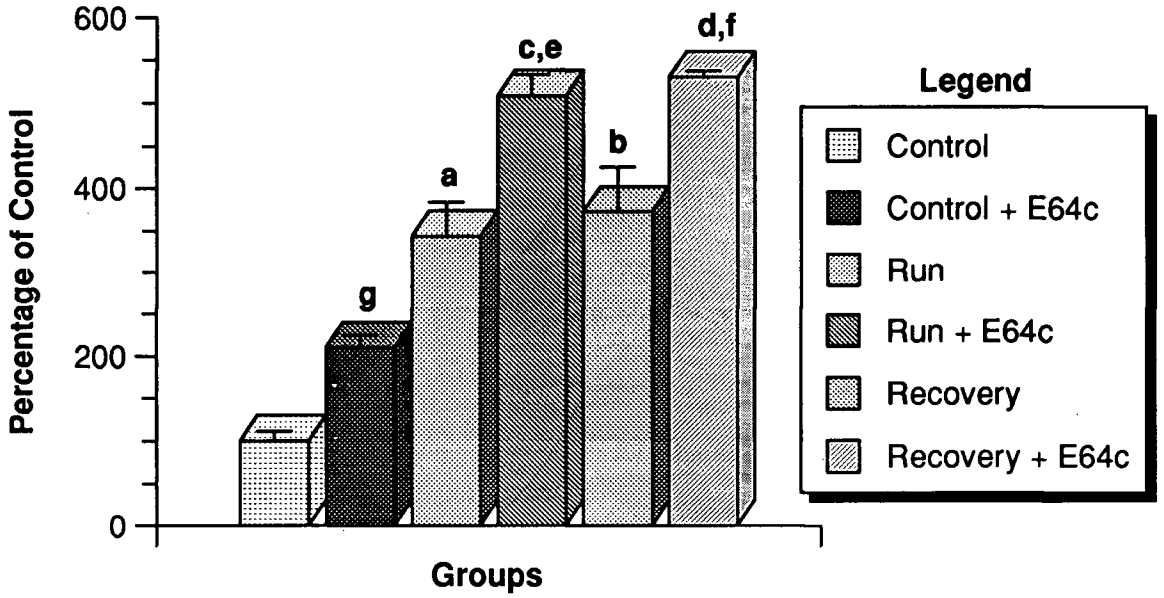

Figure 26.

Bar-plot representing cytoplasmic sTnT band intensities (these values were obtained via SDS-PAGE and quantified using the IP Lab Gel program). Results are presented as means ± S.D.; "a" denotes a significant difference between Run and Control groups ($p<0.05$)(the running protocol consisted of 25 m/min downhill treadmill running (- 16°) for 45 minutes); "b" denotes a significant difference between Recovery and Control groups ($p<0.05$); "c" denotes a significant difference between Run and Control groups treated with E64c ($p<0.05$)(E64c treatment consisted of a subcutaneous 1 mg/kg injection); "d" denotes a significant difference between Recovery and Control groups treated with E64c ($p<0.05$); "e" denotes a significant difference between Run and Run + E64c treated groups ($p<0.05$); "f" denotes a significant difference between Recovery and Recovery + E64c treated groups ($p<0.05$); "g" denotes a significant difference between Control and Control + E64c treated groups ($p<0.05$); "h" denotes a significant difference between Run and Recovery groups ($p<0.05$); "i" denotes a significant difference between Run + E64c and Recovery + E64c treated groups ($p<0.05$).

application of E64c, the myofibrillar decreases in sTnT were arrested, however the cytoplasmic increases were enhanced.

Skeletal Tropomyosin (TM) Distributional Changes

A small decrease of 9.9% in myofibrillar tropomyosin band intensity was noted with exercise (control: 22.1 $\pm$ 12.6 units vs. exercise: 19.9 $\pm$ 17.6 units)($p>0.05$)(Fig. 27). Upon recovery however, a much larger 19.7% decrease was evident versus controls (17.75 $\pm$ 4.9 units)($p>0.05$). With E64c treatment, no difference among groups was observed ($p>0.05$). Treated exercise and recovery groups actually increased to 110.7% (24.47 $\pm$ 6.60 intensity units) and 113.8% (25.16 $\pm$ 12.41 intensity units) of control ($p>0.05$). The control + E64c group also remained unchanged, increasing to 101.9% of control values (22.51 $\pm$ 4.29 intensity units)($p>0.05$).

The results for the cytoplasmic quotient of tropomyosin emulated the results of the other myofibrillar proteins (Fig. 28). Band densities for the control group (129.36 $\pm$ 5.47 units) increased to 430.1% of their values post-exercise (556.38 $\pm$ 63.9 units)($p<0.05$). After the 6 hour recovery, the cytoplasmic values remained at 473.2% of control (612.16 $\pm$ 60.9 units)($p<0.05$). E64c prompted each of the groups to increase over its equivalent non-treated groups ($p<0.05$). The treated exercise and recovery groups increased to 478.6% (619.06 $\pm$ 112.8 intensity units) and 514.9% (666.08 $\pm$ 28.6 intensity units) of control levels ($p<0.05$). Likewise, the treated control group increased to 206.5% of untreated control values (267.13 $\pm$ 14.18 intensity units)($p<0.05$).

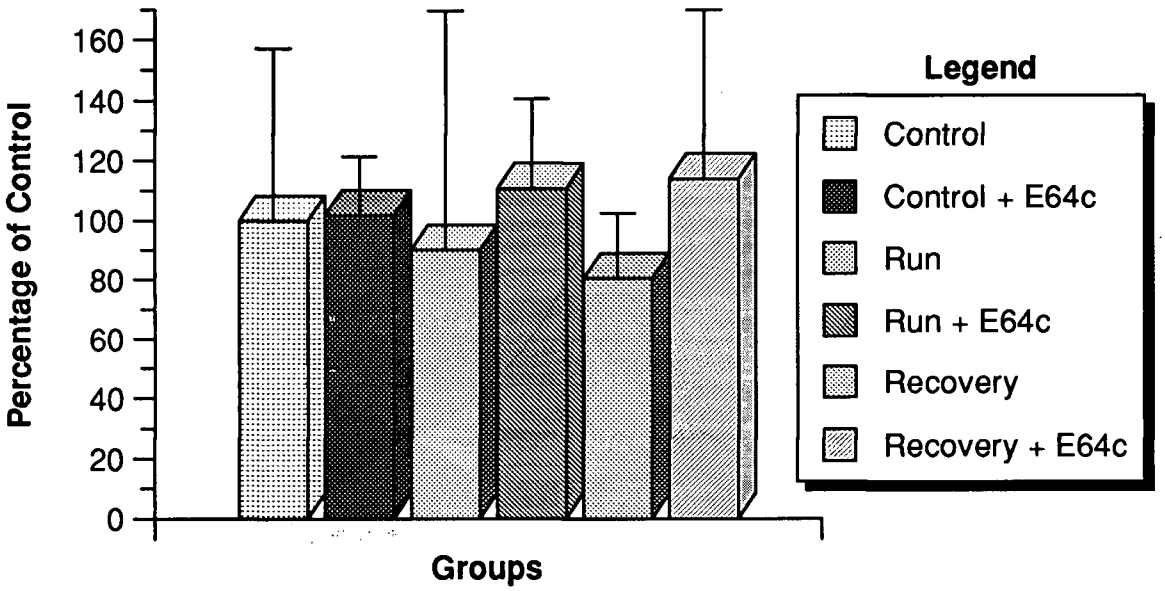

Figure 27.

Bar-plot representing myofibrillar TM band intensities (these values were obtained via SDS-PAGE and quantified using the IP Lab Gel program). Results are presented as means ± S.D. No statistically significant differences were evident between any of the groups (Control, Run or Recovery, with or without E64c).

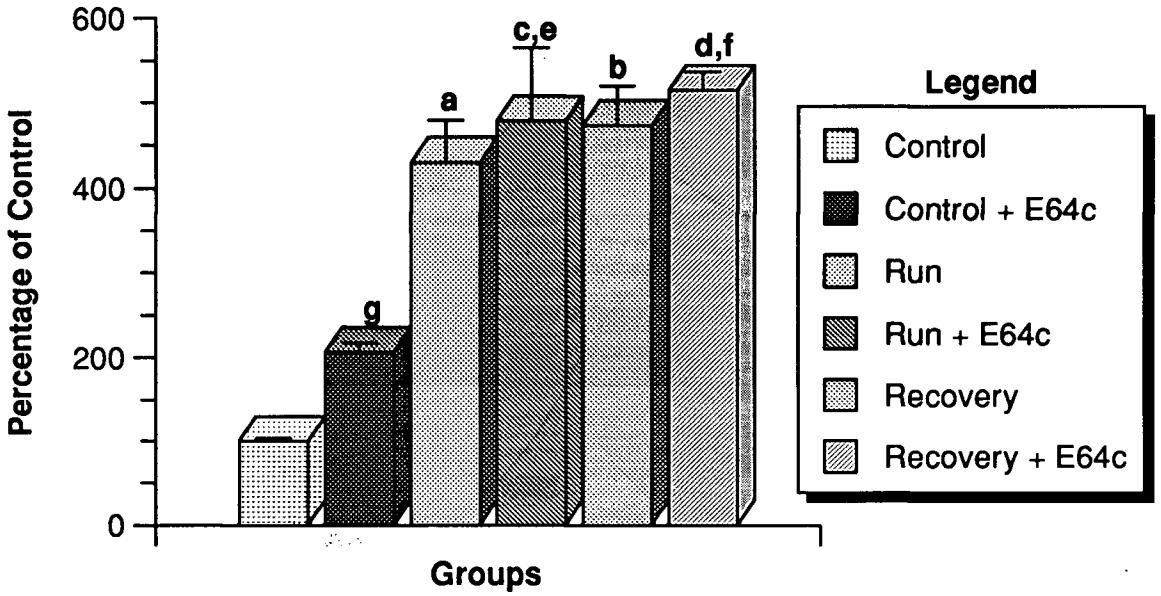

Figure 28.

Bar-plot representing cytoplasmic TM band intensities (these values were obtained via SDS-PAGE and quantified using the IP Lab Gel program). Results are presented as means ± S.D.; "a" denotes a significant difference between Run and Control groups ($p<0.05$)(the running protocol consisted of 25 m/min downhill treadmill running (- 16°) for 45 minutes); "b" denotes a significant difference between Recovery and Control groups ($p<0.05$); "c" denotes a significant difference between Run and Control groups treated with E64c ($p<0.05$)(E64c treatment consisted of a subcutaneous 1 mg/kg injection); "d" denotes a significant difference between Recovery and Control groups treated with E64c ($p<0.05$); "e" denotes a significant difference between Run and Run + E64c treated groups ($p<0.05$); "f" denotes a significant difference between Recovery and Recovery + E64c treated groups ($p<0.05$); "g" denotes a significant difference between Control and Control + E64c treated groups ($p<0.05$); "h" denotes a significant difference between Run and Recovery groups ($p<0.05$); "i" denotes a significant difference between Run + E64c and Recovery + E64c treated groups ($p<0.05$).

In comparison with the other myofibrillar proteins (sTnI, sTnT), the decrease in myofibrillar tropomyosin band density was extremely low until the recovery time point ($p>0.05$). The cytoplasmic tropomyosin content however, increased immediately with exercise and remained elevated after the recovery. As a result of the E64c treatment, each of the groups displayed an increase in absolute cytoplasmic tropomyosin quantities. The myofibrillar tropomyosin decrease was also inhibited with E64c application.

CHAPTER 5: DISCUSSION:

The primary objective in this study was to determine the functional relationship between the activation of the calcium stimulated cysteine protease, calpain, and that of its endogenous inhibitor, calpastatin, as well as their potential role in exercise-induced myofibrillar protein breakdown (i.e. changes in protein composition). Therefore calpain isoform (I and II) and calpastatin activities, and the relative quantities of proteins such as sTnI, sTnT and tropomyosin, were quantified in both the presence and absence of a cysteine protease inhibitor (E64c) prior to exercise.

Acute eccentric exercise resulted in the increased activation of both calpain I and calpain II (Figs. 9,10) in agreement with previous observations of heightened protease activities during periods of increased contractile activity (Belcastro, 1993; Cottin et al., 1994). This increase in calpain isoform protease activity is not surprising in light of the known loss of Ca^{2+} homeostasis and consequential increase in $[Ca^{2+}]_i$ which accompanies intense physical exercise. Whether the flux in $[Ca^{2+}]_i$ is primarily a function of a decreased rate in Ca^{2+} sequestration by the sarcomplasmic reticulum (SR)(Byrd, 1992) or an increased influx of Ca^{2+} from the extracellular environment and/or Ca^{2+} storage sites is open to conjecture, however the associated results in overall muscle regulation are clear. Compromised function of the myofibrillar contractile apparatus through decreased actin-myosin interaction (Vollestad & Stejersted, 1988), temporarily reduced SR-ATPase activity (Byrd et al., 1989), decreased mitochondrial energy production and SR vacuolization (Armstrong, 1990; Belcastro et al., 1985) are all descendants of increased $[Ca^{2+}]_i$. It is interesting to note that the observed calpain activity increases for isoforms I

(36.1%) and II (37.5%) were somewhat larger than those previously reported by Belcastro (1993) (18% and 22% respectively). This may be a result of the animals in the Belcastro (1993) study being exercised at a 0° grade, which might have been less damaging than the downhill running protocol used in the present study.

