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Exercise-induced muscle damage in humans:

Heat shock proteins and inflammation in recovery, regeneration and adaptation

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LIST OF PAPERS

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II. Paulsen G, Egner IM, Drange M, Langberg H, Benestad HB, Fjeld JG, Hallén J, Raastad T. A COX-2 inhibitor reduces muscle soreness, but does not influence recovery and adaptation after eccentric exercise. *Scand.J Med.Sci.Sports* 2009: In press

III. Paulsen G, Vissing K, Kalhovde JM, Ugelstad I, Bayer ML, Kadi F, Schjerling P, Hallen J, Raastad T. Maximal eccentric exercise induces a rapid accumulation of small heat shock proteins on myofibrils and a delayed HSP70 response in humans. *Am.J Physiol Regul.Integr.Comp Physiol* 2007 Aug;293(2):R844-R853.

IV. Paulsen G, Lauritzen F, Bayer ML, Kalhovde JM, Ugelstad I, Owe GS, Bergersen LH, Hallén J, Raastad T. Involvement of HSP27, αB-crystallin and HSP70 in the repeated-bout effect after eccentric exercise in humans. *J Appl. Physiol;* In revision

Note that Paper II is available online in its final form (Scand.J Med.Sci.Sports, June 2009; http://www.wiley.com/bw/journal.asp?ref=0905-7188&site=1). Paper IV is available online in its accepted form (J. Appl. Physiol, 4. June 2009; http://jap.physiology.org/). The title of Paper IV has been changed to: “Subcellular movement and expression of HSP27, αB-crystallin, and HSP70 after two bouts eccentric exercise in humans”.

1.0 INTRODUCTION

The purpose of this introduction is to give a brief overview of exercise-induced muscle damage, with special focus on inflammation and heat shock proteins in human skeletal muscle.

A special feature of our skeletal muscles is the remarkable adaptability[1] to physical demands. However, another important characteristic of skeletal muscles is that during strenuous physical activity (exercise), the force-generating capacity becomes temporarily reduced. The duration of recovery from this fatigue varies from minutes to several days, depending on the type, intensity, and duration of the exercise. For example, moderately intense running exercise (~70% of VO_{2max}) for 45 minutes may not compromise the force-generating capacity detectably in young active subjects (278), a bout of high-force resistance exercise (lifting weights) seems to require 1-2 days of recovery in trained subjects (333), while recovery from extreme types of exercise, such as running a marathon, can take more than one week (371). Long-lasting recovery is associated with muscle damage that evidently takes time to repair (60; 154; 179; 430), and tissue damage is generally linked to development of a local inflammatory reaction (310). A large body of animal studies gives support to such reasoning, and accordingly, many relevant reviews discuss "exercise-induced muscle damage"[2] and inflammation as integrated events in the process of recovery from demanding exercise, especially high-force eccentric exercise[3] (8; 30; 102; 105; 124; 189; 220; 241; 328; 331; 374; 382). Notably, no universal definition of exercise-induced muscle damage exists (105; 437),

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[1] Adaptability in physiological terms implies the ability to adjust and to improve certain qualities. "Adaptation" is used here to describe physiological improvements, e.g., increased tolerance against exercise-induced muscle damage.

[2] Exercise-induced muscle damage differs from the more severe form of muscle damage inflicted by a strain injury: A sudden, painful, rupture (tear) of myofibres, capillaries, and connective tissue in a localised area, characterises muscle strain injuries (176; 294; 298), but not exercise-induced muscle damage (235; 439). Nevertheless, the regeneration processes observed after exercise-induced muscle damage and strain injuries, as well as other forms of mechanical overloading models (e.g., unloading-reloading models), appear quite stereotypical (406; 407).

[3] Eccentric exercise entails a series of eccentric actions. "Eccentric actions" is used here synonymously with the term "lengthening contractions", which is also commonly used in the literature (104).

but it seems largely agreed upon that large reductions in force-generating capacity is the most reliable marker of muscle damage (47; 105; 277; 437). However, histological techniques are required for direct verification (105). Long-lasting (i.e., days) reduction of the force-generating capacity following eccentric exercise-induced muscle damage seems to be caused by three central mechanisms:

1. Mechanical disruptions, oxidation, and degradation of the myofibrillar structure, i.e., force-generating and force-transmitting structures
2. Mechanical disruptions, oxidation, and degradation that lead to malfunction of the excitation-contraction coupling (activation failure of the contractile filaments)
3. Myofibre necrosis (complete degradation of myofibre segments)

The interaction between these events and their relative contribution to the loss of force-generating capacity are, however, debated (3; 105; 274; 323; 328; 375; 435; 436). Still, such events occur not without a struggle, as myofibres possess defence systems by virtue of antioxidants and heat shock proteins (HSPs; or stress proteins; see introduction below).

Solidly founded on animal studies, the paradigm of exercise-induced muscle damage appears robust. However, critical voices have recently questioned the existing evidence of *muscle damage and inflammation in humans after voluntary[4] exercise* (78; 250; 251; 453; 455). The rationale of this scepticism is based on: 1) doubt as to whether results from animal models (using mice, rats, and rabbits) are transferable to humans; 2) methodological circumstances (concerning the biopsy procedure) in some of the "classical" humans studies cast doubt on certain conclusions that have been drawn (discussed further below); and, 3) the histological interpretations of "muscle damage" have been challenged, and it has been claimed that the changes in the myofibrillar structure observed after eccentric exercise in humans may in fact be signs of remodelling and adaptation, not damage[5].

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[4] As opposed to (external) electrical stimulation of the muscles – commonly used to "exercise" muscles in animal models.
[5] This will not be discussed in detail, but to some degree, this might be a question of semantics. Hence, Yu *et al.* (454; 455) used loss of desmin, necrosis, and inflammation, as signs of "damage", (the footnote continues on the next page...)

1.1 Exercise-induced local inflammation

One of the main aims of this thesis was to study the local inflammatory reaction[6] in human muscles after maximal eccentric exercise. Before delving into central methodological issues, an overview of findings from experiments investigating the presence of leukocytes in human muscles after exercise is warranted. What quickly becomes evident when we look into relevant research is the inconsistent findings concerning leukocyte accumulation. The discrepancy between studies might seem ambiguous, but when we organise them after the reduction in force-generating capacity, it becomes apparent that the reduction in force is associated with the detection of leukocytes in the exercised muscles (Appendix I, Table A-C). Indeed, in studies where no leukocyte accumulation was found, the reduction in force-generating capacity was relatively moderate (< 30%; (39; 78; 107; 130; 253)) and recovery of the force-generating capacity was completed within one week, thus indicating minor or moderate damage (105; 437). Conversely, in studies where leukocytes were found in large numbers, the type of exercises applied evidently caused extensive damage, as judged from the large loss of force-generating capacity (~50% reduction) and long-lasting recovery (> 1 week), as well as high levels of creatine kinase (CK) in the circulation (> 10 000 $IU \cdot L^{-1}$; e.g.: (66; 152; 154; 179; 351)). Unfortunately, in many of the relevant studies changes in muscle function was not monitored. However, by evaluating the exercise protocols that were used, indices of the degree of damage inflicted may be found. Based on *in vitro*, *in situ* and *in vivo* studies, the most crucial factors for exercise-induced muscle damage and large reductions in force-generating capacity are: 1) high-force (maximal) eccentric actions (35; 67; 180; 245; 258; 302; 314; 432) and 2) large strain[7] (19; 68; 144; 180; 219; 244; 289; 305; 306; 398; 399). In line with this, concentric and isometric exercise (81; 180; 290), and conventional

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whereas irregularities (disruptions) in the myofibrillar structure were interpreted as remodelling – not damage. However, it seems reasonable to say that there has to be some levels of myofibrillar damage (myofibrillar disruptions) before the stage of complete degradation of desmin and necrosis – i.e., cell death. Intuitively, something has to trigger the remodelling process. Note also that Yu *et al.* (453; 455) did not measure changes in muscle function, which makes comparisons to other studies difficult.

[6] The local inflammatory reaction (inflammation) is primarily characterised by accumulation of different types of leukocytes in the affected tissue (367).

[7] The muscles are actively stretched beyond the optimal length for force-generation.

resistance exercise (coupled concentric-eccentric muscle actions; (39; 87; 329))[8], as well as eccentrically biased exercise[9], such as downhill and downstairs running (107; 129; 253; 309; 455), seem to not cause severe muscle damage, and consequently, no significant leukocyte accumulation in the exercised muscles (Appendix I, Table D). On the contrary, tissue accumulation of leukocytes has more consistently been observed after single joint, voluntary maximal eccentric exercise with large range of motion ((26; 27; 66; 179; 247; 383); Appendix I, Table E). Intriguingly, the relationship between the degree of muscle damage and leukocyte accumulation seems to be less clear for rodents: In experimental rodents, leukocyte accumulation has been reported after "non-damaging" passive stretches and isometric actions (195; 234; 322), as well as low mechanical impact exercises, such as swimming (276) and level running (338). Hence, there appears to be important differences between men and (caged) mice in this matter.