It is also apparent that when a moderate recovery (6 hours) post-exercise is provided, there is a substantial decrease in both calpain I and calpain II activity (Figs. 9,10). Although the activity is still well above baseline, there are significant questions as to what mechanism(s) are driving this overall reduction in calpain proteolysis. While the simple solution relates to the potential recovery in the $[Ca^{2+}]_i$ sequestering ability of the muscle cell, and hence a decrease in $[Ca^{2+}]_i$, calpain autolysis and calpain activity, a more thorough conclusion surrounds the observed dominance of calpastatin activity. Upon recovery, calpastatin activity increased dramatically (Fig. 11), outpacing protease activity to such an extent that it dominated the system. The lack of concert between the protease (calpain) and its inhibitor (calpastatin) in the recovering muscle pushes the system towards a net protein maintenance (or decrease in breakdown)(Figs. 12,13).

While the absolute activity of both calpain I and calpain II in the recovery group remains elevated in relation to the controls, indicating many of the activating factors necessary for activity are still present during recovery, the muscle microcosm can no longer be described as an environment favourable to calpain-induced muscle breakdown. This type of muscle surrounding seems to fit with the concept that Ca^{2+}-activated proteases, such as calpain, are primarily responsible for the initial and immediate changes associated with eccentrically exercised muscle damage (Cottin et al., 1994). Exercise-

induced calpain activity increases are present by 5-10 minutes after the initiation of eccentric work (unpublished data). In fact, calpain is regarded more as a regulatory enzyme, responsible for limited proteolytic actions (producing large peptide fragments) resulting in the destabilisation and loss of cellular structural integrity (Saido et al., 1994) than an indiscriminate and complete catabolic protease system. The attenuation of the calpain-induced muscle breakdown potential by 6 hours of recovery, indicates the switch from a calpain enzyme to a lysosomal enzyme driven proteolytic system may occur somewhat earlier in the recovery process than previously believed (i.e. prior to 24-48 hrs. post exercise). This sequence of events does never the less, fit with the concept that the non-calpain protease systems (i.e. lysosomal) are thought to be active and dominant in the protein turnover during the later events in muscle injury.

It is also interesting to speculate as to exactly what factors may be responsible for such an imbalance in the relative activation of calpain versus calpastatin. The most obvious of these factors in the decrease of calpain activity surrounds the Ca^{2+} ion itself. As the muscle recovers, the SR's ability to sequester Ca^{2+} increases dramatically and hence a gradual return to homeostatic $[Ca^{2+}]_i$ levels by the 6 hour time point would, on its own, reduce calpain isoform activity by attenuating autolysis. It is also theoretically possible that as a result of reduced phospholipase activation by the same return to normal $[Ca^{2+}]_i$ levels, a decrease in intracellular phospholipids may also occur (i.e. a decreased lipid oxidation). Goll et al. (1991) have proposed that phospholipids lower the $[Ca^{2+}]_i$ requirement for calpain autolysis and the proenzyme's conversion to an active protease. In such a system, a reduction in phospholipid concentrations, via sufficient recovery, would prevent this

conversion and reduce overall calpain activity. Other activating factors may also include the hormonal, ionic and nutritional status of the muscle in an exercise-stressed situation (i.e. increased ionic concenrations and reduced glycogen levels). Each of these, as well as a lowered calpain pCa_{50}, have been shown to increase calpain activity (Belcastro, 1993). In regards to the immense increase in calpastatin activity upon recovery from exercise, it is likely due to a reduction in calpain's degradation of the calpastatin molecule itself (i.e. via a reduced calpain activity). In a physiological sense, it would be crucial to inhibit the breakdown capacity of the calpain protease at some point during the recovery/regeneration state in a rapid and efficient manner; a reduction in calpain activation and an increase in calpastatin activity appear to be the agents.

In addition to the immense increase in calpastatin activity following recovery, the moderate increase in its activity immediately after exercise is also informative ($p>0.05$)(Fig. 11). Although the activities for both calpain I and calpain II rose dramatically with exercise, the net potential of the system immediately after exercise showed no significant change (i.e. net potential CI/calpastatin and CII/calpastatin ratios are the same with and without exercise). While the absolute increases in calpastatin activity were not statistically significant on their own, they were large enough to offset the rise in calpain activities (for both isoforms). This indicates that although there was no change in the net potential towards either a predominantly breakdown or maintenance conducive environment following exercise, there was an increase in the rate of the calpain-calpastatin system (i.e. an increase in the cycling or turnover of the system). This concept is intriguing as it suggests the response to an intense exercise bout is not only a factor-

induced increase in protease activity, but also an increase in the accompanying inhibitor as well (which acts as a "brake" on the protease system). Again, the precise factors initiating these exercise-induced activities are unclear, but may include increases in intracellular Ca^{2+} and phospholipid concentrations and hence a rise in calpain I and calpain II activation (i.e. via Ca^{2+} binding to sites specific for subunit dissociation, autolysis and proteolytic activity (Yoshizawa et al., 1995; Saido et al., 1994)). The moderate and offsetting rise in calpastatin activity in spite of the immense calpain activity, is likely a function of two factors. Exercise-induced phosphorylation of the calpastatin molecule is known to have a "protective effect" which attenuates calpain's degradation of calpastatin. It may also be a function of Ca^{2+} binding as well. Calpastatin acts to completely inhibit all isozymes of calpain at a $[Ca^{2+}]_i$ lower than that required for either calpain subunit autolysis or proteolytic activity (Goll et al., 1992). Perhaps the $[Ca^{2+}]_i$ during the recovery time frame is sufficient to increase calpastatin activity in such a dramatic fashion, but insufficient to promote calpain isoform activity. Furthermore, it is apparent that calpain activity as a whole is regulated by programmed responses to the binding of Ca^{2+} to specific sites on the molecule. One such site, and consequential response, is thought to be unique for calpastatin binding (Goll et al., 1991). Because calpastatin's measured activity is based on its ability to inhibit the action of calpain, an exercise-induced increase in $[Ca^{2+}]_i$ would likely increase the inhibition capabilities of calpastatin.

By comparing the calpain isoform activities themselves (calpain I / calpain II ratio)(Fig. 14), it appears that this factor-induced increase in calpain activity is not isoform

specific. Both calpain I and calpain II respond similarly to eccentric exercise and its associated activating factors.

Upon the pre-exercise application of the cysteine protease inhibitor E64c to the exercise and recovery groups, an attenuation of the run-induced calpain isoform activation was evident (Figs. 9,10). This has also been reported in rat striated muscle by both Wang (1990) and Arthur & Belcastro (1997). It should also be stated that the use of *i.p.* injected (2s,3s)-trans-epoxysuccinyl-L-leucylamido-3-methyl-butane (E64c) was preferred in comparison to oral E64d, even though Wang (1990) showed the *d* isoform to be acceptable, for a number of reasons. It requires a much shorter time course for calpain inhibition versus E64d (i.e. less than 4 weeks)(Parkes et al., 1985); E64d is a pro-drug of E64c and therefore must be converted into E64c prior to calpain inhibition (Wang, 1990); and its effects have also been well characterised in rat skeletal muscle (Arthur & Belcastro, 1997; Wang, 1990). Also, E64c has no significant effect on sedentary animals, and therefore on any potential calpain initiated, normal and necessary protein turnover in subjects (Figs. 9,10).

It is also noted that E64c treatment does not inhibit all calpain isoform activity in either exercise or recovery groups (Figs. 9,10). While the breakdown of myofibrillar protein substrates such as sTnI, sTnT and TM is arrested with E64c, it is possible that this remaining lesser activity could still be sufficient to promote the degradation of other cell proteins. The large variance in calpain substrate susceptibility (i.e. unique Km) based on factors such as phosphorylation (DiLisa et al., 1995), oxidation status (Rivett, 1986), carbohydrate metabolism (Belcastro et al., 1991) and sulfhydrl group content (Belcastro et

al., 1994), virtually guarantees dramatic differences in calpain activity requirements for its extensive list of substrates. This line of reasoning may also explain the large increase in both calpain isoform activities immediately after exercise (Figs. 9,10). Perhaps the physiological purpose of this elevated activity is outlined by the sheer number of substrate proteins calpain may actively regulate/degrade with exercise (i.e. myofibrillar proteins, cytoskeletal complex, cell signal pathway proteins, transcription factors and cytokines)(Saido et al., 1994).

In regards to the net potential (i.e. towards protein breakdown or maintenance) for the E64c-treated subjects, the same patterns were noted as those for the untreated groups (Figs. 12,13). E64c does not inhibit calpastatin activity and actually seems to enhance it (Fig. 11). The mechanistic link between the synthetic and natural cysteine protease inhibitors is postulated to be E64c's inhibition of calpain activation via attenuating autolysis, and hence a decrease in calpain's degradation of calpastatin. Again, with E64c treatment in the recovery group, there is a dramatic shift towards a maintenance (non-breakdown) mode for the muscle in relation to both the sedentary and exercise subjects (Figs. 12,13). As E64c does not appear to completely inhibit either calpain or calpastatin activity, many of the same activating factors may still influence their activation (i.e. the differing $[Ca^{2+}]_i$ requirements for calpain autolysis and calpastatin activation as well as $[phospholipids]_i$ and phosphorylation events).

As a whole (i.e. across all groups), the administration of E64c decreases the net potential ratio (Figs. 12,13). This fact alone provides evidence that E64c is not limited to the inhibition of calpain protease activity, but actually depresses the overall net potential

for substrate breakdown. It is shifting the environment towards one of maintenance or protein restoration. Had the ratio values remained similar to the non-E64c treated subjects, the effect on the system's net potential may have been altered.

The response by various isoforms of calpain to exercise, while treated with E64c, is identical to those which go untreated (Fig. 14). The similarity between the ratios provides support for the conclusion that E64c is not preferentially selective for either of the calpain isoforms.

As an aside, it is important to discuss the purity and accuracy of the calpain isoform isolation and quantification procedure (Gopalakrishna & Barsky, 1985). While antibodies specific for calpain I and calpain II were not utilised in this study to ensure calpain presence in our assay quantification, SDS-PAGE results support the precision of the protocol. Calpain isoforms (I and II) are heterodimers consisting of a unique catalytic subunit (80 kDa) and an identical regulatory subunit (30 kDa)(Saido et al. 1994; Sorimachi et al., 1990). Prior to proteolytic activation however, subunit dissociation and subsequent autolysis is an absolute requirement (Sorimachi et al., 1990; Saido et al., 1994). These two calpain subunits are autolyzed into 76 kDa and 17 kDa fragments respectively. In both the exercise and recovery groups, a significant decrease in the intensity of the 80 kDa band and increase in that of a 76 kDa band was noted for both calpain isoform isolations (Figs. 15,16,17,18,19). While the identification of these bands as the autolyzed and unautolyzed calpain subunits is not certain, their location, as well as their exercise-induced changes in density provide strong support for such an association.

Interestingly, the intensity of the 80 kDa band is already increasing in intensity (though not statistically) by the 6 hour recovery in relation to the exercise group. This indicates that perhaps the resynthesis rate of the calpain isoform itself is quite rapid or that a local 80 kDa precursor pool is replenishing the supply of the unautolyzed subunit. Likewise, there is a significant reduction in the 76 kDa band after the recovery time point (Figs. 18,19). This fits with the concept of a relatively immediate removal in the autolyzed subunit, post-muscle damage. It also seems physiologically correct, as the decreased calpain activity, increased calpastatin activity and overall shift towards a muscle maintenance environment would necessitate the removal of the autolyzed (and hence more proteolytically active) calpain fragment. While the same conclusions can not be stated for the disappearance of a 30 kDa band (i.e. because no single 30 kDa band could be differentiated), they can be made for the increase in density of the 17 kDa band (and hence potentially of the small, autolyzed calpain subunit)(Figs. 20,21). The densities of both the 76 kDa and 17 kDa bands were almost nil prior to the exercise and recovery protocols. This is again supported by the literature, as the basal activity of calpain is quite small and therefore a large concentration of autolyzed calpain subunits would not be predicted.

Until now, the precise mechanism(s) of E64c inhibition on calpain activity has been unknown. Based on the stability of the 80 kDa band (i.e. ~165 density units) as well as the 76 kDa band (~2-3 density units) intensity with E64c treatment, it appears that E64c acts to inhibit the autolysis step prior to calpain activation (Figs. 15,16,17,18,19). Across all groups, E64c prevents any decrease in the density of the 80 kDa band and hence likely the unautolyzed large calpain subunit. E64c also inhibits any increase in the 76 kDa and 17

kDa bands and therefore the autolyzed subunits. The necessity for subunit dissociation and autolysis to ensure calpain activation is unavoidable (Goll et al., 1992). Although it is not definitive by any means, this observation provides some insight into the mechanism(s) by which E64c intimately inhibits increased calpain isoform activity and hence promotes calpastatin activity and a shift toward a net protein maintenance potential immediately following exercise or in a recovery state.

In addition to calpain's "key role in the disassembly and remodelling of the cytoskeletal matrix" (Belcastro et al., 1997), it is also thought to be largely responsible for the metabolic turnover (i.e. proteolysis) of the myofibrillar proteins from their filamentous structure (especially during periods of increased contractile activity). Not only is calpain found in close physical proximity to both the I (desmin) and T (α-actinin) bands, but within the myofiber as a whole (i.e. within the same muscle compartments). Calpain's *in vitro* degradation of myofibrillar proteins (i.e. TnI, TnT, TM)(DiLisa et al., 1995; Saido et al., 1994) coupled with observations of "transient and specific z-line removal" from striated muscles following injury, indirectly link calpain activity to muscle damage (Belcastro et al., 1994; Goll et al., 1992). Furthermore, purified myofibrils prepared from exercised muscle also display myofibrillar disruption (i.e. z-line streaming) reminiscent of calpain's action (Belcastro et al., 1988; Goll et al., 1991). Even the muscle environment itself is optimal for protease activation during sustained exercise "as the hormonal, ionic and nutritional status of the muscle cell" is conducive to calpain activity (Belcastro, 1992; Fagan et al., 1992). It is also evident, based on experiments employing lysosomotropic agents, that lysosomal initiated proteolysis is primarily responsible for the protein turnover

at a later time point during recovery from exercise, as opposed to the initial and limited regulatory calpain proteolysis (Baracos et al., 1986; Zemon et al., 1985).

The susceptibility of the myofibrillar protein substrates themselves is also noted to increase dramatically with exercise (Belcastro, 1993), effectively improving calpain's degradative activity. Factors which contribute to this greater rate of calpain-mediated proteolysis are unclear, but an altered oxidation as well as covalent modification status is thought to play an important role (Belcastro et al., 1991; Chung et al., 1990; Rivett, 1986). Even sulfhydrl content, protein charge status and carbohydrate metabolism state seem to affect calpain activity (Belcastro et al., 1994). There is also evidence for enhanced Ca^{2+} sensitivity by calpain isoforms (i.e. lowered pCa_{50})(Belcastro, 1993). This could plausibly have a tremendous effect on calpain activation (i.e. the very large increases in calpain activity due to exercise).

While calpain activation is associated with the specific peptide cleavage of a vast number of enzymes (PKC, MLK, PK), cytoskeletal proteins (desmin, nebulin, titin), adhesion molecules (integrin, cahedrin), growth factor receptors (EGFr), ion transporters, phosphatases, phospholipases and cytokines, its degradation of various myofibrillar proteins is the best characterised (and the most obviously linked to structurally related muscle injury)(Goll et al., 1992; Saido et al., 1994). Of the myofibrillar proteins, troponin I is a particularly good *in vitro* and *in situ* substrate for calpain (DiLisa et al., 1995). Also, Sorichter et al. (1997) have quantified significant increases in plasma sTnI levels following exercise-induced muscle damage, an environment where calpain proteolysis is optimal. This intimate relationship is further exemplified in myocardial stunning, where Dong Gao

et al. (1997) recently demonstrated that calpain can reproduce partial and selective cTnI degradation specific to this condition. This proteolysis was prevented by the endogenous calpain inhibitor, calpastatin.

Although not statistically significant, a decrease in the intensity of the bands corresponding to myofibrillar sTnI was observed immediately after exercise (Fig. 23). Furthermore, this depression remained consistent through the recovery phase. Concurrently, a dramatic increase in the density of the cytosolic bands corresponding to sTnI was noted in both exercise and recovery groups ($p<0.05$)(Fig. 24). Utilising a three compartment model for myofibrillar protein breakdown and release, the intact peptides are typically degraded by a protease system (i.e. calpain) and then released from their filamentous structures into the muscle cell cytoplasm (Fig. 1a,b). These unbound (free) cytoplasmic peptides can then leak into the blood plasma if the plasma membrane separating the two has been mechanically or enzymatically disrupted (Fig. 1b). Although no plasma protein concentrations were quantified in this study, one might suggest that in light of the apparent myofibrillar TnI breakdown and subsequent increase in cytoplasmic sTnI, the plasma sTnI concentration may also be expected to rise with exercise. This is plausible not only based on the mechanical deformation of the plasma membrane that is resident in an eccentric-exercise bout, but also on its proteolytic degradation by exercise-induced increases in calpain isoform activity (Fig. 1a,b). The activation of calpain has been implicated in the degradation of the muscle's plasmalemma and therefore may invoke a passage for degraded myofibrillar proteins to leak into the circulatory bloodstream (Mellgren, 1987; Zaida & Narahara, 1989). While there appears to be a significant flow of

degraded sTnI from the intact myofiber to its cytoplasm, it is also telling to investigate the relative amount changes with exercise. The more than tripling of the cytosolic band intensity corresponding to TnI in comparison to an approximate 1/4 reduction in sTnI-associated myofibrillar bands confirms that the vast majority of the myofiber sTnI protein pool remains structurally bound to the myofibrillar apparatus. The dramatic increase in cytosolic sTnI is also predictable. With such a small initial pool (quantified by Sorichter et al. (1997) at only 3.4% of the total muscle sTnI pool), almost any influx from myofibrillar sTnI breakdown would increase values immensely.

To implicate calpain isoform involvement in the exercise-induced breakdown of myofibrillar proteins such as sTnI, E64c, a cysteine protease inhibitor was employed. This inhibitor has been shown to inhibit the exercise-induced increase in both calpain I and calpain II activity to a significant extent (i.e. via attenuation of calpain autolysis)(Figs. 9,10). Upon the administration of E64c, an inhibition of the reduction in bands corresponding to myofibrillar TnI was observed ($p>0.05$)(Fig. 23). The band intensity actually increased with E64c treatment in the exercise and recovery groups. While it is plausible that the E64c may inhibit even the basal calpain activity employed in normal protein turnover (i.e. and hence prevent 100% of sTnI breakdown), the absence of complete inhibition by E64c treatment on control animals suggests otherwise. This evidence supports the concept that calpain is directly involved in regulating myofibrillar sTnI breakdown in an exercise-induced muscle injury model (it is noted that although trends for bands corresponding to myofibrillar breakdown were evident, statistical significance was not reached likely due to two factors: first, the myofibril preparation itself

reduced the intensity of the myofibril band and hence it was difficult to obtain consistent measurements; second, the variance was also very large (i.e. because the protein amounts were so small, it took very little change to make a dramatic impact)).

Cytosolic sTnI-corresponding band values also increased dramatically with E64c application in both exercise and recovery groups (Fig. 24). Although unexpected, this increase can still be explained by the three compartment muscle breakdown model (Fig. 1a,b). While it may be predicted that a decrease in cytoplasmic sTnI concentrations would result (i.e. because calpain-induced myofibrillar sTnI breakdown is eliminated by inhibiting cysteine protease autolysis and subsequent activity), it is plausible that this increase is actually a reflection of attenuated sTnI leakage into the plasma and therefore out of the muscle. This is again supported by the decreased calpain isoform activity associated with E64c treatment and hence a potential reduction in the degradation of the muscle plasmalemma (Fig. 1a). By damming up this "drainage route", the sTnI from normal protein turnover (i.e. not via calpain proteolysis) may remain in the cytoplasm for a longer duration of time. As with the myofibrillar results, E64c injections increased the relative concentrations of the bands corresponding to cytoplasmic sTnI in control subjects. These increases, like those in the exercise and recovery groups, can be explained by the retention of sTnI in the cytoplasm, due to an inhibition in the disruption of the plasma membrane by calpain.

This study supports the concept then, that calpain is intimately involved in the breakdown and release of myofibrillar sTnI in an exercise-induced muscle state (Fig. 1a,b). It also suggests that perhaps calpain activity is essential to the disruption of the plasma

membrane necessary for the removal of sTnI from the cytoplasm and into the blood plasma (Fig. 1a,b).

The results eluding to calpain's potential influence in exercise-induced TnT breakdown are virtually identical to those for sTnI. Like TnI, sTnT is a good *in vitro* substrate for calpain (Saido et al., 1994). Exercise-induced reductions in myofibrillar band intensities corresponding to sTnT ($p>0.05$) were inhibited by treatment with E64c, however cytoplasmic sTnT-corresponding band intensities were increased significantly ($p<0.05$). It is once again postulated that the inhibition of calpain activity prevents the required plasma membrane breakdown necessary to permit myofibrillar protein leakage into the circulation (Fig. 1a,b). The E64c-induced inhibition of sTnT breakdown adds further support to the importance of cysteine proteases (calpains) in the myofibrillar breakdown process of muscle injury and recovery.

The final band corresponding to a myofibrillar protein was the ~37 kDa peptide, tropomyosin. Although there was a slight decrease in the band intensity with exercise, the decrease was much smaller than for either myofibrillar TnI or TnT associated bands, indicating that perhaps calpain may not be involved at the same level in TM degradation, as it is in the others immediately after running (Fig. 27). Unlike sTnI and sTnT whose *in vivo* data agrees with their observed *in vitro* myofibrillar degradation, the calpain-induced *in vitro* tropomyosin degradation does not seem to hold to the same extent in a whole body setting. There does appear however, to be a more delayed increase in tropomyosin degradation (i.e. increase in band intensity corresponding to TM) following a 6 hour recovery (Fig. 27). This increase is also inhibited by E64c. These results are especially

intriguing when compounded upon changes in cytosolic TM-corresponding band densities which emulate the findings for TnI and TnT (Fig. 28). Whether the decrease in myofibrillar TM breakdown immediately post-exercise (i.e. relative to the other myofibrillar proteins), is a statistical anomaly or genuinely reflects a lesser role of calpain in initiating TM breakdown is unclear. Because the remaining results are so similar to the other myofibrillar proteins (i.e. TnI and TnT), the former seems more likely.

In addition to calpain's apparent involvement in myofibrillar protein breakdown, this protease may also be active in the exercise-induced degradation of other proteins such as metabolic by-product enzymes like CK (Komulainen et al., 1994; Komulainen et al., 1995), LDH (Green et al., 1992) and even adenylates (Green et al., 1992; Simpson & Phillis, 1992). Because the breakdown of these proteins is believed to coincide with accompanying changes in pH, core temperature and electrolyte shifts that manipulate both metabolic turnover (Byrd et al., 1989; Vollested & Sejersted, 1988) as well as calpain activity, calpain's involvement was thought to be possible. Calpain isoforms, CK and LDH are also found in close muscle intracellular quarters. Although statistically insignificant due to extremely large variances (which is a common problem with using CK as an indicator for muscle damage), there was an increase in blood CK concentrations with exercise (Fig. 7).

Furthermore, this level decreased somewhat post-recovery. The inability of E64c to inhibit the exercise-induced CK increase to any extent, indicates that calpain isoform activity may not be a factor in CK release. The relative increase in CK activity with exercise however, did help to support the statement that the animals were physically taxed

and exercise-induced muscle injury had occurred. The presence of significant structural and histological damage utilising an exercise protocol similar to the one in this study has also been previously quantified and shown to be significant by Belcastro (1993).

Calpain is also known to degrade a number of specific enzymes in the exercise-induced breakdown of glycogen (Saido et al. 1994). To investigate its possible association with glycogen, muscle glycogen concentrations were quantified. It was apparent that the reduction in muscle glycogen caused by exercise was not inhibited by the cysteine protease inhibitor E64c, immediately following exercise or after recovery (Fig. 8). Although both glycogen phosphorylase (GP) and pyruvate kinase (PK) are substrates for calpain, and crucial to the glycogen breakdown process, it is suspected that by decreasing calpain activity, an increase in the effective Km of PK results. This would necessitate an increase in the amount of substrate (glycogen) required to provide the same quantity of ATP. While the un-treated (i.e. no E64c) exercise-induced glycogen depletion may be explained by an increased requirement for the absolute amount of glycogen (hence an increased metabolic demand and subsequent release breakdown), the E64c-treated decrease may be a result of a raised PK Km.

In summary, although a vast array of research has helped elucidate the response of calpain to exercise, the literature lacks clear and specific mechanism(s) to explain the onset of these exercise-induced changes. This study has confirmed that the activity of both calpain isoforms increases with exercise and remains elevated over control values following a short recovery (which can be inhibited by E64c via attenuating calpain autolysis). It also reports a significant increase in calpastatin activity post-recovery which

dominates this protease system, pushing it from a net breakdown potential towards a net maintenance potential. In addition, although absolute calpain activity increases with exercise, calpastatin activity keeps pace, resulting in an increase of the overall rate of cycling in the system without changing its net potential. Furthermore, calpain appears to play an vital role in the exercise-induced myofibrillar breakdown and cytoplasmic build-up of proteins such as TnI, TnT and possibly tropomyosin. Not only did E64c inhibit their myofibrillar degradation, but also enhance their cytoplasmic concentrations. The proposed model for this exercise-stressed system encompasses an increased activation of calpain isoforms which proceeds to breakdown myofibrillar peptides and release them into the muscle cell cytoplasm and eventually the blood plasma (Fig. 1a,b). It also seems that calpain acts to degrade the plasma membrane to ensure muscle peptide leakage into the blood (Fig. 1a,b).