The only way to directly assess leukocytes in exercised muscles in humans is by obtaining and analysing muscle biopsies, but concerns have been raised about the suitability of this method (28; 77; 158; 250; 251; 350; 381; 423; 437). Malm *et al.* (252) demonstrated methodological limitations with repeated biopsies (from the same muscle) by observing increased numbers of leukocytes in the repeat biopsy samples from both exercised and non-exercised, control muscles. Consequently, one can question the relevance of previous studies that have obtained repeated biopsies from the exercised muscles, but did not collect biopsies with the same frequency from the control muscles (e.g.: (66; 113; 152; 179; 201; 351)). Nevertheless, it seems reasonable to state that high-force eccentric exercise *can* lead to accumulation of leukocytes in the exercised muscles, as higher numbers of leukocytes have been observed in controlled experiments including just one biopsy time point (e.g.: (383; 389)). Repeated biopsies are, however, necessary to investigate the time course of the

[8] A bout of conventional strength training; using equal loads in both the concentric and eccentric phase of each repetition. The loads applied are usually > 60% of 1 RM (one repetition maximum).
[9] The exercise is dominated by eccentric muscle ("negative") work, but includes also concentric/isometric actions.

inflammatory reaction in exercised muscles. Other methodological challenges with the biopsy procedure are the inherently low reliability and the restrictions with respect to sampling areas (28; 216; 217; 253; 437). For instance, obtaining and analysing a biopsy from the middle part of a muscle may not reflect leukocyte accumulation in the muscle-tendon junction (7; 9).

Another issue recently addressed is the presence of neutrophilic granulocytes (neutrophils) in damaged muscle tissue. After reviewing the literature, Schneider and Tiidus (366) concluded that neutrophils are inconsistently observed after exercise – in both animal and human studies. The development of a "classical" inflammatory reaction follows certain steps, and accumulation of neutrophils is expected as an early sign (353; 365). MacIntyre *et al.* (239; 240) and Raastad *et al.* (335) used radionuclide imaging, i.e., radiolabelling of autologous leukocytes (primarily neutrophils) and scintigraphy, and documented an early accumulation of leukocytes in exercised muscles in humans. However, with this non-invasive procedure it cannot be ascertained whether the leukocytes are located inside the tissue, or in the micro-vessels. Only Fielding *et al.* (113) have reported histological observation of neutrophils among the myofibres shortly (45 minutes) after eccentric exercise[10]. In several other investigations no significant tissue accumulation of neutrophils (or leukocytes in general) was observed during the first six hours after exercise (27; 126; 152; 247; 252; 253; 311; 455), which is in contrast to mechanically overloaded muscles of rats and mice (118; 323). Increased numbers of neutrophils have been reported 24 and 48 hours after eccentric exercise in humans (247; 389). However, the detection of neutrophils can be methodologically challenging; e.g., most antibodies used in previous human studies are not adequately specific for neutrophils (366).

In contrast to neutrophils, the presence of macrophages is a more consistent finding in eccentrically exercised muscles (Appendix I). Hubal *et al.* (166) reported an increased

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[10] The presence of neutrophils was observed via histological evaluation – not by means of immunohistochemistry.

number of monocytes/macrophages already six hours after exercise. However, after extensive exercise-induced muscle damage (including necrosis), the highest numbers of mononuclear cells and macrophages have been found several (7-14) days after exercise (66; 179; 351). Note that these three latter cited studies were not fully controlled with repeated biopsies from a non-exercised muscle.

In summary: In the study of leukocyte accumulation and inflammation in human muscles exposed to exercise, there is a paucity of fully controlled studies that have obtained repeated biopsies and used types of exercise that cause extensive muscle damage (large reduction in the force-generating capacity). The biopsy procedure and radionuclide imaging (radiolabelling of leukocytes) are valuable techniques, but on their own, they both have certain limitations. Thus, methodological shortcomings have hitherto made it difficult to draw firm conclusions about the time course and magnitude of the inflammatory reaction in human skeletal muscles exposed to strenuous exercise. Moreover, the role of neutrophils in exercise-induced muscle damage is especially unclear.

In paper I and II we addressed these issues by 1) applying highly muscle-damaging exercises, 2) obtaining multiple biopsies from both the exercised muscles and the control muscles, combined with radionuclide imaging (scintigraphy), and 3) using a specific antibody against neutrophils.

1.2 Recovery of muscle function after high-force exercise

The first phase of recovery from intensive high-force exercise is normally fast (240; 333; 335), as the restoration from accumulated metabolites is completed within minutes (4; 115; 442). Moreover, some of the myofibrillar disturbances (damage) inflicted during exercise might reintegrate shortly after exercise as well (178) – possibly facilitated by HSPs (see below). However, this first phase of recovery, lasting for a few hours, may become interrupted by a phase of halted recovery, or even a secondary reduction in force-generating capacity (105; 240; 333; 335).

1.2.1 Secondary loss of force-generating capacity

In a study by McCully and Faulkner (257) the m. extensor digitorum longus in mice was subjected to 450 eccentric actions. The force-generating capacity was reduced to ~38% of baseline values ten minutes after exercise, but declined further to ~22% three days after exercise (Figure 1.1). Such a secondary loss of force-generating capacity seems to be almost a hallmark after unaccustomed mechanical loading of skeletal muscles, such as high-force eccentric exercise (106; 209; 222; 255; 257; 457) and unloading-reloading[11] (120). A biphasic recovery time course, with a secondary loss of force-generating capacity (or halted recovery) 1-2 days after exercise, has also been observed in human studies after unaccustomed eccentric exercise (Figure 1.1; (48; 240)) and exhaustive stretch-shortening cycle exercise (91; 202; 291), as well as after resistance exercise (333; 335).

The secondary reduction in force-generating capacity has been related to time-corresponding exacerbated tissue damage, seen both at the cellular and subcellular levels (105; 220; 437). Furthermore, the exacerbated damage is in turn associated with the inflammatory reaction (105; 241; 411). To illustrate, in Figure 1.1 the force-generating capacity was at its lowest

[11] Animal model: A limb is immobilised, e.g., by hindlimb suspension, before mechanically reloaded by free ambulation (118; 321).

concomitantly with massive accumulation of leukocytes (“day 3”). The damaging aspect of the leukocyte accumulation is believed to primarily involve increased generation of reactive oxygen species (ROS[12]; (44; 45; 263; 457)). If there is a causal relationship between leukocyte accumulation and delayed changes in force-generating capacity, interventions that block or reduce the inflammatory reaction should improve muscle function in this early phase of the recovery process. In line with this, Pizza *et al.* (323) reported less muscle damage and weakness, as well as faster recovery, after eccentric actions in CD18 knockout mice (a mutation that inhibits neutrophil extravasation). Intervening with antibodies against CD11b[13] (M1/70) has resulted in analogous findings in rabbits (45). Yet another approach, which is applicable to human subjects, is administration of non-steroidal anti-inflammatory drugs (NSAIDs).

[12] ROS: superoxide anion, hydrogen peroxide and the hydroxyl radical.
[13] CD11b (integrin αM) is part of the integrin $\alpha M\beta_2$ (Mac-1) together with CD18 (integrin β_2), which is important for the tissue migration of blood borne granulocytes and monocytes (82). CD11b has an iC3b binding site (complement receptor 3), which mediates phagocytosis and “respiratory burst” (massive release of oxidants).