CONCLUSION:

The calcium activated neutral protease, calpain, is known to increase in activity immediately following exercise, and is thought to be responsible for initial exercise-enhanced myofibrillar degradation. While this study has pointed out the importance of calpastatin in the regulation of the calpain proteolytic system, it has also provided evidence that calpain plays a crucial regulatory role in the breakdown and turnover of myofibrillar peptides following intense exercise. The vital nature of calpastatin activity in manipulating the rate of the protease system, as well as its movement between environments of net breakdown and net maintenance potentials are especially interesting due to the exploding use of calpain inhibitors for therapeutic purposes.

FUTURE DIRECTIONS:

- To examine the blood plasma levels for sTnI, sTnT, TM and other myofibrillar proteins potentially susceptible to degradation by calpain. This would provide insight into the functioning of the three compartment model, as well as calpain's effect on it.
- To explore the breakdown and release of peptide pieces and/or fragments in an exercise-induced muscle damage model, as well as calpain's role in their production.
- To investigate other activating factors responsible for differential activation of calpain and calpastatin in an exercise-induced model.

BIBLIOGRAPHY:

Adachi, Y.; Ishida-Takahashi, C. Takano, E.; Murachi, T. & Hatanaka, M. (1991) Phosdphorylation and subcellular distribution of calpastatin in human hempoetic system cells. *J. Biol. Chem.* 266:3968-3972.

Adams, J.; Schechtman, K.; Landt, Y.; Ladenson, J. & Jaffe, A. (1994) Comparable detection of acute myocardial infarction by creatine kinase MB isoenzyme and cardiac troponin I. *Clin. Chem.* 40: 1291-1295.

Armstrong, R.; Ogilvie, R. & Schwane, J. (1983) Eccentric exercise-induced injury to rat skeletal muscle. *J. Appl. Physiol.* 54: 80-93.

Armstrong, R. (1984) Mechanisms of exercise-induced delayed onset muscle soreness: a brief review. *Med. and Sci. in Sports and Ex.* 16: 529-538.

Armstrong, R. (1990) Initial events in exercise-induced muscular injury. *Med. and Sci. in Sports and Ex.* 22: 429-435.

Armstrong, R.; Warren, G. & Warren, J. (1991) Mechanisms of exercise-induced muscle fiber injury. *Sports Med.* 12: 184-207.

Aurthur, G. & Belcastro, A. (1997) A calcium stimulated protease involved in isoproteronol-induced cardiac hypertrophy. *Mol. Cell. Bioch.* 176: 241-248.

Baracos, V.; Greenberg, R. & Goldberg, A. (1984) Calcium ions and the regulation of intracellular protein breakdown in muscle. In: Calcium regulation in biological systems (Endo, M. & Ebashi, S., eds.), Takeda Science Foundation.

Baracos, V.; Greenberg, R. & Goldberg, A. (1986) Influence of calcium and other divalent cations on protein turnover in rat skeletal muscle. *Am. J. Physiol. Endocrinol. Metab.* 250: E701-E710.

Belcastro, A.; Rossiter, M.; Low, M & Sopper, M. (1981) Calcium activation of sarcoplasmic reticulum ATPase following strenuous activity. *Can. J. Physiol. Pharmacol.* 59: 1214-1218.

Belcastro, A.; MacLean, I. & Gilchrist, J. (1985) Biochemical basis of muscle fatigue associated with repetitious contractions of skeletal muscle. *Int. J. Biochem.* 17: 447-453.

Belcastro, A.; Parkhouse, W.; Dobson, G. & Gilchrist, J . (1988) Influence of exercise on cardiac and skeletal muscle myofibrillar proteins. *Mol. Cell Biochem.* 83:27-36.

Belcastro, A. (1993) Skeletal muscle calcium activated neutral protease (calpain) with exercise. *J. Appl. Physiol.* 74:1381-1386.

Belcastro, A.; Machan, C. & Gilchrist, J.(1991) Diabetes enhances calpain degradation of cardiac myofibrils and easily releasable myofilaments. In: *The Diabetic Heart*, edited by M. Hagano and N. Dhalla. New York: Raven, p301-310.

Belcastro, A.; Gilchrist, J.; Scrubb, J. & Arthur, G. (1994) Calcium-supported calpain degradation rates for cardiac myofibrils in diabetes: sulfhydryl and hydrophobic interactions. *Mol. Cell Biochem.* 135:51-60.

Belcastro, A.; Albisser, T. & Littlejohn, B. (1996) The role of calcium activated neutral protease (calpain) with diet and exercise. *Can. J. Appl. Physiol.* 21: 328-346.

Belcastro, A.; Arthur, G.; Albisser, T. & Raj, D. (1996) Heart, liver and skeletal muscle myeloperoxidase activity during exercise. *J. Appl. Physiol.* 80: 1331-1335.

Belcastro, A.; Shewchuk, L. & Raj, D. (1997) Exercise-induced muscle damage: A calcium-calpain hypothesis, *Mol. Cell Biochem., in press.*

Bindoli, A.; Balente, M. & Cavallini, L. (1983) Effects of palmitoyl coenzyme A on rat skeletal muscle sarcoplasmic reticulum. *Int. J. Biochem.* 15:1219-1223.

Bodor, G.; Porter, S.; Landt, Y. & Ladenson, J. (1992) The development of monoclonal antibodies and an assay for cardiac troponin I with preliminary results in suspected myocardial infarction. *Clin. Chem.* 11:2203-2214.

Bond, J. & Butler, P. (1987) Intracellular proteases. *Ann. Rev. Biochem* 56:333-364.

Byrd, S.; McCutcheon, K; Hodgson, D. & Gollnick, P. (1989) Altered SR function after high-intensity exercise. *J. Appl. Physiol.* 67:2072-2077.

Byrd, S.; Bode, A. & Klug, G. (1989) Effects of exercise of varying duration on SR function. *J. Appl. Physiol.* 66: 1383-1388.

Byrd, S. (1992) Alterations in the sarcoplasmic reticulum: a possible link to exercise-induced muscle damage. *Med. Sci. Sports Exerc.* 24: 531-536.

Canadian Council on Animal Care. Guide to the Care and Use of Experimental Animals. 2 vols. Ottawa, Ont.: CCAC, 1980-1984.

Cannon, J.; Meydani, S.; Fielding, R.; Fiatarone, M; Meydani, M.; Farhangmehr, M.; Orencole, S.; Blumberg, J. & Evans, W. (1991) Acute phase response in exercise II. Associations between vitamin E, cytokines, and muscle proteolysis. *Am. J. Physiol*: Regulatory Integrative Comp. Physiol. 260: R1235-R1240.

Chalovich, J. (1992) Actin-mediated regulation of muscle contraction. *Pharmacol. Ther.* 55: 95-148.

Chung, S.; Kwak, B.; Lee, J.; Ha, D. & Chung, C. (1991) Preferential degradation of the $KMnO_4$-oxidized or N-ethyl malenide modified form of SR-ATPase by calpain from chick skeletal muscle. *Biochem. Biophys. Acta.* 1041: 160-163.

Clarkson, P.; Byrnes, W.; McCormick, K.; Turcotte, L. & White, J. (1986) Soreness and serum CK activity following isometric, eccentric and concentric exercise. *Int. J. Sports. Med.* 7:152-155.

Clarkson, P. & Tremblay, I. (1988) Exercise-induced muscle damage, repair, and adaptations in humans. *J. Appl. Physiol.* 65: 1-6.

Cottin, P.; Brustis, J.; Poussard, S.; Elamrani, N.; Broncard, S. & Ducastaing, A. (1994) Calcium dependent proteinases (calpains) and muscle cell differentiation. *Biochem. Biophys. Acta.* 1223: 170-178.

Davies, C. & White, M. (1981) Muscle weakness following eccentric work in man. *Pflugers Archiv.* 392: 168-171.

Dayton, W.; Goll, D.; Zeece, M.; Robson, M. & Reville, W. (1976) A Ca^{2+}-protease possibly involved in myofibrillar protein turnover. Purification from porcine muscle. *Biochem.* 15: 2150.

DiLisa, F.; DeTullio, R.; Salamino, F.; Barbato, R.; Melloni, E.; Siliprandi, N.; Schiffino, S. & Pontremoli, S. (1995) Specific degradation of TnT and I by u-calpain and its modification by substrate phosphorylation. *Biochem. J.* 308:57-61.

Dong Gao, W.; Atar, D.; Liu, Y.; Perez, N.; Murphy, A. & Marban, E. (1997) Role of troponin I in the pathogenesis of stunned myocardium. *Circ. Res.* 80,3: 393-399.

Duan, C.; Delp, M.; Hayes, D., Delp, P.& Armstrong, R. (1990) Rat skeletal muscle mitochondrial [Ca] and injury from downhill walking. *J. Appl. Physiol.* 68: 1241-1251.

Farah, C. & Reinach, F. (1995) The troponin complex and regulation of muscle contraction. *FASEB J.* 9: 755-766.

Friden, J.; Seger, J. & Ekblom, B. (1988) Sublethal muscle fiber injuries after high-tension anaerobic exercise. *Eur. J. Appl. Physiol. Occup. Physiol.* 57: 360-368.

Friden, J.; Sjostrom, M. & Ekblom, B. (1981) A morphological study of delayed onset muscle soreness. *Experiential Basel.* 37: 506-507.

Friden, J.; Sfakianos, P. & Hargens, A. (1989) Blood indices of muscle injury associated with eccentric muscle contractions. *J. Orthopaedic Res.* 7: 142-145.

Golapakrishna, R. & Barsky, S. (1985) Quantification of tissue calpain activity after isolation by hydrophobic chromatography. *Anal. Biochem.* 148: 413-423.

Goll, D.; Dayton, W.; Singh, I. & Robson, R. (1991) Studies of the alpha-actinin/actin interaction in the z-disk by using calpain. *J. Biol. Chem.* 266: 8501-8510.

Goll, D.; Thompson, V.; Taylor, R. & Zalewska, T. (1992) Is calpain regulated by membranes and autolysis or by calcium and calpastatin. *BioEssays* 14: 549-556.

Greaser, M. & Gergely, J. (1971) Reconstitution of troponin activity from three protein components. *J. Biol. Chem.* 246: 4226-4233.

Green, H.; Dusterhoft, S.; Dux, L. & Pette, D. (1992) Metabolic patterns released to exhaustion, recovery and transformation of chronically stimulated rabbit fast-twitch muscle. *Pflugers Arch.* 420: 359-366.

Gregoriou, A.; Pearson, M & Crawford, C. (1994) The calpain cleavage sits in the epidermal growth factor kinase domain. *Eur. J. Biochem.* 223: 455-464.

Ingraham, R. & Swenson, C. (1984) Binary interactions of troponin subunits. *J. Biol.Chem.* 259:9544-9548.

Johnson, P. (1990) Calpain: structure-activity relationship and involvement in normal and abnormal cellular metabolism *Int. J. Biochem.* 263: 823-828.

Kapprell, H. & Goll, D. (1989) Effect of Ca^{2+} on binding of calpains to calpastatin. *J. Biol. Chem* 264: 17888-17896.

Kawasaki, H.; Emori, Y.; Imajoh-Ohmi, S. Minami, Y. & Suzuki, K. (1989) Identification and characterization of inhibitory sequences in 4 repeating domains of the endogenous inhibitor for calcium-dependent protease. *J. Biochem.* 106: 274-281.

Kayashima, S.; Ohno, H.; Fujioka, T.; Taniguchi, N. & Nagato, N. (1995) Leucocytosis as a marker of organ damage induced by chronic strenuous physical exercise. *Eur. J. Appl. Physiol. Occup. Physiol.* 70: 413-420.

Komulainen, J.; Kytola, J. & Vihko, V. (1994) Running-induced muscle injury and myocellular enzyme release in rats. *J. Appl. Physiol.* 77: 2299-2304.

Komulainen, J; Takala, T. & Vihko, V. (1995) Does increased serum CK activity reflect exercise-induced muscle damage in rats? *Int. J. Sports. Med.* 16:150-154.

Lammeli, U (1970) Cleavage of structural proteins during the assembly of head of bacteriophage T4. *Nature* 227: 680-688.

Larue, C.; Calzolari, C.; Bertinchant, J.; Leclerq, F.; Grolleau, R. & Pau, B. (1993) Cardiac-specific immunoenzymetric assay of TnI in the early phase of acute myocardial infarction. *Clin. Chem.* 39: 972-979.

Laughlin, M. & Armstrong, R. (1982) Muscular blood flow distribution patterns as a function of running speed in rats. *Am. J. Physiol.* 243 (*Heart Circ. Physiol.* 12) H296-H306.

Laughlin, M. & Armstrong, R. (1983) Rat muscle blood flow as a function of time during prolonged slow treadmill exercise *Am. J. Physiol.* 244 (*Heart Circ. Physiol.* 13) H814-H306.

Lee, W.; Ma, J.; Takano, H.; Yang, H.; Hatanaka, Q. & Maki, M. (1992) Molecular diversity of amino-terminal domains of human calpastatin by exon skipping. *J. Biol. Chem.* 267: 8437-8442.

Leger, J.; Bouvagnet, B.; Pau, B.; Roncucci, R. & Leger, J. (1985) Levels of ventricular myosin fragments in human sera after myocardial infarction, determined with monoclonal antibodies to myosin heavy chains. *Eur. J. Clin. Invest.* 15: 422-429.

Lieber, R. & Friden, J. (1988) Selective damage of fast glycolytic muscle fibers with eccentric contraction of rabbit tibialis anterior. *Acta. Physiol. Scand.* 133: 587-588.

Lieber, R.; Schmitz, M.; Mishra, D. & Friden, J. (1994) Contractile and cellular remodelling in rabbit skeletal muscle after cyclic eccentric exercise. *J. Appl. Physiol* 77:1926-1934.

Lindena, J.; Kuppner, W. & Trautschold, I. (1984) Enzyme activities in thoracic duct lymph and plasma of an anaesthetized and conscious resting and exercise and exercise dogs *Eur. J. Appl. Physiol. Occup. Physiol.* 52:188-195.

Lo, S.; Russell, J. & Taylor, A. (1970) Determination of glycogen in small tissue samples. *J. Appl. Physiol.* 28:234-236.

Lowry, O.; Rosenbrough, N.; Farr, A. & Randall, R. (1951) Quantitative protein assay. *J. Biol. Chem.* 193: 265.

Mair, J.; Koller, A.; Artner-Dworzak, E.; Haid, C.; Wicke, K.; Judmaier, W. & Puschendorf, B. (1992) Effects of exercise on plasma myosin heavy chain fragments and MRI of skeletal muscle. *Am. J. Physiol.* 656-663.

Mair, J.; Thome-Kromer, B.; Wagner, I.; Lechleitner, P.; Dienstl, F.; Puschendorf, B. & Michel, G. Concentration time course of troponin and myosin subunits after acute myocardial infarction. *Cort. Art. Dis.* 5:868-872.

Mair, J.; Mayr, M.; Muler, A.; Koller, A.; Haid, C.; Artner-Dworzak, E.; Calzolari, C.; Larue, C. & Puschendorf, B. (1995) Rapid adaption to eccentric exercise-induced muscle damage. *Int. J. Sports Med.* 16: 352-356.

Mair, J.; Genser, N.; Normdell, D.; Maier, J.; Mair, P.; Lechleitner, P.; Calzolari, C.; Larue, C.; Ambach, E.; Dienstl, F.; Pau, B. & Puschendorf, B. (1996) Cardiac troponin I in the diagnosis of myocardial injury and infarction. *Clin. Chem. Acta.* 245: 19-38.

Maki, M.; Bagci, H; Hamaguchi, K.; Ueda, M.; Murachi, T. & Hatanaka, M. (1989) Inhibition of calpain by a synthetic oligopeptide corresponding to an exon of the human calpastatin gene. *J. Biol. Chem.*, 264: 18886-18889.

Maki, M.; Takano, E. & Hatanaka, M. (1990) In: Intracellular calcium dependent proteolysis; (Mellgren, R. & Murachi, T., eds.) CRC Press, Florida, U.S.A., pp 37-54.

McNeil, P. & Khakee, R. (1992) Disruptions of muscle fiber plasma membrane : role in exercise-induced damage. *Am. J. Pathol.* 140:1097-1109.

Mellgren, R. (1987) Ca^{2+}-dependent proteases: an enzyme system active at cellular membranes? *FASEB J.* 1: 110-115.

Messineo, F.; Rathier, M.; Favreau, C.; Watra, J. & Takenaka, H. (1984) Mechanisms of fatty acids effects on the sarcoplasmic reticulum. *J. Biol. Chem.* 259: 1336-1343.

Molinari, M.; Anagali, J. & Carafoli, E. (1995) PEST sequences do not influence substrate susceptibility to calpain proteolysis. *J. Biol. Chem.* 270:2032-2035.

Morrisey, J. (1981) Silver stain for proteins in polyacrylamide gels. A modified procedure with enhanced uniform sensitivity. *Anal. Biochem.* 117: 307-310.

Nadel, E.; Bergh, U. & Staltin, B. (1972) Body temperature during negative work. *J. Appl. Physiol.* 33: 553-558.

Newham, D.; McPhail, G.; Mills, K. & Edwards, R. (1983) Ultrastructural changes after concentric and eccentric contractions of human muscle *J. Neurol. Sci.* 61: 109-122.

Ohtsuki, I.; Maruyama, K. & Ebashi, S. (1986) Regulatory and cytoskeletal proteins of vertebrate skeletal muscle. *Adv. Prot. Chem.* 38: 1-67.

Otsuka, Y. & Goll, D. (1987) Purification of Ca^{2+}-dependent protein inhibitor from bovine cardiac muscle and its interaction with millimolar Ca^{2+}-dependent proteinases. *J.Biol.Chem.* 262:5839.

Parkes, C.; Kembhavi, A. & Barrett, A. (1985) Calpain inhibition by epoxides. *Biochem J.* 230: 509-516.

Pette & Steron (1996) Cellular and molecular diversities of mammalian skeletal muscle fibers. *Rev. Physiol. Biochem. Pharmacol.* 116:1-76.

Rivett, A. (1986) Regulation of intracellular protein turnover: covalent modification as a mechanism of marking proteins for degredation. *Curr. Topics Cell Reg.* 28: 291-337.

Saggin, L.; Garza, L.; Ausoni, S. & Schiffino, S. (1990) Cardiac TnI in developing, regenerating, and denervated rat skeletal muscle. *Develop.* 110:547-54.

Saido, T.; Sorimachi, H. & Suzuki, K. (1994) Calpain: new perspective in molecular diversity and physiological-pathological involvement. *FASEB J.* 8:814-822.

Simpson, R. & Phillis, J. (1992) Adenosine in exercise adaptation. *Br. J. Sports Med.* 26: 54-58.

Smith, L. (1991) Acute inflammation: the underlying mechanism in delayed onset muscle soreness? *Med. Sci. Sports Ex.* 23: 542-551.

Sorichter, S.; Koller, A.; Haid, C.; Wicke, K.; Judmaier, W.; Werner, P. & Raas, E. (1995) Light concentric exercise and heavy eccentric loading: Effects on CK, MRI & markers of inflammation. *Int. J. Sports. Med.* 16: 288-292.

Sorichter, S.; Koller, A.; Mair, J.; Gebert, W.; Roma, D.; Calzolari, C.; Artner-Duorzak, E. & Puschendorf, B. (1997) Skeletal troponin I as a marker for exercise-induced muscle damage. *Am. Physiol. Soc.* 1076-1082.

Sorimachi, H.; Imajoh, S.; Emori, Y.; Kawasaki, H.; Ohno, S.; Minami, Y. & Suzuki, K. (1989) Molecular cloning of a novel mammalian calcium-dependent protease distinct from both u and m-types. *J. Biol. Chem* 264:20106-20111.

Sorimachi, H.; Ohmi, S.; Emori, Y.; Kawasaki, H;, Saido, J.; Ohno, S.; Mnami, Y. & Suzuki, K. (1990) A novel member of Ca-dependent cysteine protease family. *Biol. Chem. Hopre-Seylor* 371: 171-176.

Sorimachi, H. Toyama-Sorimachi N.; Saido, T.; Kawasaki, H.; Sugita, H.; Miyasaka, M.; Arhata, K.; Ishiura, S. & Suzuki, K. (1993) Muscle specific calpain, p94, is

degraded by autolysis immediately after translation, resulting in disappearance from muscle. *J. Biol. Chem.* 268: 10593-10605.

Stauber, W. (1989) Eccentric action of muscles: physiology, injury and adaptation. In: Exercise and sport sciences reviews (Pandolf K., ed.), Williams and Wilkins, Baltimore, Hong Kong, London, Sydney, pp. 157-185.

Talbot, J. & Hodges, R. (1981) Synthetic studies on the inhibitory region of rabbit skeletal troponin I. *J. Biol. Chem.* 256: 2798-2802.

Takano, E.; Hatanaka, M.; Hitomi, K. & Maki, M. (1997) Abstract from the Calpain conference at the University of Oxford, April 13, 1997.

Takano, E.; Nosaka, T.; Lee, W.; Nakamura, K.; Takahashi, T.; Funaki, M.; Okada, H.; Hatanaka, M & Maki, M (1993) *Arch. Biochem. Biophys.* 303:349-254.

Towbin, H.; Staehlin ,T. & Gordon, J. (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and applications. *Proc. Natl. Acad. Sci.* 76: 435-454.

Trump, B. & Berezesky, I. (1995) Calcium-mediated cell injury and cell death. *FASEB J.* 9:219-228.

Vollestad, N. & Sejerstad, D. (1988) Biochemical correlates of fatigue: a brief review. *Eur. J. Appl. Physiol.* 53:336-347.

Wang, K.; Villalobo, A. & Roufogalis, B. (1989) Calmodulin-binding proteins as calpain substrates. *Biochem. J.* 262:693-706.

Wang, K. (1990) Developing selective inhibitors of calpain. *Trends. Pharmacol. Sci.* 11: 139-142.

Wilkinson, J. & Grand, R. (1978) Comparison of amino acid sequence of troponin I from different striated muscles. *Nature* 271: 31-35.

Yoshimura, V. & Schlaefer, W. (1982) Membrane-associated calpain I in human erythrocytes. Detected by an immunoelecrophoretic blotting method using non-specific antibody. *Biochem.* 21:3977-2983.

Yoshizawa, T.; Sorimachi, H.; Tomioka, S.; Ishiura, S. & Suzuki, K. (1995) Calpain dissociates into subunits in the presence of calcium ions. *Biochem. Biophys. Res. Comm.* 208: 376-383.

Zaida, S. & Narahara, H. (1989) Degradation of skeletal muscle plasma membrane proteins by calpain. *J. Membrane Biol.* 110: 209-216.

Zeman, R.; Kaneyama, T.; Matsumoto, K.; Bernstein, P. & Etlinger, D. (1985) Regulation of protein degradation by Ca^{2+}. Evidence for enhanced nonlysosomal proteolysis associated with elevated cytosolic calcium. *J. Biol. Chem.* 260: 13619-13624.

Zot, A. & Potter, J. (1987) Structural aspects of troponin-tropomyosin regulation of skeletal muscle contraction. *Annu. Rev. Biophys. Chem.* 16: 535-559.

Calpain Isoform Activity-Table2

Sample #	Calpain I	Calpain II	Sample #	Calpain I	Calpain II	Sample #	Calpain I	Calpain II
Control # 1	0.59	0.66	Run + 0hrs #1	0.725	0.915	Run + 6hrs #1	0.765	0.845
Control # 2	0.585	0.650	Run + 0hrs #2	0.845	0.905	Run + 6hrs #2	0.575	0.78
Control # 3	0.59	0.650	Run + 0hrs #3	0.98	1.105	Run + 6hrs #3	0.78	0.85
Control # 4	0.55	0.715	Run + 0hrs #4	0.885	0.960	Run + 6hrs #4	0.755	0.845
Control # 5	0.705	0.780	Run + 0hrs #5	0.77	1.075	Run + 6hrs #5	0.865	0.965
Control # 6	0.71	0.815	Run + 0hrs #6	0.995	1.13	Run + 6hrs #6	0.79	0.86
Control # 7	0.705	0.795	Run + 0hrs #7	1.005	1.11	Run + 6hrs #7	0.86	0.96
Control #8	0.885	0.955	Run + 0hrs #8	1.015	1.105	Run + 6hrs #8	0.8	0.885
MEAN	0.665	0.755	MEAN	0.905	1.038	MEAN	0.77375	0.875
STD. DEV.	0.1118	0.105864	STD. DEV.	0.113641	0.0947907	STD. DEV.	0.08999	0.06220645
Sample #	Calpain I	Calpain II	Sample #	Calpain I	Calpain II	Sample #	Calpain I	Calpain II
E64C Control # 1	0.615	0.725	E64C Run + 0hrs #1	0.66	0.815	E64C Run + 6hrs #1	0.69	0.79
E64C Control # 2	0.725	0.780	E64C Run + 0hrs #2	0.775	0.865	E64C Run + 6hrs #2	0.725	0.825
E64C Control # 3	0.700	0.765	E64C Run + 0hrs #3	0.735	0.81	E64C Run + 6hrs #3	0.72	0.82
E64C Control # 4	0.7	0.76	E64C Run + 0hrs #4	0.72	0.795	E64C Run + 6hrs #4	0.7	0.79
E64C Control # 5	0.705	0.78	E64C Run + 0hrs #5	0.74	0.82	E64C Run + 6hrs #5	0.855	0.95
E64C Control # 6	0.725	0.79	E64C Run + 0hrs #6	0.725	0.815	E64C Run + 6hrs #6	0.69	0.81
E64C Control # 7	0.695	0.725	E64C Run + 0hrs #7	0.69	0.835	E64C Run + 6hrs #7	0.73	0.84
E64C Control # 8	N/A	N/A	E64C Run + 0hrs #8	N/A	N/A	E64C Run + 6hrs #8	0.745	0.95
MEAN	0.695	0.761	MEAN	0.720714	0.8221429	MEAN	0.73188	0.8337
STD. DEV.	0.04025	0.026367	STD. DEV.	0.036904	0.022334	STD. DEV.	0.05351	0.05122