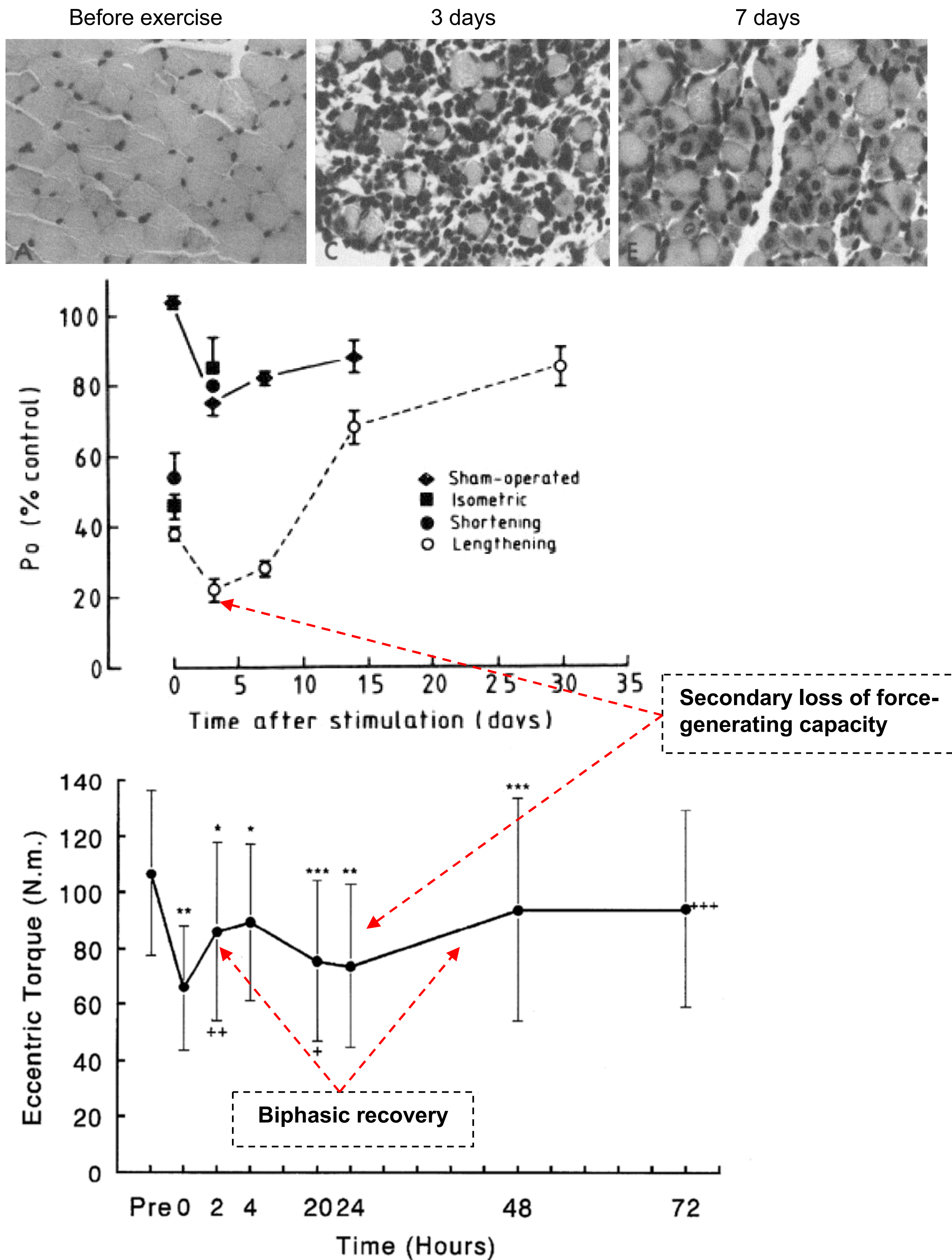

Figure 1.1. Upper panel: Histological changes (light micrographs) and changes in force-generating capacity in mice m. extensor digitorum longus after electrical stimulated lengthening actions *in situ* ("eccentric exercise"). Note the great number of mononuclear cells accumulated at "3 days"; a large proportion of the myofibres are infiltrated and probably necrotic. Lower panel: Biphasic recovery of the knee extensors after maximal eccentric exercise in humans. The acute (immediate) loss of force-generating capacity is followed by a fast recovery the next hours, but then the force-generating capacity drops again, in a secondary loss of force. Upper graph data are from McCully and Faulkner (257), whereas lower graph data are from MacIntyre *et al.* (240); both used with permission from the American Physiological Society.

1.2.2 The effects of non-steroidal anti-inflammatory drugs (NSAIDs) on muscle damage and recovery

In animal models, NSAIDs have been shown to reduce inflammation and facilitate early recovery of muscle function after exercise-induced muscle damage (208; 269). In rats, Lapointe *et al.* (209) found that administration of the NSAID diclofenac reduced the $ED1^+$ macrophage[14] accumulation and attenuated the secondary reduction in force-generating capacity. Consequently, these investigators concluded that the presence of $ED1^+$ macrophages was related to exacerbated muscle damage and the secondary reduction in force-generating capacity.

The effect of NSAIDs on recovery of muscle function has been investigated in several human studies, and in some studies enhanced recovery was found (e.g., by administration of naproxen (95; 213)). However, reviews of the literature (5; 18; 23; 65; 76) have concluded that the efficacy of NSAIDs on recovery of force-generating capacity is equivocal; in fact, the anti-inflammatory properties of NSAIDs have never been properly documented in exercise-induced inflammation in human skeletal muscles.

A new group of NSAIDs[15] was developed in the 1990s: the selective COX-2[16] inhibitors (337; 429). In contrast to the "old" NSAIDs (e.g., acetylsalicylic acid, ibuprofen and naproxen), which inhibit both COX-1 and COX-2, the selective COX-2 inhibitors (e.g., celecoxib) were supposed to efficiently dampen inflammatory reactions, but without severely inhibiting

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[14] $ED1^+$ (positive) macrophages, i.e., inflammatory macrophages (M1), infiltrate necrotic myofibres and display phagocytotic activity. In contrast to ED2 positive (anti-inflammatory) macrophages (M2) severe the regeneration process from outside the myofibres (380).

[15] The main effects of NSAIDs are mediated through reduced generation of eicosanoids, encompassing prostanoids (prostaglandins, prostacyclins and tromboxanes) and leukotrienes (LOX; (2; 38; 131; 372; 419)). Most NSAIDs inhibit the cyclooxygenase (COX)-1 and -2 (e.g., naproxen), some only COX-2 (the selective COX-inhibitors, e.g., celecoxib), whereas some inhibit both COX-1 and -2 plus lipoxygenase (LOX), e.g., ketoprofen (215; 248; 429). Prostaglandins are *inter alia* involved in pain signalling, the inflammatory reaction and fever; and NSAIDs are clinically used to dampen all these conditions (38; 337). Furthermore, NSAIDs seem to have COX/LOX-independent effects as well (2; 400).

[16] Cyclooxygenase exists in (at least) three isoforms: COX-1, COX-2, and COX-3 (390).

physiological COX-1 dependent processes, e.g., in the stomach (373)[17]. However, like the old NSAIDs (5; 6), COX-2 inhibitors have been found to impair the regeneration[18] process after muscle damage in animal models (37; 369)[19]. This negative effect seems first of all to be mediated through reduced activation of satellite cells (37; 370). Such negative effects have not been clearly shown in humans, even though Mackey *et al.* (243) found an attenuated satellite cell response after long distance running in subjects who received indomethacin (a COX-1 and 2 inhibitor) in the days before and after the run. To date the effects of selective COX-2 inhibitors have not been studied after muscle-damaging exercise in humans.

In summary: Animal studies support the notion that accumulation of leukocytes may cause muscle damage and reduce the force-generating capacity during the first days after exercise. Reduced inflammation by NSAIDs might speed up the recovery process initially, but the subsequent regeneration may eventually suffer. There is, however, no convincing evidence from human studies for a relationship between accumulation of leukocytes in the exercised muscle and changes in force-generating capacity. It is therefore unclear whether NSAIDs can accelerate early recovery through anti-inflammatory properties and whether these drugs adversely affect the later phase of regeneration. Moreover, no one has thus far investigated the effects of selective COX-2 inhibitors on recovery from exercise-induced muscle damage in humans.

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[17] Note that COX-2 inhibitors have been claimed to increase the risk of cardiovascular insults in human, and some drugs have been taken off the market (Bextra and Vioxx). Such effects for other COX-2 inhibitors, e.g., celecoxib, is not clear, but short-term use by healthy people (i.e., with no cardiovascular risk factors) appears not to be associated with significantly increased risk of dangerous adverse effects (188; 272; 376).

[18] "Regeneration" is used here as the ability of a tissue to restore damaged parts.

[19] The negative effect on regeneration has been demonstrated also in bone, tendon and ligaments, primarily in animal models. Yet, these findings are not unequivocal. In humans, less negative effects have been documented, although some signs indicate reduced bone healing (344; 427). Note that NSAIDs are widely used in connection with exercise and training and in sports medicine (414; 431).

In paper I and II we addressed these issues by exploring the time course of events by scintigraphy and repeated biopsies in both exercised and control muscle, and by administering a COX-2 inhibitor to reduce the inflammatory reaction.

1.3 Muscle soreness after high-force exercise

Hough (161) was the first researcher in modern history to describe delayed onset muscle soreness (DOMS) in detail. Since then, investigators have discussed the aetiology of DOMS that first of all seems to be a consequence of unaccustomed exercise, and especially eccentric exercise (reviews: (1; 7; 15; 54; 55; 65; 75; 76; 146; 155; 161; 164; 221; 241; 289; 331; 349; 374)). Typically, the soreness starts to develop some hours after exercise, and the soreness (tenderness) is primarily felt when moving and palpating the exercised muscles (72). The severity of soreness usually peaks 24-72 hours after exercise and subsides within one week (374).

Several mechanisms for DOMS have been suggested (1; 221; 287), including:

- Lactic acid accumulation (unlikely; (7))
- Reflex muscle activity (unlikely; (33; 287))
- Myofibrillar disruptions (not *per se*, because the intracellular environment cannot be sensed directly by nerves)
- Connective tissue strain/damage (likely; (1; 15; 78))
- Intramuscular pressure (swelling; not *per se*, but possibly in synergy with nerve sensitising stimuli; (164; 287))
- Inflammation (possibly; (76; 240; 241; 374))
- Free radicals (possibly; (75))

However, none of these mechanisms seem to explain the phenomenon adequately.

Several substances, such as adenosine, bradykinin, histamine, prostaglandins (especially PGE_2), TNFα, serotonin, substance P, glutamate, calcitonin gene related peptide (CGRP), and neuropeptide Y (NPY) can potentially activate and/or sensitise nociceptors (type III [Aδ] and IV [C] nerves) in the muscle tissue (7; 92; 174; 181; 221; 347; 401; 445). However, no single culprit has been identified. The sources of production are uncertain as well, but several cell types could be involved, including the myofibres themselves, nerve cells, fibroblasts, mast cells, endothelial cells, and inflammatory cells. Interestingly, recent experimental findings in humans experiencing DOMS indicate involvement of mechanoreceptors and changes in the central nervous system (16; 22; 396; 397; 440; 441). This could explain the fact that DOMS is a kind of allodynia, where non-noxious stimuli, such as a gentle stretch, evoke pain. Proske and Allen (327) suggested that changes in processing at the level of the spinal cord allow signals from mechanoreceptors, served by large-diameter afferents, to access the pain pathway.

Inflammation has been suggested to be the principal mechanism behind DOMS (76; 154; 241; 374), and in terms of generation of pain, a local inflammation might indeed explain both the situation in the muscle and the (subsequent) changes in the central nervous system (395). However, investigators have questioned the role of inflammation in DOMS, because it has been difficult to detect significant numbers of leukocytes in the exercised muscles at the time when subjects experience DOMS (39; 128). Furthermore, in studies where leukocyte accumulation has been observed (concomitant with myofibre necrosis) in the exercised muscle tissue, leukocyte infiltration was temporally out of phase with DOMS (287). A causal relationship between the inflammatory reaction and DOMS has also been questioned based on the inconsistent effect of anti-inflammatory drugs on DOMS (18; 65; 76; 201). On the other hand, MacIntyre *et al.* (240) found a temporal relationship between increased radioactivity, from accumulated radiolabelled leukocytes, and muscle soreness in eccentrically exercised muscles. Consequently, the authors suggested that leukocytes contribute to DOMS. Thus, the relationship between DOMS and inflammation is ambiguous.

In summary: The study of DOMS has a long history (Hough 1902), but surprisingly, we are still unable to explain the mechanism behind this phenomenon. Based on the commonness of DOMS – experienced after virtually every bout of unaccustomed exercise – it would be of great value to unravel this biological mystery. One unresolved question in the aetiology of DOMS is the role of inflammation in the exercised muscle.

In paper I and II we addressed this issue by investigating the spatial and temporal accumulation of leukocytes in relation to the development of DOMS. An additional goal in paper II was to investigate the role of the inflammation more directly by reducing the inflammation with a COX-2 inhibitor.

1.4 Heat shock protein response to exercise

Heat shock proteins (HSPs), or stress proteins, are a protein family organised after their molecular weight, e.g.: the small HSPs (15-30 kDa), HSP32, HSP40, HSP60, HSP70, HSP90 and HSP110 (231; 280; 296; 297). Some of the HSPs are found in several compartments of a cell (e.g., HSP27), whereas others are restricted to a certain compartment (e.g., HSP75/GRP75 – in the mitochondria). Furthermore, two homologous, but differentially regulated sets of genes exist: the "heat shock proteins" and the "glucose-regulated proteins" (GRP). The GRP's promoters do not contain the heat shock element (HSE), and GRPs are not induced by heat stress (231). GRPs are rather induced by exercise stress in relation to energy turnover (254).

Together the HSPs display a diversity of cellular effects and functions, not just in stressful situations, but in housekeeping chores as protein chaperones as well (49; 156; 392; 393; 425). In essence, their role is to promote correct protein folding and transport; taking care of both denatured (damaged) proteins and newly synthesised polypeptide chains (99; 100;

148). The HSPs assist *de novo* generated proteins to obtain correct folding for proper function, whereas denatured proteins need to be refolded or degraded, because unguarded denatured proteins aggregate and become a serious threat to a cell (99; 182; 295). The HSPs, especially HSP70, work in concert with the ubiquitin-proteasome system in the degradation of non-refoldable proteins (Figure 1.2). During cellular stress (of diverse types) the HSPs display cytoprotective effects, and an important defence mechanism for increasing the stress tolerance is to rapidly increase the cellular content of HSPs (231; 295). HSPs, such as HSP27 and HSP70, also display strong anti-apoptotic features (69; 177; 183). In many ways the HSPs are involved in both physiological and pathological cellular events (for reviews, see: (177; 230; 295; 413)).

Recent research has demonstrated that extracellular HSPs (endogenous [self] and exogenous; e.g., produced by bacteria) have immune-regulatory functions (11; 324). Thus, HSP70 demonstrates chaperokine activity[20] by being released into extracellular compartments and binding receptors (e.g., TLR2 and TLR4)[21] on immune cells (13). This causes intracellular signalling (including NFκB[22]), which initiates an immune response, characterised by altered immune cell activation and up-regulation of inflammatory cytokines (12; 14; 56; 191; 413; 443). Interestingly, HSPs, such as HSP70, may function as important extracellular signalling molecules during myofibre necrosis, by activating surrounding immune cells (such as dendritic cells and macrophages), which will orchestrate an inflammatory reaction for removing the remnants of the dying cells (25; 132).

The HSPs are together with certain antioxidants parts of a well conserved, ubiquitous, endogenous protective system against cellular stress (111; 231; 315). Exercise may certainly

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[20] Chaperokine describes the function of HSPs, especially HSP70, as both a chaperone and a cytokine.

[21] Toll-like receptors are part of the pattern recognition receptors that are used by immune cells/inflammatory cells to recognize broadly shared pathogens on microbes, as well as certain "danger molecules" such as HSP60 and HSP70 (12; 368).

[22] Nuclear factor kappa-light-chain-enhancer of activated B cells. NFκB has *inter alia* a governing position in controlling activity level of immune cells (203).

cause considerable cellular stress, and a single bout of exercise is sufficient to elevate the muscle levels of HSPs (Appendix II; (187; 226; 297)) and antioxidants, such as superoxide dismutase (297; 315). Fascinatingly, the HSP response can be extremely fast: increased mRNA levels of HSP70[23] have been observed during and immediately after exercise (108; 330), and increased protein levels have been found just 30 minutes after exercise in the skeletal muscles of rats (233). Both resistance and endurance training have been shown to increase the levels of both HSP27 and HSP70 in the exercised muscles (228; 229; 279; 421), and the increase of HSP70 seems to be related to the exercise intensity (135; 227).

In the field of exercise physiology, the study of HSPs started in the early 80s, but it was first in the 90s the interest for these proteins increased (231). Reichsman *et al.* (341) and Thompson and Scordilis (404) conducted the first studies on human skeletal muscle[24] that indicated a HSP response after high-force eccentric exercise (elbow flexors). Reichsman *et al.* (341) observed increases in certain protein bands suggested to be HSPs (on sodium dodecyl sulfate [SDS] gels), whereas Thompson and Scordilis found increased protein levels of ubiquitin[25]. Some years later, Puntschart *et al.* (330) and Liu *et al.* (229) found evidence for increased transcription (HSP70 mRNA) and expression of HSP70 in muscles exposed to running exercise and rowing (training). Thompson *et al.* (403; 405) observed increased expression of both HSP27 and HSP70 after high-force exercise, and the HSP response seemed to be related to increased MAPK-activity[26]. Of the MAPK pathways, p38[27] seems to be involved both in the phosphorylation and in promoting increased gene expression of the small HSPs (HSP27 and αB-crystallin; Figure 1.2).

---

[23] Note that inducible HSP70 lack introns (231).
[24] Several human studies have assessed the exercise-induced HSP response in blood borne leukocytes, hepatosplanchnic viscera and the brain (e.g.: (109; 112; 205)), but here we focus on the HSP response in skeletal muscles.
[25] Ubiquitin expression is stress sensitive, and, thus, considered a small HSP (8.5 kDa; (296)).
[26] Mitogen-activated protein (MAP) kinases; important intracellular signal transduction pathways (317).
[27] A mitogen-activated protein kinase (MAPK) responsive to several stimuli, especially “stress” stimuli such as pro-inflammatory cytokines (364).

The HSP that has received most attention is HSP70[28] (226; 231; 449). One reason for this is probably the low response threshold to different types of exercise (including both non-damaging and damaging exercise). Since exercise generally induces some kind of stress, either metabolic or mechanical, and increases protein turnover (268; 343), it seems reasonable that training leads to augmented levels of HSP70, in virtue of its cytoprotective and chaperon properties (135; 229; 246; 405). The small HSPs, particularly HSP27 and αB-crystallin, seem to be less easily up-regulated by "non-damaging" exercise than HSP70 (278; 279). However, these small HSPs may have special functions during and after high-force exercise, since increased levels have been detected in the days after such type of strenuous exercise (107; 403; 405). Koh (192) suggested that the small HSPs have a central function in stabilisation of the cytoskeleton during high-force eccentric actions. This hypothesis is based on observations indicating that the small HSPs are involved in actin dynamics (e.g., in fibroblasts; (212; 212; 280)) and desmin-related myopathies induced by mutation in the αB-crystallin gene (40; 422). Indeed, the small HSPs seem to translocate to the cytoskeleton and myofibrillar structures during ischemic heart and skeletal muscle stress (140; 451). Furthermore, the small HSPs accumulate in foci of disorganised myofibrillar structures in certain myopathies (114). Koh and Escobedo (194) confirmed the suggested function of HSP25 (HSP27 homolog) and αB-crystallin by demonstrating that the small HSPs translocated from the cytosol (soluble fraction) to the myofibrillar (insoluble) fraction in mice immediately after eccentric actions (but not after isometric actions). In addition, the small HSPs seem to be part of the remodelling process of growing skeletal muscles (169). Concerning humans, the total protein levels of HSP27 and HSP70 are reported to increase after eccentric exercise (107; 405), but the changes in the subcellular location of HSPs have scarcely been investigated after high-force exercise (424). The induction of HSPs seems complex, but some putative stimuli and signalling pathways are summarised in Figure 1.2.

[28] Several genes for HSP70 exists (HSPA1-14), and at the protein level HSC70/71/73 (Heat shock cognate [HSPA8]), HSP72 (HSPA1/2), GRP75 (Glucose regulated protein 75 [HSPA9]) and GRP78 (HSPA5) can be measured by immunological techniques in skeletal muscle (136; 143; 153; 403). Note that the "inducible" HSP72 is constitutively expressed in many cells (226).

In summary: The study of HSPs in human exercise physiology is a young field of science, and although many important studies have been conducted, the role of HSPs during and after exercise is far from understood. Especially interesting is the movement of HSPs between cellular compartments (192). Additionally, up-regulated levels of HSPs after an unaccustomed bout of high-force exercise can plausibly be part of the repeated-bout effect (as discussed below).

In paper III and IV, we addressed these issues by investigating the HSP27, αB-crystallin, and HSP70 with several immunological techniques in order to detect movement of the HSPs between cellular compartments, i.e., cytosol and the cytoskeleton and myofibrilar structures. Moreover, we aimed to study changes in mRNA and protein expression of these HSPs after maximal eccentric exercise.