Calpastatin Activity- Table 3

Sample #	Calpastatin	Sample #	Cal.	Sample #	Cal.
Control # 1	0.23	Run + 0hrs #1	1.05	Run + 6hrs #1	1.01
Control # 2	1.0288	Run + 0hrs #2	N/A	Run + 6hrs #2	1.095
Control # 3	0.7635	Run + 0hrs #3	N/A	Run + 6hrs #3	N/A
Control # 4	0.46	Run + 0hrs #4	0.605	Run + 6hrs #4	1.45
Control # 5	N/A	Run + 0hrs #5	0.9	Run + 6hrs #5	1.3
Control # 6	0.61	Run + 0hrs #6	0.62	Run + 6hrs #6	1.4
Control # 7	0.775	Run + 0hrs #7	0.61	Run + 6hrs #7	0.65
Control #8	0.45	Run + 0hrs #8	0.665	Run + 6hrs #8	0.65
MEAN	0.615	MEAN	0.740	MEAN	1.079286
STD. DEV.	0.264058414	STD. DEV.	0.18806	STD. DEV.	0.33232
Sample #	**Calpastatin**	**Sample #**	**Cal.**	**Sample #**	**Cal.**
E64C Control # 1	1.105	E64C Run + 0hrs #1	1.135	E64C Run + 6hrs #1	2
E64C Control # 2	0.45	E64C Run + 0hrs #2	0.645	E64C Run + 6hrs #2	1.035
E64C Control # 3	0.755	E64C Run + 0hrs #3	0.615	E64C Run + 6hrs #3	1.12
E64C Control # 4	0.770	E64C Run + 0hrs #4	1.055	E64C Run + 6hrs #4	N/A
E64C Control # 5	0.92	E64C Run + 0hrs #5	N/A	E64C Run + 6hrs #5	1.96
E64C Control # 6	0.765	E64C Run + 0hrs #6	0.61	E64C Run + 6hrs #6	1.66
E64C Control # 7	0.65	E64C Run + 0hrs #7	0.615	E64C Run + 6hrs #7	N/A
E64C Control # 8	0.75	E64C Run + 0hrs #8	N/A	E64C Run + 6hrs #8	1.3495
MEAN	0.771	MEAN	0.779167	MEAN	1.52075
STD. DEV.	0.189801353	STD. DEV.	0.24626	STD. DEV.	0.416627

Net Potential Ratios-Table 4

Sample #	CI:CAL	CII:CAL	Sample #	CI:CAL	CII:CAL	Sample #	CI:CAL	CII:CAL
Control # 1	2.5652	2.86956	Run + 0hrs #1	0.6904	0.87142	Run + 6hrs #1	0.7574	0.83663
Control # 2	0.5686	0.632	Run + 0hrs #2	N/A	N/A	Run + 6hrs #2	0.5251	0.71232
Control # 3	0.77275	0.851	Run + 0hrs #3	N/A	N/A	Run + 6hrs #3	N/A	N/A
Control # 4	1.19565	1.55434	Run + 0hrs #4	1.4628	1.587	Run + 6hrs #4	0.52068	0.58275
Control # 5	N/A	N/A	Run + 0hrs #5	0.85555	1.19444	Run + 6hrs #5	0.6653	0.7423
Control # 6	1.1639	1.33606	Run + 0hrs #6	1.6048	1.82258	Run + 6hrs #6	0.56428	0.61428
Control # 7	0.90967	1.0258	Run + 0hrs #7	1.6475	1.81967	Run + 6hrs #7	1.32307	1.47692
Control #8	1.9777	2.12222	Run + 0hrs #8	1.5263	1.66165	Run + 6hrs #8	1.23076	1.361538
MEAN	1.308	1.484	MEAN	1.298	1.493	MEAN	0.79808	0.90381971
STD. DEV.	0.7134783	0.78426	STD. DEV.	0.414848	0.3815909	STD. DEV.	0.33868	0.36335111
Sample #	CI:CAL	CII:CAL	Sample #	CI:CAL	CII:CAL	Sample #	CI:CAL	CII:CAL
E64C Control # 1	0.5475	0.6561	E64C Run + 0hrs #1	0.58149	0.718061	E64C Run + 6hrs #1	0.345	0.355
E64C Control # 2	1.6111	1.611	E64C Run + 0hrs #2	1.20155	1.341085	E64C Run + 6hrs #2	0.7	0.797101
E64C Control # 3	0.927	1.013	E64C Run + 0hrs #3	1.19512	1.317073	E64C Run + 6hrs #3	0.64285	0.73214
E64C Control # 4	0.90909	0.987012	E64C Run + 0hrs #4	0.68246	0.75355	E64C Run + 6hrs #4	N/A	N/A
E64C Control # 5	0.7663	0.847826	E64C Run + 0hrs #5	N/A	N/A	E64C Run + 6hrs #5	0.43622	0.48469
E64C Control # 6	0.94117	1.03679	E64C Run + 0hrs #6	1.18857	1.33606	E64C Run + 6hrs #6	0.41566	0.48751
E64C Control # 7	1.06923	1.115384	E64C Run + 0hrs #7	1.12195	1.357723	E64C Run + 6hrs #7	N/A	N/A
E64C Control # 8	N/A	N/A	E64C Run + 0hrs #8	N/A	N/A	E64C Run + 6hrs #8	0.55205	0.626157
MEAN	0.967	1.038	MEAN	0.99519	1.1372587	MEAN	0.5153	0.580433
STD. DEV.	0.3284266	0.294299	STD. DEV.	0.284594	0.3114366	STD. DEV.	0.13922	0.16773322

Calpain Activity Ratios-Table 5

Sample #	CI:CII	Sample #	CI:CII	Sample #	CI:CII
Control # 1	0.893939	Run + 0hrs #1	0.79234	Run + 6hrs #1	0.905325
Control # 2	0.9	Run + 0hrs #2	0.9337	Run + 6hrs #2	0.737179
Control # 3	0.90769	Run + 0hrs #3	0.88687	Run + 6hrs #3	0.917647
Control # 4	0.76923	Run + 0hrs #4	0.92187	Run + 6hrs #4	0.893491
Control # 5	0.9166666	Run + 0hrs #5	0.716279	Run + 6hrs #5	0.896373
Control # 6	0.87116	Run + 0hrs #6	0.88053	Run + 6hrs #6	0.918604
Control # 7	0.88679	Run + 0hrs #7	0.905405	Run + 6hrs #7	0.89583
Control #8	0.931937	Run + 0hrs #8	0.918552	Run + 6hrs #8	0.903954
MEAN	0.885	MEAN	0.869	MEAN	0.883550375
STD. DEV.	0.050174272	STD. DEV.	0.075982345	STD. DEV.	0.059905143
Sample #	CI:CII	Sample #	CI:CII	Sample #	CI:CII
E64C Control # 1	0.83448	E64C Run + 0hrs #1	0.80981	E64C Run + 6hrs #1	0.8734177
E64C Control # 2	0.92948	E64C Run + 0hrs #2	0.89595	E64C Run + 6hrs #2	0.878787
E64C Control # 3	0.91503	E64C Run + 0hrs #3	0.907407	E64C Run + 6hrs #3	0.87804
E64C Control # 4	0.921052	E64C Run + 0hrs #4	0.90566	E64C Run + 6hrs #4	0.886075
E64C Control # 5	0.903846	E64C Run + 0hrs #5	0.902431	E64C Run + 6hrs #5	0.9
E64C Control # 6	0.9113924	E64C Run + 0hrs #6	0.88957	E64C Run + 6hrs #6	0.85185
E64C Control # 7	0.9586	E64C Run + 0hrs #7	0.826347	E64C Run + 6hrs #7	0.869047
E64C Control # 8	N/A	E64C Run + 0hrs #8	N/A	E64C Run + 6hrs #8	0.881656
MEAN	0.911	MEAN	0.876739286	MEAN	0.87735
STD. DEV.	0.037924943	STD. DEV.	0.040803536	STD. DEV.	0.013860957

80 kDa Band Intensity-Table 6

Sample #	Cal. I	Cal. II	Sample #	Cal. I	Cal. II	Sample #	Cal. I	Cal. II
Control # 1	163	162	Run + 0hrs #1	N/A	N/A	Run + 6hrs #1	N/A	N/A
Control # 2	160	163.000	Run + 0hrs #2	123	120	Run + 6hrs #2	N/A	N/A
Control # 3	N/A	N/A	Run + 0hrs #3	120	121.000	Run + 6hrs #3	144	149.000
Control # 4	N/A	N/A	Run + 0hrs #4	N/A	N/A	Run + 6hrs #4	N/A	N/A
Control # 5	N/A	N/A	Run + 0hrs #5	127.000	133.000	Run + 6hrs #5	148.000	130.000
Control # 6	168	159	Run + 0hrs #6	139	129	Run + 6hrs #6	N/A	N/A
Control # 7	166	166	Run + 0hrs #7	N/A	N/A	Run + 6hrs #7	137	143
Control #8	N/A	N/A	Run + 0hrs #8	N/A	N/A	Run + 6hrs #8	141	143
MEAN	164.250	162.500	MEAN	127.250	125.750	MEAN	142.5	141.25
STD. DEV.	3.5	2.886751	STD. DEV.	8.341663	6.2915287	STD. DEV.	4.65475	8.01560977
Sample #	**Cal. I**	**Cal. II**	**Sample #**	**Cal. I**	**Cal. II**	**Sample #**	**Cal. I**	**Cal. II**
E64C Control # 1	166	160	E64C Run + 0hrs #1	153	155	E64C Run + 6hrs #1	168	156
E64C Control # 2	165	173.000	E64C Run + 0hrs #2	162	152	E64C Run + 6hrs #2	172	159
E64C Control # 3	N/A	N/A	E64C Run + 0hrs #3	168	158	E64C Run + 6hrs #3	N/A	N/A
E64C Control # 4	161	171	E64C Run + 0hrs #4	N/A	N/A	E64C Run + 6hrs #4	N/A	N/A
E64C Control # 5	155	154	E64C Run + 0hrs #5	N/A	N/A	E64C Run + 6hrs #5	148	164
E64C Control # 6	168	158	E64C Run + 0hrs #6	N/A	N/A	E64C Run + 6hrs #6	N/A	N/A
E64C Control # 7	N/A	N/A	E64C Run + 0hrs #7	N/A	N/A	E64C Run + 6hrs #7	N/A	N/A
E64C Control # 8	N/A	N/A	E64C Run + 0hrs #8	160	173	E64C Run + 6hrs #8	159	163
MEAN	163.000	163.200	MEAN	160.75	159.5	MEAN	161.75	160.5
STD. DEV.	5.1478151	8.348653	STD. DEV.	6.184658	9.3273791	STD. DEV.	10.6575	3.6968455

76 kDa Band Intensity-Table 7

Sample #	Cal. I	Cal. II	Sample #	Cal. I	Cal. II	Sample #	Cal. I	Cal. II
Control # 1	N/A	N/A	Run + 0hrs #1	64	67	Run + 6hrs #1	N/A	N/A
Control # 2	0	8.000	Run + 0hrs #2	69	73	Run + 6hrs #2	N/A	N/A
Control # 3	N/A	N/A	Run + 0hrs #3	N/A	N/A	Run + 6hrs #3	N/A	N/A
Control # 4	12	0	Run + 0hrs #4	N/A	N/A	Run + 6hrs #4	N/A	N/A
Control # 5	0.000	0.000	Run + 0hrs #5	N/A	N/A	Run + 6hrs #5	32.000	21.000
Control # 6	N/A	N/A	Run + 0hrs #6	63	71	Run + 6hrs #6	27	29
Control # 7	N/A	N/A	Run + 0hrs #7	70	61	Run + 6hrs #7	28	29
Control #8	N/A	N/A	Run + 0hrs #8	N/A	N/A	Run + 6hrs #8	37	25
MEAN	4.000	2.667	MEAN	66.500	68.000	MEAN	31	26
STD. DEV.	6.9282032	4.618802	STD. DEV.	3.511885	5.2915026	STD. DEV.	4.54606	3.82970843
Sample #	Cal. I	Cal. II	Sample #	Cal. I	Cal. II	Sample #	Cal. I	Cal. II
E64C Control # 1	0	2	E64C Run + 0hrs #1	N/A	N/A	E64C Run + 6hrs #1	0	0
E64C Control # 2	N/A	N/A	E64C Run + 0hrs #2	N/A	N/A	E64C Run + 6hrs #2	6	0
E64C Control # 3	N/A	N/A	E64C Run + 0hrs #3	0	0	E64C Run + 6hrs #3	7	0
E64C Control # 4	O	0	E64C Run + 0hrs #4	0	0	E64C Run + 6hrs #4	N/A	N/A
E64C Control # 5	3	0	E64C Run + 0hrs #5	0	0	E64C Run + 6hrs #5	N/A	N/A
E64C Control # 6	4	0	E64C Run + 0hrs #6	1	0	E64C Run + 6hrs #6	N/A	N/A
E64C Control # 7	N/A	N/A	E64C Run + 0hrs #7	1	1	E64C Run + 6hrs #7	3	0
E64C Control # 8	N/A	N/A	E64C Run + 0hrs #8	N/A	N/A	E64C Run + 6hrs #8	N/A	N/A
MEAN	2.333	0.500	MEAN	0.4	0.2	MEAN	4	0
STD. DEV.	2.081666	1	STD. DEV.	0.547723	0.4472136	STD. DEV.	3.16228	0