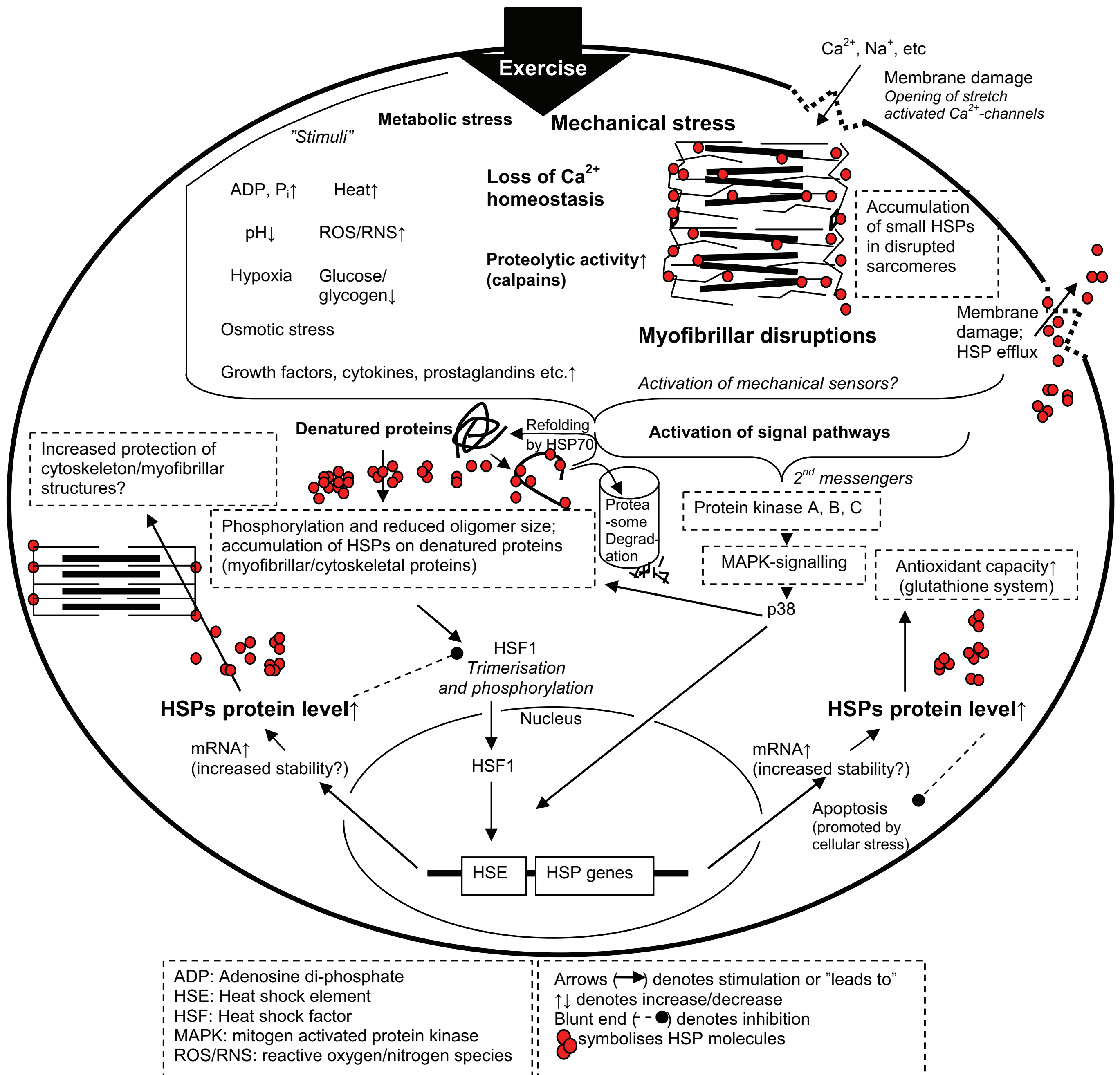

Figure 1.2. Exercise generates several stimuli for increased HSP expression. Although many pathways are shared, they are complex and not equivalent for all HSPs. However, activation of HSF1 seems to be of superior importance. Increased occupation of HSP70 (e.g., refolding activity) will promote trimerisation of HSF1 and translocation to the nucleus. MAPK-signalling through p38 appears to be involved in both phosphorylation (affecting oligomeric size and activity) and gene expression of the small HSPs (HSP27 and αB-crystallin). Increased levels and altered signalling activity in different pathways might increase the stress tolerance of the myofibres. For instance, increased levels of HSP27 and αB-crystallin can be important for protecting cytoskeletal/myofibrillar structures and HSP27 can contribute to increased antioxidant capacity through interaction with the glutathione system. Changes in the permeability of the membrane may cause diffusion of HSPs to extracellular compartments. HSP70 may also be actively released (possibly in exosomes). Cellular stress can initiate programmed cell death (apoptosis), but this process is inhibited by HSPs. The figure is based on the following references: (110; 111; 173; 177; 186; 191; 192; 200; 206; 225; 231; 285; 286; 297; 354; 425; 449).

1.5 Adaptation to unaccustomed high-force exercise

1.5.1 The repeated-bout effect

A rather amazing chapter of skeletal muscle physiology is the comprehensive adaptation seen after a single bout of unaccustomed high-force exercise. This feature has been named the "repeated-bout effect" (71), and it is manifested by attenuation of the exercise-induced loss of force-generating capacity, as well as muscle soreness and swelling after a second (repeated) bout, compared to the initial bout of exercise (300; 307). Less myofibrillar disruption and reduced levels of circulating creatine kinase after the second bout signify attenuation of muscle damage, which probably explains the increased rate of recovery (71; 160; 308; 389). Moreover, the repeated-bout effect is evident as soon as a few days after the initial bout and may last as long as nine months (249; 303; 307; 313). Several hypotheses have been put forward to explain the repeated-bout effect, but no unequivocal verifications have been established. Most likely several mechanisms are at work, comprising altered neural activation patterns of the exercised muscles (e.g., indicated by reduced EMG median frequency)[29] (162; 163; 433), as well as subcellular changes in the myofibres (259; 260). Because a repeated-bout effect has been clearly found after electrically evoked muscle actions in both animals and humans, it seems reasonable that local changes in the exercised muscles are of major importance (36; 208; 304). At the subcellular level recent studies points towards remodelling and strengthening of the myofibrillar structure, including increased numbers of sarcomeres in series (21; 52; 57; 58; 107; 214; 237; 446; 447; 452; 455). However, the involvement of such changes in the repeated-bout effect needs verification in human models. Increased numbers of sarcomeres will in theory reduce the strain stress and protect against over-stretching ("popping") of sarcomeres (3; 273-275). Signs of adaptations, such as increased expression of pro-collagen type III, collagen IV, and tenascin C, occur in the extracellular matrix as well (78; 80; 199; 242). However, the functional effects of these adaptations are unknown.

[29] Reduced median frequency could indicate increased reliance on slow motor units (433).

Two interesting putative mechanisms behind the repeated-bout effect are the inflammatory reaction and the HSP response (259).

Inflammation

McHugh (259) suggested that the inflammatory reaction could be involved in the repeated-bout effect. The rationale for this assumption was the finding by Pizza *et al.* (322) that non-damaging passive stretches and isometric actions resulted in accumulation of neutrophils in the exposed muscles, which two weeks later appeared to give protection against damage from eccentric actions. Follow-up studies (195; 234) have confirmed the findings by Pizza *et al.* (322). Although no direct evidence has been gathered, the mechanisms behind these findings might be leukocyte-derived ROS generation and ROS signalling in the myofibres (234). Thus, increased generation of ROS may be favourable under certain circumstances (encompassing minor initial muscle damage and not fully activated neutrophils), but as discussed above (in chapter 1.2.1), ROS are also capable of causing cellular damage (264). Furthermore, in a study by Lapointe *et al.* (208), rats were exposed to an eccentric muscle-damaging exercise protocol and diclofenac (an NSAID) was successfully used to reduce the post-exercise inflammation (i.e., the number of $ED1^+$ macrophages). Fourteen days later, when the rats were exposed to the exercise bout again, but without any drug administration, the rats that had received NSAIDs after the first bout clearly showed an inferior repeated-bout effect compared with placebo rats: The diclofenac rats had larger force deficits and larger accumulation of leukocytes in the muscle tissue after the repeated bout. Additionally, rats that received NSAIDs for seven days after the first bout demonstrated less adaptation than rats that received drugs for only two days[30]. Consequently, it seems like inflammation, regeneration, and adaptation processes go hand in hand.

[30] Because the muscles (ankle dorsiflexors) were activated by external electrical stimulation during the exercise protocol, the authors claimed that the major adaptations to eccentric exercise are due to changes within the muscle (not dependent on neural components) and that inflammation is part of this process.

The study design used by Lapointe *et al.* (208) has not previously been applied on human subjects, and it is suitable for testing both early and late effects of NSAIDs, as well as for investigating the mechanisms behind the repeated-bout effect. The design of the "arm experiment" in this thesis was influenced by this work (as described in the Methods).

Heat shock proteins

A single bout of unaccustomed exercise (a kind of cellular stress) can augment the HSP levels in the myofibres (231). Such "preconditioning"[31] has been shown to increase a cell's tolerance for different kinds of stress (85; 246; 256; 449). Consequently, it seems reasonable to suggest that HSPs could be part of the mechanisms behind the repeated-bout effect in humans (259).

Thompson *et al.* (402) investigated the response of HSP27 and HSP70 after two bouts of maximal eccentric exercise (elbow flexors) separated by four weeks. The repeated-bout effect was successfully manifested by attenuated exercise-induced muscle weakness and soreness. However, no change in the HSP response was found. The HSP27 and HSP70 protein content increased 48 hours after both the first and second bout. Intriguingly, Thompson *et al.* reported indications of lower basal levels of these HSPs at the second bout, which do not support HSPs as important players in the repeated-bout effect. Vissing *et al.* (424) observed faster recovery of muscle function after a second bout of eccentric exercise (bench-stepping) and a blunted translocation of HSP27 to cytoskeletal structures (eight weeks between bouts). However, neither the HSP27 nor HSP70 protein levels increased detectably after the initial bout, indicating no "preconditioning" effect. Therefore, no evidence for a role of the HSP system in the repeated-bout effect could be detected. Interestingly,

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[31] The "classical" way to induce increased expression of HSPs is by heat stress. The augmented HSP levels will give a cell increased protection when encountering the same stress again, but also towards other types of stress (i.e., "cross-tolerance"; (232)).

recent findings demonstrate that passive heating (microwave diathermy treatment) of muscles one day before unaccustomed eccentric exercise allegedly give some protection against muscle damage – as indicated by enhanced recovery of muscle function (301). Nosaka *et al.* (301) did not measure the HSP levels in this study, but suggested a possible role of HSPs in the preconditioning effect observed. Thus, it is apparent that involvement of HSPs in the repeated-bout effect is possible, but such evidence does not exist for human subjects.

In summary: The repeated-bout effect is well documented, but the aetiology is elusive. This might be due to a complex interaction between several mechanisms. Nevertheless, an exercise-induced inflammation and HSP response are two plausible mechanisms involved in the repeated-bout effect.

In paper II and IV we addressed these issues by investigating the repeated-bout effect in light of the inflammatory reaction and the HSP response.

2.0 RESEARCH AIMS

The aim of this thesis was to investigate the recovery from maximal eccentric exercise for the knee extensors (m. quadriceps) and the elbow flexor muscles (including m. biceps brachii), in order to build on previous research from our laboratories (332; 333; 335; 345). The overall goal was to better understand the underlying mechanisms of the recovery from long-lasting exercised-induced reduction in force-generating capacity, with special focus on: 1) the inflammatory reaction and regeneration processes in the exercised muscles (paper I and II); and 2) the heat shock proteins (HSPs) in the exercised muscles (paper III and IV). These systems were investigated after a single bout of unaccustomed exercise (paper I-IV) and after a repeated bout of exercise (paper II and IV).

The hypotheses concerning the inflammatory reaction were:

1. Maximal eccentric exercise causes accumulation of leukocytes in the exercised muscles
2. Neutrophils precede the tissue accumulation of macrophages
3. Accumulation of leukocytes affects the recovery of muscle function during the first days after exercise and causes the secondary loss of force
4. NSAID intake (celecoxib; a COX-2 inhibitor) enhances early recovery by attenuating the inflammatory reaction, but delays the final restoration of muscle structure and function
5. Celecoxib intake during recovery from an unaccustomed bout of maximal eccentric exercise reduces the repeated-bout effect
6. Accumulation of leukocytes and development of the inflammatory reaction cause delayed onset muscle soreness (DOMS)

The hypotheses concerning the HSP response were:

1. HSP27 and αB-crystallin translocate from the cytosol to cytoskeletal and myofibrillar structures during maximal eccentric exercise
2. Maximal eccentric exercise initiates increased expression of HSPs
3. Increased protection against muscle damage during a repeated-bout of exercise will be associated with increased content of HSP in the exercised muscles

Additionally, we investigated muscle damage at the ultrastructural level (by electron microscopy), calpain content and activity (in homogenated muscle samples), changes in the protein expression in the extracellular matrix, low-frequency fatigue and changes in EMG[32], as well as changes in the levels of cytokines and neutrophils in the circulation. Some of these data have been published (316), two papers have recently been submitted to international journals (210; 334), and some data are still in preliminary format. Some of these data are used to support findings presented in the papers herein. Figure 2.1 lists some of our research questions and hypotheses during planning of the experiments with respect to the phases of recovery from high-force exercise.

[32] Electromyography

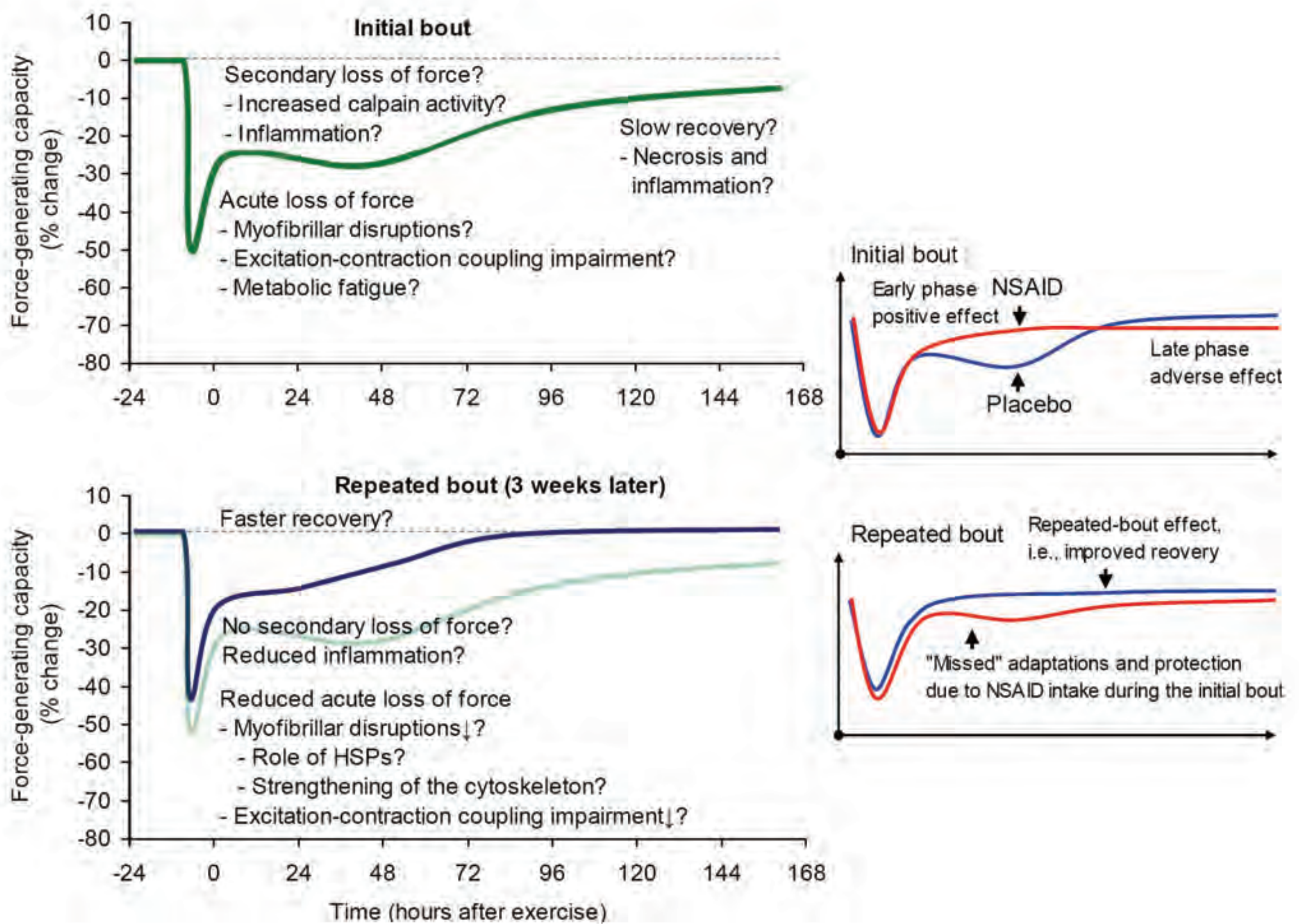

Figure 2.1. The upper graph illustrates recovery from an unaccustomed bout of high-force eccentric exercise; while the lower graph illustrates a faster recovery from a second bout (pale green denotes the initial bout). Research questions and hypothesises are listed in relevant phases of the recovery. The framed graphs illustrate the hypothesised effects of NSAIDs during the initial bout, and the consequences for the repeated-bout effect. Thus, by reducing inflammation, the early phase of recovery may be enhanced, but complete recovery may be hindered together with the adaptations necessary for the acquiring full repeated-bout effect.

3.0 METHODS

This thesis presents data from two consecutive studies. The first study, the “leg experiment”, was carried out in 2002, and the “arm experiment” started in 2005 and was completed in 2007. Analyses of muscle tissue samples are still running. The leg experiment involved monitoring of recovery from maximal eccentric exercise using the knee extensors, while the arm experiment involved the elbow flexors muscles (Figure 3.1).

3.1 Subjects

A total of forty-four healthy male (33) and female (11) students and employees at the Norwegian School of Sport Sciences (NSSS; 21-37 years of age) gave written, informed consent to participate in the experiments. Most subjects were physically active and involved in different activities, such as running and cycling, and team sports, such as basketball, handball, and soccer, but some were rather sedentary. None of the subjects were familiar with maximal eccentric exercise. No exercise was allowed during the three days prior to the experiments. The subjects were asked to continue their regular diet and not to take any form of medication (except contraceptives) or prescription-free supplements (such as anti-oxidants), as well as avoid exercise, stretching, and massage therapy (etc.) during the experimental periods. The study complied with the standards set by the Declaration of Helsinki and was approved by the Regional Ethics Committee of Southern Norway.