17 kDa Band Intensity-Table 8

Sample #	Cal. I	Cal. II	Sample #	Cal. I	Cal. II	Sample #	Cal. I	Cal. II
Control # 1	0	0	Run + 0hrs #1	38	33	Run + 6hrs #1	8	16
Control # 2	0	2.000	Run + 0hrs #2	30	32	Run + 6hrs #2	0	22
Control # 3	N/A	N/A	Run + 0hrs #3	29	34.000	Run + 6hrs #3	N/A	N/A
Control # 4	N/A	N/A	Run + 0hrs #4	N/A	N/A	Run + 6hrs #4	N/A	N/A
Control # 5	N/A	N/A	Run + 0hrs #5	N/A	N/A	Run + 6hrs #5	N/A	N/A
Control # 6	0	2	Run + 0hrs #6	28	29	Run + 6hrs #6	N/A	N/A
Control # 7	7	0	Run + 0hrs #7	N/A	N/A	Run + 6hrs #7	N/A	N/A
Control #8	4	0	Run + 0hrs #8	N/A	N/A	Run + 6hrs #8	4	0
MEAN	2.200	0.800	MEAN	31.250	32.000	MEAN	4	12.6666667
STD. DEV.	3.1937439	1.095445	STD. DEV.	4.573474	2.1602469	STD. DEV.	4	11.3724814
Sample #	Cal. I	Cal. II	Sample #	Cal. I	Cal. II	Sample #	Cal. I	Cal. II
E64C Control # 1	0	2	E64C Run + 0hrs #1	0	0	E64C Run + 6hrs #1	N/A	N/A
E64C Control # 2	0	1.000	E64C Run + 0hrs #2	N/A	N/A	E64C Run + 6hrs #2	0	0
E64C Control # 3	0.000	0.000	E64C Run + 0hrs #3	N/A	N/A	E64C Run + 6hrs #3	N/A	N/A
E64C Control # 4	0	0	E64C Run + 0hrs #4	N/A	N/A	E64C Run + 6hrs #4	0	9.9
E64C Control # 5	0	0	E64C Run + 0hrs #5	0	5	E64C Run + 6hrs #5	0	0
E64C Control # 6	N/A	N/A	E64C Run + 0hrs #6	0	7	E64C Run + 6hrs #6	N/A	N/A
E64C Control # 7	N/A	N/A	E64C Run + 0hrs #7	23	0	E64C Run + 6hrs #7	11	0
E64C Control # 8	N/A	N/A	E64C Run + 0hrs #8	N/A	N/A	E64C Run + 6hrs #8	N/A	N/A
MEAN	0.000	0.600	MEAN	5.75	3	MEAN	2.75	2.475
STD. DEV.	0	0.894427	STD. DEV.	11.5	3.5590261	STD. DEV.	5.5	4.95

CK Activity - Table 9

Sample #	CK	Sample #	CK	Sample #	CK
Control # 1	1340.75	Run + 0hrs #1	1670.6	Run + 6hrs #1	1163.45
Control # 2	1329.8	Run + 0hrs #2	6287.25	Run + 6hrs #2	1331.15
Control # 3	1447.95	Run + 0hrs #3	1193.85	Run + 6hrs #3	N/A
Control # 4	455.67	Run + 0hrs #4	1711.720	Run + 6hrs #4	738.8
Control # 5	824.005	Run + 0hrs #5	1530.45	Run + 6hrs #5	1843.8
Control # 6	602.725	Run + 0hrs #6	582.12	Run + 6hrs #6	2444.4
Control # 7	605.48	Run + 0hrs #7	1669.25	Run + 6hrs #7	2236.9
Control #8	1328.873	Run + 0hrs #8	4648.95	Run + 6hrs #8	1226.65
MEAN	991.907	MEAN	2411.774	MEAN	1569.307
STD. DEV.	409.5143602	STD. DEV.	1972.045	STD. DEV.	621.2097
Sample #	**CK**	**Sample #**	**CK**	**Sample #**	**CK**
E64C Control # 1	976.59	E64C Run + 0hrs #1	2631.4	E64C Run + 6hrs #1	3439.45
E64C Control # 2	976.56	E64C Run + 0hrs #2	2900.7	E64C Run + 6hrs #2	N/A
E64C Control # 3	2294.2	E64C Run + 0hrs #3	4550.58	E64C Run + 6hrs #3	1720.1
E64C Control # 4	1203.350	E64C Run + 0hrs #4	1261.05	E64C Run + 6hrs #4	821.255
E64C Control # 5	954.58	E64C Run + 0hrs #5	N/A	E64C Run + 6hrs #5	1698.1
E64C Control # 6	676.95	E64C Run + 0hrs #6	2412.8	E64C Run + 6hrs #6	3887.5
E64C Control # 7	667.33	E64C Run + 0hrs #7	4343.8	E64C Run + 6hrs #7	1974.05
E64C Control # 8	1886.4	E64C Run + 0hrs #8	3332.804	E64C Run + 6hrs #8	3121.95
MEAN	1204.495	MEAN	3061.876	MEAN	2380.344
STD. DEV.	583.7875411	STD. DEV.	1140.799	STD. DEV.	1113.584

Glycogen Concentrations-Table10

Sample #	Glycogen(abs)	[Glycogen]	Glycogen(ug/g)	Sample #	Glycogen(abs	[Glycogen]	Glycogen(ug/g)
Control # 1	0.168	13.77352	1449.84	Run + 0hrs #1	0.172	14.21408	607.43
Control # 2	0.136	10.24904	1078.84	Run + 0hrs #2	0.165	13.4431	584.47
Control # 3	0.135	10.1389	792.1	Run + 0hrs #3	0.186	15.75604	N/A
Control # 4	0.12	8.4868	898.53	Run + 0hrs #4	0.126	9.14764	609.84
Control # 5	0.160	12.8924	1371.53	Run + 0hrs #5	0.122	8.70708	414.62
Control # 6	0.113	7.71582	989.21	Run + 0hrs #6	0.19	16.1966	686.29
Control # 7	0.065	2.4291	N/A	Run + 0hrs #7	0.108	7.16512	261.49
Control #8	0.089	5.07246	694.79	Run + 0hrs #8	0.118	8.26652	409.21
MEAN	0.123	8.845	1039.263	MEAN	0.148	11.612	510.479
STD. DEV.	0.034507763	3.800685	283.6087233	STD. DEV.	0.03323482	3.660484	151.1600481
Sample #	Glycogen(abs)	[Glycogen]	Glycogen(ug/g)	Sample #	Glycogen(abs	[Glycogen]	Glycogen(ug/g)
E64C Control # 1	0.18	15.0952	1324.14	64C Run + 0hrs	0.183	15.42562	712.62
E64C Control # 2	0.115	7.9361	655.87	64C Run + 0hrs	0.185	15.6459	662.92
E64C Control # 3	0.124	8.92736	671.22	64C Run + 0hrs	0.089	5.07246	352.25
E64C Control # 4	0.263	24.23682	N/A	64C Run + 0hrs	0.161	13.00254	528.55
E64C Control # 5	0.304	28.75256	N/A	64C Run + 0hrs	0.092	5.40288	278.49
E64C Control # 6	0.108	7.16512	823.56	64C Run + 0hrs	0.081	4.19134	203.46
E64C Control # 7	0.116	8.04624	718.41	64C Run + 0hrs	0.094	5.62316	446.28
E64C Control # 8	0.133	9.91862	708.47	64C Run + 0hrs	0.227	20.27178	N/A
MEAN	0.168	13.760	816.945	MEAN	0.139	10.579	454.939
STD. DEV.	0.075535115	8.319438	255.3223176	STD. DEV.	0.05654076	6.227399	191.5159347

Glycogen Concentrations-Table10

Sample #	Glycogen(abs)	[Glycogen]	Glycogen(ug/g)
Run + 6hrs #1	0.12	8.4868	597.660
Run + 6hrs #2	0.204	17.73856	974.64
Run + 6hrs #3	0.334	N/A	N/A
Run + 6hrs #4	0.204	17.73856	N/A
Run + 6hrs #5	0.143	11.02002	680.24
Run + 6hrs #6	0.168	13.77352	612.94
Run + 6hrs #7	0.146	11.35044	591.14
Run + 6hrs #8	0.119	8.37666	837.6
MEAN	0.17975	12.641	715.703
STD. DEV.	0.070566585	3.936748	156.9555338
Sample #	Glycogen(abs)	[Glycogen]	Glycogen(ug/g)
54C Run + 6hrs	0.139	10.57946	503.78
54C Run + 6hrs	0.209	18.28926	692.76
54C Run + 6hrs	0.119	8.37666	550.66
54C Run + 6hrs	0.156	12.45184	598.65
54C Run + 6hrs	0.364	N/A	N/A
54C Run + 6hrs	0.444	N/A	N/A
54C Run + 6hrs	0.293	27.54102	N/A
54C Run + 6hrs	0.153	12.12142	577.19
MEAN	0.234625	14.893	584.608
STD. DEV.	0.119521591	7.017367	70.0619224

Fibril TnI Band Dens.-Table 11

Sample #	TnI	Sample #	TnI	Sample #	TnI
Control # 1	21.63	Run + 0hrs #1	15.96	Run + 6hrs #1	24
Control # 2	17.51	Run + 0hrs #2	N/A	Run + 6hrs #2	52.8
Control # 3	39.14	Run + 0hrs #3	49.17	Run + 6hrs #3	4.8
Control # 4	23.69	Run + 0hrs #4	8.940	Run + 6hrs #4	4.8
Control # 5	22.660	Run + 0hrs #5	8.94	Run + 6hrs #5	9.6
Control # 6	N/A	Run + 0hrs #6	.4.47	Run + 6hrs #6	4.8
Control # 7	3.09	Run + 0hrs #7	13.41	Run + 6hrs #7	9.6
Control #8	21.63	Run + 0hrs #8	13.41	Run + 6hrs #8	14.4
MEAN	21.336	MEAN	16.034	MEAN	15.6
STD. DEV.	10.576632	STD. DEV.	14.9752	STD. DEV.	16.37838
Sample #	**TnI**	**Sample #**	**TnI**	**Sample #**	**TnI**
E64C Control # 1	6.98	E64C Run + 0hrs #1	37.68	E64C Run + 6hrs #1	23
E64C Control # 2	20.94	E64C Run + 0hrs #2	18.84	E64C Run + 6hrs #2	23
E64C Control # 3	27.92	E64C Run + 0hrs #3	23.55	E64C Run + 6hrs #3	59.8
E64C Control # 4	10.470	E64C Run + 0hrs #4	9.41	E64C Run + 6hrs #4	9.2
E64C Control # 5	31.41	E64C Run + 0hrs #5	14.13	E64C Run + 6hrs #5	13.8
E64C Control # 6	17.45	E64C Run + 0hrs #6	37.68	E64C Run + 6hrs #6	4.6
E64C Control # 7	27.92	E64C Run + 0hrs #7	18.84	E64C Run + 6hrs #7	N/A
E64C Control # 8	N/A	E64C Run + 0hrs #8	23.55	E64C Run + 6hrs #8	N/A
MEAN	20.441	MEAN	22.96	MEAN	22.23333
STD. DEV.	9.327417343	STD. DEV.	10.1746	STD. DEV.	19.7845

Cyto. TnI Band Density-Table 12

Sample #	TnI	Sample #	TnI	Sample #	TnI
Control # 1	117.645	Run + 0hrs #1	412.877	Run + 6hrs #1	380.77
Control # 2	126.914	Run + 0hrs #2	N/A	Run + 6hrs #2	463.71
Control # 3	116.219	Run + 0hrs #3	451.889	Run + 6hrs #3	456.17
Control # 4	124.062	Run + 0hrs #4	429.132	Run + 6hrs #4	618.28
Control # 5	113.367	Run + 0hrs #5	526.662	Run + 6hrs #5	448.63
Control # 6	N/A	Run + 0hrs #6	393.371	Run + 6hrs #6	452.4
Control # 7	119.784	Run + 0hrs #7	419.379	Run + 6hrs #7	429.78
Control #8	118.358	Run + 0hrs #8	409.626	Run + 6hrs #8	441.09
MEAN	119.478	MEAN	434.705	MEAN	461.679
STD. DEV.	4.636458377	STD. DEV.	43.8853	STD. DEV.	68.45394
Sample #	**TnI**	**Sample #**	**TnI**	**Sample #**	**TnI**
E64C Control # 1	259.26	E64C Run + 0hrs #1	626.58	E64C Run + 6hrs #1	588.8
E64C Control # 2	265.22	E64C Run + 0hrs #2	672.6	E64C Run + 6hrs #2	691.84
E64C Control # 3	248.83	E64C Run + 0hrs #3	343.38	E64C Run + 6hrs #3	673.44
E64C Control # 4	245.850	E64C Run + 0hrs #4	665.52	E64C Run + 6hrs #4	614.56
E64C Control # 5	245.85	E64C Run + 0hrs #5	700.92	E64C Run + 6hrs #5	625.6
E64C Control # 6	257.77	E64C Run + 0hrs #6	690.3	E64C Run + 6hrs #6	651.36
E64C Control # 7	253.3	E64C Run + 0hrs #7	654.9	E64C Run + 6hrs #7	N/A
E64C Control # 8	N/A	E64C Run + 0hrs #8	661.98	E64C Run + 6hrs #8	N/A
MEAN	254.524	MEAN	636.0843	MEAN	642.2843
STD. DEV.	7.385866812	STD. DEV.	116.7884	STD. DEV.	38.4849

Fibril TnT Band Density-Table13

Sample #	TnT	Sample #	TnT	Sample #	TnT
Control # 1	24.3	Run + 0hrs #1	14.56	Run + 6hrs #1	11.33
Control # 2	20.81	Run + 0hrs #2	N/A	Run + 6hrs #2	51
Control # 3	28.2	Run + 0hrs #3	26.8	Run + 6hrs #3	7.8
Control # 4	20.5	Run + 0hrs #4	12.890	Run + 6hrs #4	6.54
Control # 5	30.100	Run + 0hrs #5	9.78	Run + 6hrs #5	6.23
Control # 6	N/A	Run + 0hrs #6	25.6	Run + 6hrs #6	19.67
Control # 7	19.45	Run + 0hrs #7	32	Run + 6hrs #7	24.51
Control #8	23.7	Run + 0hrs #8	4.7	Run + 6hrs #8	16.89
MEAN	23.866	MEAN	18.047	MEAN	17.99625
STD. DEV.	4.042119317	STD. DEV.	10.1126	STD. DEV.	14.89868
Sample #	TnT	Sample #	TnT	Sample #	TnT
E64C Control # 1	17.22	E64C Run + 0hrs #1	20.47	E64C Run + 6hrs #1	54.44
E64C Control # 2	18.2	E64C Run + 0hrs #2	24.56	E64C Run + 6hrs #2	22.78
E64C Control # 3	29.3	E64C Run + 0hrs #3	12.4	E64C Run + 6hrs #3	11.04
E64C Control # 4	27.230	E64C Run + 0hrs #4	13.5	E64C Run + 6hrs #4	21.445
E64C Control # 5	29.31	E64C Run + 0hrs #5	45.96	E64C Run + 6hrs #5	16.43
E64C Control # 6	23.446	E64C Run + 0hrs #6	43.21	E64C Run + 6hrs #6	19.65
E64C Control # 7	26.99	E64C Run + 0hrs #7	24.55	E64C Run + 6hrs #7	N/A
E64C Control # 8	N/A	E64C Run + 0hrs #8	16.86	E64C Run + 6hrs #8	N/A
MEAN	24.528	MEAN	25.18875	MEAN	24.2975
STD. DEV.	5.060021871	STD. DEV.	12.81086	STD. DEV.	15.35101

Cyto. TnT Band Density-Table 14

Sample #	TnT	Sample #	TnT	Sample #	TnT
Control # 1	139.748	Run + 0hrs #1	464.893	Run + 6hrs #1	422.24
Control # 2	135.47	Run + 0hrs #2	N/A	Run + 6hrs #2	490.1
Control # 3	103.385	Run + 0hrs #3	575.427	Run + 6hrs #3	501.41
Control # 4	135.47	Run + 0hrs #4	432.383	Run + 6hrs #4	414.7
Control # 5	N/A	Run + 0hrs #5	409.626	Run + 6hrs #5	640.9
Control # 6	137.609	Run + 0hrs #6	451.889	Run + 6hrs #6	542.88
Control # 7	149.017	Run + 0hrs #7	438.885	Run + 6hrs #7	516.49
Control #8	141.887	Run + 0hrs #8	464.893	Run + 6hrs #8	486.33
MEAN	134.655	MEAN	462.571	MEAN	501.8813
STD. DEV.	14.56236578	STD. DEV.	53.44293	STD. DEV.	71.25877
Sample #	**TnT**	**Sample #**	**TnT**	**Sample #**	**TnT**
E64C Control # 1	287.62	E64C Run + 0hrs #1	679.68	E64C Run + 6hrs #1	747.04
E64C Control # 2	299.49	E64C Run + 0hrs #2	704.46	E64C Run + 6hrs #2	850.08
E64C Control # 3	302.47	E64C Run + 0hrs #3	686.76	E64C Run + 6hrs #3	736
E64C Control # 4	280.120	E64C Run + 0hrs #4	746.94	E64C Run + 6hrs #4	585.12
E64C Control # 5	248.83	E64C Run + 0hrs #5	697.38	E64C Run + 6hrs #5	647.68
E64C Control # 6	274.16	E64C Run + 0hrs #6	651.36	E64C Run + 6hrs #6	721.28
E64C Control # 7	286.08	E64C Run + 0hrs #7	637.2	E64C Run + 6hrs #7	N/A
E64C Control # 8	N/A	E64C Run + 0hrs #8	679.68	E64C Run + 6hrs #8	N/A
MEAN	283.526	MEAN	685.4325	MEAN	714.5333
STD. DEV.	18.36221	STD. DEV.	33.47326	STD. DEV.	9.7642

Fibril TM Band Density-Table 15

Sample #	Tropomyosin	Sample #	Tropo	Sample #	Tropo
Control # 1	22.76	Run + 0hrs #1	53	Run + 6hrs #1	22.2
Control # 2	22.39	Run + 0hrs #2	N/A	Run + 6hrs #2	12.78
Control # 3	44.53	Run + 0hrs #3	35.69	Run + 6hrs #3	13.22
Control # 4	14.99	Run + 0hrs #4	11.410	Run + 6hrs #4	12.43
Control # 5	N/A	Run + 0hrs #5	5.9	Run + 6hrs #5	17.55
Control # 6	16.5	Run + 0hrs #6	9.56	Run + 6hrs #6	25.97
Control # 7	4.2	Run + 0hrs #7	10.44	Run + 6hrs #7	20.45
Control #8	29.33	Run + 0hrs #8	13.3	Run + 6hrs #8	17.4
MEAN	22.100	MEAN	19.900	MEAN	17.75
STD. DEV.	12.63737578	STD. DEV.	17.57357	STD. DEV.	4.904045
Sample #	**Tropomyosin**	**Sample #**	**Tropo**	**Sample #**	**Tropo**
E64C Control # 1	20.09	E64C Run + 0hrs #1	29.76	E64C Run + 6hrs #1	10.22
E64C Control # 2	22.467	E64C Run + 0hrs #2	27.22	E64C Run + 6hrs #2	26.44
E64C Control # 3	16.78	E64C Run + 0hrs #3	14.76	E64C Run + 6hrs #3	29.05
E64C Control # 4	18.940	E64C Run + 0hrs #4	25.88	E64C Run + 6hrs #4	36.52
E64C Control # 5	27	E64C Run + 0hrs #5	34.38	E64C Run + 6hrs #5	23.5
E64C Control # 6	28.66	E64C Run + 0hrs #6	21.11	E64C Run + 6hrs #6	10.21
E64C Control # 7	23.6	E64C Run + 0hrs #7	16.62	E64C Run + 6hrs #7	N/A
E64C Control # 8	N/A	E64C Run + 0hrs #8	26.02	E64C Run + 6hrs #8	N/A
MEAN	22.505	MEAN	24.46875	MEAN	22.65667
STD. DEV.	4.29353377	STD. DEV.	6.60456	STD. DEV.	10.56108

Cyto. TM Band Density-Table 16

Sample #	Tropo	Sample #	Tropo	Sample #	Tropo
Control # 1	126.201	Run + 0hrs #1	594.933	Run + 6hrs #1	557.96
Control # 2	124.775	Run + 0hrs #2	N/A	Run + 6hrs #2	561.73
Control # 3	121.21	Run + 0hrs #3	565.674	Run + 6hrs #3	754
Control # 4	131.192	Run + 0hrs #4	552.670	Run + 6hrs #4	599.43
Control # 5	N/A	Run + 0hrs #5	416.128	Run + 6hrs #5	606.97
Control # 6	134.757	Run + 0hrs #6	578.678	Run + 6hrs #6	603.2
Control # 7	136.183	Run + 0hrs #7	588.431	Run + 6hrs #7	610.74
Control #8	131.192	Run + 0hrs #8	598.184	Run + 6hrs #8	603.2
MEAN	129.359	MEAN	556.385	MEAN	612.1538
STD. DEV.	5.47444636	STD. DEV.	63.93896	STD. DEV.	60.88746
Sample #	**Tropo**	**Sample #**	**Tropo**	**Sample #**	**Tropo**
E64C Control # 1	274.16	E64C Run + 0hrs #1	637.2	E64C Run + 6hrs #1	651.36
E64C Control # 2	245.85	E64C Run + 0hrs #2	683.22	E64C Run + 6hrs #2	655.04
E64C Control # 3	251.81	E64C Run + 0hrs #3	661.98	E64C Run + 6hrs #3	621.92
E64C Control # 4	281.610	E64C Run + 0hrs #4	637.2	E64C Run + 6hrs #4	699.2
E64C Control # 5	265.22	E64C Run + 0hrs #5	647.82	E64C Run + 6hrs #5	688.16
E64C Control # 6	268.2	E64C Run + 0hrs #6	658.44	E64C Run + 6hrs #6	680.8
E64C Control # 7	283.1	E64C Run + 0hrs #7	343.38	E64C Run + 6hrs #7	N/A
E64C Control # 8	N/A	E64C Run + 0hrs #8	683.22	E64C Run + 6hrs #8	N/A
MEAN	267.136	MEAN	619.0575	MEAN	666.08
STD. DEV.	14.18018436	STD. DEV.	112.8277	STD. DEV.	28.60002

Protein Concentrations-Table 17

SAMPLE #	[PROT] homo	[PROT] cyto-1	[PROT] cyto-2	[PROT] fibrls
CONTROL 1	1.470	0.677	0.217	0.951
CONTROL 2	1.495	0.683	0.151	1.035
CONTROL 3	1.456	0.692	0.131	1.252
CONTROL 4	1.515	0.634	0.165	1.051
CONTROL 5	0.000	0.732	0.165	1.011
CONTROL 6	0.000	0.696	0.190	1.038
CONTROL 7	0.000	0.829	0.168	0.918
CONTROL 8	0.000	0.761	0.177	0.974
CONTROL E64C 1	1.356	0.700	0.115	0.898
CONTROL E64C 2	1.403	0.679	0.099	0.918
CONTROL E64C 3	1.246	0.638	0.123	0.949
CONTROL E64C 4	1.419	0.714	0.140	0.957
CONTROL E64C 5	0.000	0.000	0.000	0.000
CONTROL E64C 6	0.000	0.000	0.794	7.968
CONTROL E64C 7	6.434	2.926	0.731	5.524
CONTROL E64C 8	7.266	3.411	0.973	7.109
SAMPLE #	[PROT] homo	[[PROT] cyto-1	[PROT] cyto-2	[PROT] fibrls
RUN 1 + 0hrs	7.325	3.188	0.645	6.167
RUN 2 + 0hrs	7.392	3.205	0.687	3.967
RUN 3 + 0hrs	8.123	3.052	0.537	5.388
RUN 4 + 0hrs	7.784	3.295	0.683	4.545
RUN 5 + 0hrs	7.790	3.093	0.475	3.992
RUN 6 + 0hrs	4.070	3.626	0.208	1.461
RUN 7 + 0hrs	7.904	3.118	0.681	5.042
RUN 8 + 0hrs	0.000	3.531	0.828	5.214
RUN 1 + E64C + 0hrs	7.944	3.700	0.804	6.900
RUN 2 + E64C + 0hrs	7.643	3.017	0.429	6.606
RUN 3 + E64C + 0hrs	8.757	3.844	0.581	3.721
RUN 4 + E64C + 0hrs	7.843	4.103	0.466	3.369
RUN 5 + E64C + 0hrs	9.774	3.356	0.559	5.675
RUN 6 + E64C + 0hrs	7.456	3.457	0.511	4.497
RUN 7 + E64C + 0hrs	6.414	2.834	0.448	2.998
RUN 8 + E64C + 0hrs	7.704	4.418	0.515	3.819

Protein Concentrations-Table 17

SAMPLE #	[PROT] homo	[[PROT] cyto-1	[PROT] cyto-2	[PROT] fibrls
RUN 1 + 6hrs	6.049	3.151	0.583	4.669
RUN 2 + 6hrs	5.839	4.548	0.000	5.768
RUN 3 + 6hrs	0.000	0.000	0.000	0.000
RUN 4 + 6hrs	6.709	3.369	0.624	4.980
RUN 5 + 6hrs	5.295	4.167	0.148	4.810
RUN 6 + 6hrs	6.507	3.470	0.119	4.827
RUN 7 + 6hrs	6.515	3.609	0.192	5.057
RUN 8 + 6hrs	6.038	4.094	0.258	5.131
RUN 1 + E64C + 6hrs	7.448	3.263	0.737	5.135
RUN 2 + E64C + 6hrs	7.715	3.260	0.579	5.658
RUN 3 + E64C + 6hrs	0.000	0.000	0.000	0.000
RUN 4 + E64C + 6hrs	8.828	4.144	0.744	4.597
RUN 5 + E64C + 6hrs	6.857	4.160	0.611	5.131
RUN 6 + E64C + 6hrs	7.220	3.525	0.618	4.800
RUN 7 + E64C + 6hrs	8.373	3.900	0.635	3.800
RUN 8 + E64C + 6hrs	7.600	3.562	0.657	4.215