No subjects withdrew from the study.

Figure. 3.1. The leg and arm exercises, as well as tests of muscle function before and after exercise, were performed with a dynamometer. In the leg experiment we used a Cybex^{6000} (left image), while in the arm experiment we used a Technogym REV 9000 (right image).

3.2 Study design

Both the leg experiment (n = 11) and the arm experiment (n = 33) were carried out with pre-tests, the exercise protocol, and repeated post-tests; i.e., a pre-post design (Figure 3.2 and 3.3). Exceptions were collection of biopsies, scintigraphy, and microdialysis that were conducted only after exercise (exercised *vs*. control). Both the leg and arm exercises were performed with either the dominant or the non-dominant leg/arm (randomly chosen). The contralateral, non-exercised, leg/arm functioned as control for all tests and measurements

Changes in the force-generating capacity were followed daily for seven and nine days, after the leg and arm experiments, respectively. In both types of experiments, we monitored autologous radiolabelled leukocytes *in vivo.* This procedure started early in the morning on day 1 of the experiments, so that the radiolabelled leukocytes were re-infused before the exercise. Muscle soreness, and swelling, passive tension, as well as blood sampling were measured and collected just before tests of force-generating capacity. An exception was immediately after the exercises, when muscle function was tested as fast as possible after the last repetition of the exercise protocols. All tests of muscle function were conducted within 5-15 minutes after the exercises. Biopsies from both the exercised leg/arm and the control leg/arm were obtained ~30-60 minutes after other tests and measurements.

Figure 3.2. Leg experiment outlined with time points for tests and measurements.

		Pre exercise							Post exercise								
			Day 1									Day 2		Day 3	Day 4	Day 5	Day8
Hours		*-1*	*0*	*0.5*	*3*	*4*	*6*	*7*	*8*	*21*	*23*	*24*	*28*	*48*	*72*	*96*	*168*
Tests:	Fam. tests	**Exercise**															
Muscle function	•	•	•				•				•		•	•	•	•	•
Muscle soreness	x	x	x				x				x		x	x	x	x	x
Muscle swelling	•	•	•				•				•		•	•	•	•	•
Blood sample		x	x				x				x		x	x	x	x	x
Scintigraphy					•			•		•		•					
Biopsy				#1		#2			#3			#4				#5	#6

Figure 3.3. Arm experiment outlined with time points for tests and measurements.

BOUT 1		Pre exercise								Post exercise				*1 week*		*3 weeks*
				Day 1						Day 2	Day 3	Day 4	Day 5	Day 8	Day 10	Day 22 (**Bout 2**)
Hours		*-1*	*0*	*1*	*2*	*5*	*6*	*8*	*20*	*24*	*48*	*72*	*96*	*168*	*216*	*503*
Drug administration																
Tests:	Fam. tests	**Exercise Bout 1**														
Muscle function	•	•	•			•				•	•	•	•	•	•	•
Muscle soreness	x	x	x			x				x	x	x	x	x	x	x
Muscle swelling	•	•	•			•				•	•	•	•	•	•	•
Resting arm angle	x	x	x			x				x	x	x	x	x	x	x
Blood sample		•		•				•	•	•	•	•	•	•	•	•
Scintigraphy							x		x							
Biopsy				#1							#2		#3	#4		
Microdialysis					x					x	x					

BOUT 2	Pre exercise							Post exercise				*1 week*	
			Day 1					Day 2	Day 3	Day 4	Day 5	Day 8	Day 10
Hours	*-1*	*0*	*1*	*5*	*6*	*8*	*20*	*24*	*48*	*72*	*96*	*168*	*216*
Tests:	**Exercise Bout 2**												
Muscle function	•	•		•				•	•	•	•	•	•
Muscle soreness	x	x		x				x	x	x	x	x	x
Muscle swelling	•	•		•				•	•	•	•	•	•
Resting arm angle	x	x		x				x	x	x	x	x	x
Blood sample	•		•			•	•	•	•	•	•	•	•
Scintigraphy					x		x						
Biopsy			#5						#6				

Radiolabelled autologous leukocytes were monitored repeatedly during the 24 hours following the exercises to investigate early signs of the inflammatory reaction (Figure 3.2 and 3.3). The biopsy collection was spread over a one-week period: in the leg experiment, biopsies were collected 0.5, 4, 8 hours, and 1, 4, and 7 days after exercise, whereas in the arm experiment the biopsies were collected 1 hour and 2, 4, and 7 days after exercise (Figure 3.2 and 3.3). The purpose of this sampling frequency was to capture early events (0.5-48 hours) and later events (four and seven days) of the inflammatory reaction and the heat shock protein (HSP) response, as well as satellite cell response and regeneration processes.

In the arm experiment, we administered celecoxib (a cyclooxygenase-2 [COX-2] inhibitor) to half of the subjects in the recovery phase after exercise (further details are given below [3.2.2]). The experiment was double-blinded, i.e., the subjects received either celecoxib or visually similar placebo pills and the investigators did not know to which group each subject belonged. Additionally, the arm exercise was performed twice with the same arm (bout 1 and bout 2; Figure 3.3). The bouts were separated by three weeks. Note that the drug (celecoxib) was administered only after the first bout (not after bout 2). This study design was adapted from an animal study (208) and we aimed to investigate 1) the effect of an anti-inflammatory drug on the recovery from unaccustomed eccentric exercise, and 2) the effect of this anti-inflammatory drug on adaptations to the first bout evaluated by the recovery from a repeated bout of exercise.

To test the effect of the COX-2 inhibitor (celecoxib) in the exercised muscles, we applied microdialysis to gather tissue fluid for analysis of prostaglandins (PGE_2; see below) in a subgroup of subjects. This was done 2, 24, and 48 hours after bout 1 (Figure 3.3).

We aimed to investigate events in the exercised muscles by several methodological approaches (as mentioned above), and this implied that we had to recruit a certain number of subjects to each method, as outlined in Figure 3.4.

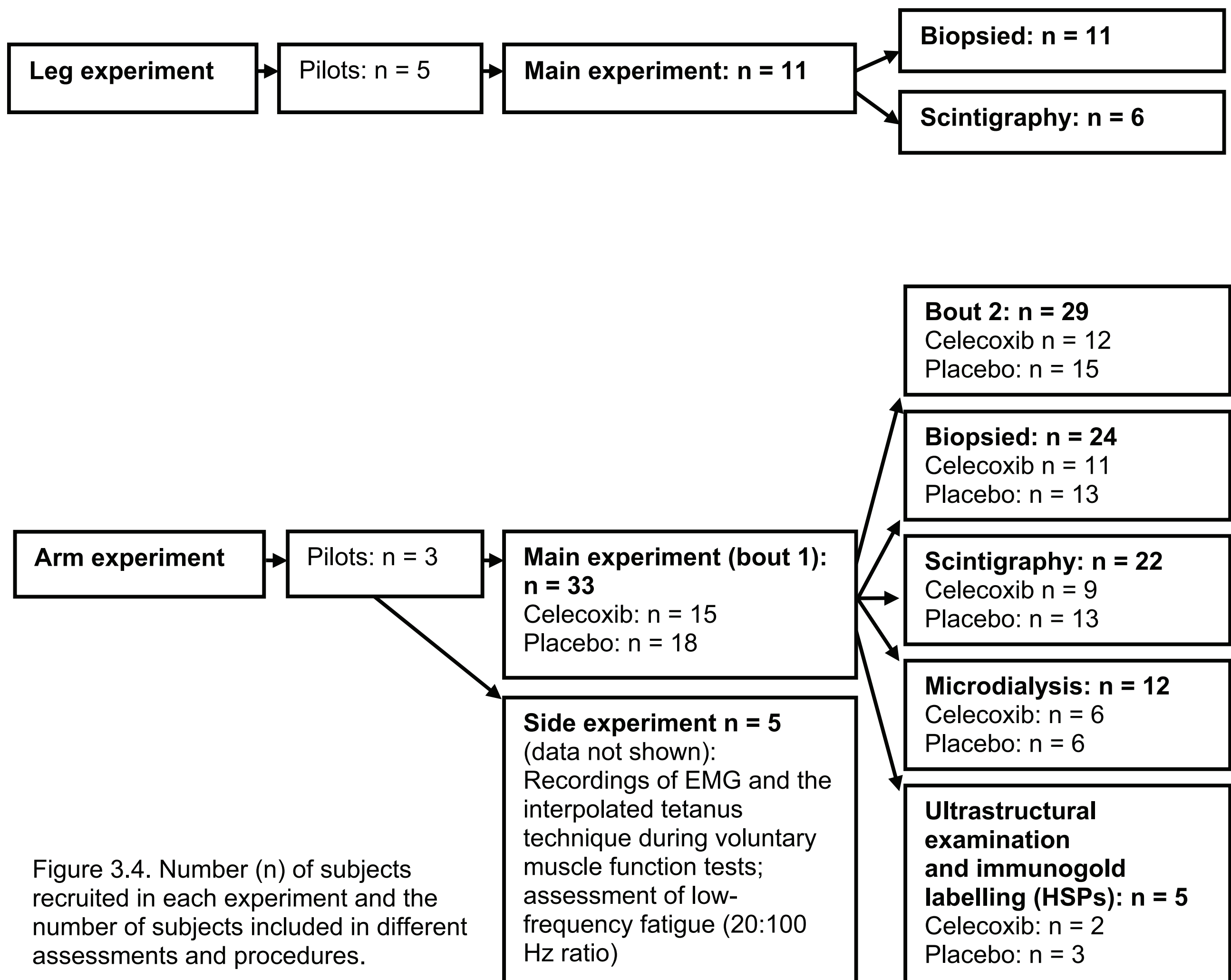

Figure 3.4. Number (n) of subjects recruited in each experiment and the number of subjects included in different assessments and procedures.

3.2.1 Choice of experimental muscles

The knee extensors are an important muscle group for human locomotion and are a well-used experimental muscle group in exercise physiology research. As previous studies by our group focused on the knee extensors (333; 335), we chose an exercise protocol for the knee extensors in the first project as well (the leg experiment). To represent the knee extensors (m. quadriceps) we obtained tissue samples from m. vastus lateralis, which is common when using the knee extensor as the experimental muscle group. However, as described in paper I, we apparently did not collect tissue samples from the part of m. quadriceps that had suffered most damage. Our motivation to use the upper arm elbow flexors in the following experiment was based on this observation. Hence, we anticipated that the chance of obtaining tissue samples from a more representative part of the exercised muscles was better in the m. biceps brachii than in m. vastus lateralis. Pilot testing confirmed this (Figure 3.4).

3.2.2 Nutrients

The subjects ate a light breakfast without coffee at home and met in the laboratory at 7.00 am on day 1 and day 2 of each experimental period (Figure 3.2. and 3.3). Subjects were provided food for the following meals during these days.

3.2.3 Drug administration (arm experiment)

The celecoxib group (Figure 3.4) was administered 400 mg (celecoxib; Celebra [Pfizer, Oslo, Norway]; 200 mg morning and evening) for nine days, with the first dose taken approximately 45 minutes before the exercise (bout 1). Maximal blood concentration of the drug is reached after 2-3 hours and the half-life of the drug is 8-12 hours (The Norwegian Pharmaceutical

Product Compendium). The placebo group received lactose pills. Drug compliance was high; only five reports of missed intake of a single pill from different subjects were received.

3.3 Exercise protocols

The leg experiment protocol was adapted from MacIntyre *et al.* (240), while the arm exercise was adapted from Stauber *et al.* (383). Both protocols were tested in pilot experiments (Figure 3.4) and the range of motion (ROM) was adjusted so that the eccentric action started at the ascending limb of the elbow angle-torque curve and ended as far as possible[33] down the descending limb. Thus, we let the muscles work eccentrically beyond the optimal length of force-generation. The force generated and the strain (degree of lengthening) have been shown to be the most important factors behind exercise-induced muscle damage (105; 124; 274).

The “work” (J), peak torque (Nm) and angle of peak torque (°) were registered during each eccentric action. The amount of work per set and total work per exercise were calculated as well. All values registered during exercise and tests (see below) were corrected for gravity and passive tension throughout the full ROM.

In both exercise protocols, we instructed the subjects to resist the movement of the dynamometer arm maximally through the full ROM in every repetition. The subjects received real time visual feedback on their performance on a computer screen and they were verbally encouraged during the exercise to ensure maximal effort.

[33] This was technically restricted by the exercise machine used in the leg experiment, whereas in the arm exercise we had to make sure that the elbow joint did not become hyperextended.

3.3.1 Leg exercise

Three-hundred (300) unilateral, voluntary maximal, isokinetic, eccentric actions ($30^{\circ}\cdot s^{-1}$) were performed using the knee extensors (m. quadriceps femoris) on a Cybex^{6000} (Lumex, Ronkonkoma, NY, USA). The subjects sat with 90˚ at the hip joint, fastened with seat belts and arms held crossed over the chest (Figure 3.1). The ROM was 35-105˚ (0˚ equals full extension in the knee joint), and the workout consisted of 30 sets of 10 repetitions with 30 seconds rest between sets.

3.3.2 Arm exercise

Seventy (70) unilateral, voluntary maximal, isokinetic, eccentric actions ($30^{\circ}\cdot s^{-1}$) were performed with the elbow flexors on a Technogym REV 9000 (Gambettola, Italy). The subjects were positioned on the chair of the REV 9000 and fastened with belts over the hip, chest, and shoulder; and a cushion supported the upper arm (Figure 3.1). Thus, the shoulder joint was kept in a slightly flexed position (30-35° from the vertical axis) and fixed during the elbow exercise. The subjects grabbed a handle connected to the lever arm of the dynamometer. The handle could be rotated about the longitudinal axis, so the subjects were instructed to supinate their forearm (elbow joint) for maximal activation of m. biceps brachii (175). The exercise protocol consisted of 14 x 5 repetitions, with 30-35 seconds rest between sets. The ROM in the elbow joint was 40-175° (180° = full extension) and the velocity was $30^{\circ}\cdot sec^{-1}$.

3.4 Monitoring changes of force-generating capacity

We included only a concentric test in the leg experiment and both concentric and isometric tests in the arm experiment, because we considered eccentric tests to be too strenuous. Eccentric tests could potentially have affected not only the recovery of the exercised leg/arm, but also that of the control leg/arm. In fact, only two maximal eccentric actions can lead to

reduced force-generating capacity for several days (308). This would have rendered the contralateral leg/arm muscles useless as a control.

Except for immediately after exercise, the test of force-generating capacity was always preceded by 3-5 minutes warm-up by cycling (100-150 W; Monark 818E, Stockholm, Sweden) or arm-cranking (30-50 W, Lode B.V., Groningen, The Netherlands), in addition to four submaximal concentric isokinetic actions on the dynamometer. All subjects participated in 1-4 familiarisation sessions on separate days before they entered the experiments. In the arm experiment, two pre-tests were conducted on the first day of the experiment, and the mean value was used the in further analyses. The intra-individual coefficient of variation (CV) for the strength tests was less than 5%.

In the leg experiment, the force-generating capacity was measured (on the Cybex^{6000}) as voluntary maximal, isokinetic, concentric knee extension peak torque at $60^{\circ}\cdot s^{-1}$ (best of three consecutive attempts). In the arm experiment, the force-generating capacity was measured (on the Technogym, REV 9000) as voluntary maximal, isokinetic, concentric elbow flexion peak torque at $60^{\circ}\cdot s^{-1}$ (best of two consecutive attempts) and as voluntary maximal, isometric peak torque at 90˚ in the elbow joint (5-second actions; best of two attempts).

During voluntary tests of concentric force-generating capacity, the work (J), peak torque (Nm) and angle of peak torque (˚) were registered in each repetition. The best attempt (with the highest peak torque) from each test was used in the further analyses. The subjects were verbally encouraged to perform maximally in each test.

3.5 Scintigraphy with radiolabeled autologous leukocytes

Leukocyte scintigraphy is a non-invasive method for detection of leukocyte accumulation *in vivo*, used clinically to search for infections and inflammatory reactions. With the procedure described below, granulocytes[34] are preferentially labelled (84; 103; 318). Neutrophilic granulocytes are usually the dominating cell type recruited to regions where acute infectious or inflammatory reactions take place (31; 353).

Fifty millilitres of blood were drawn from each of the subjects (from the cubital vein). Leukocytes were isolated by gradient centrifugation and incubated for 15 minutes with 500 MBq of ^{99m}Technetium(Tc)-hexamethylpropylene amine oxime (^{99m}Tc-HMPAO) solution. Non-cell-bound ^{99m}Tc-HMPAO and ^{99m}Tc-pertechnetate were washed off with autologous plasma. The activity injected was ~250 MBq. The half-life of the ^{99m}Tc is six hours, which limits the tracking of labelled leukocytes to about 24 hours (with a single infusion). Accumulation of radiolabelled leukocytes in the subjects' thighs and upper-arms was quantified with a gamma camera, using custom-made software (GE healthcare, Oslo, Norway) applied on anterior and lateral[35] view scintigrams (Figure 4.2). The accumulation of radioactivity within a region of interest (ROI) in the exercised muscles was related to radioactivity in the same ROI in the control muscles, corrected for background radiation. Care was taken when choosing ROI to represent the whole or parts of the muscles. In the leg experiment, we used a photograph to more easily locate the different parts of m. quadriceps (Figure 4.2).

The radiolabelling and scintigraphy was carried out at the Department of Nuclear Medicine, Rikshospitalet University Hospital (Oslo, Norway).

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[34] Since the blood concentration of neutrophilic granulocytes is much higher than eosinophils and basophils (40-75% *vs.* < 7%; (312)), and because granulocytes outnumber lymphocytes in the leukocyte fraction separated from whole blood, it is primarily neutrophils that are labelled.
[35] Obtained only in the leg experiment.

3.6 Biopsies

Biopsies were always collected from both the exercised muscle and the contralateral, non-exercised (control) muscle. The intention with of obtaining samples from a non-exercised muscle was to control for the potential muscle damaging effect of repeated biopsies *per se* (252; 381; 423), as well as the possible influence of other tests and measurements on the biopsy analyses. In both the leg and the arm experiments, we performed repeated biopsies with the intention of exploring the time courses of cellular events related to the recovery process. To obtain a reasonable time resolution to test our hypotheses, we decided on six biopsy time points in both types of experiments. However, we restricted the number of biopsies of each muscle (exercise and control) to four (leg experiment) and three (arm experiment). This meant that we had to distribute the subjects on the different biopsy time points (Table 3.1). We restricted the number of biopsies to reduce the stress on the subjects and to reduce the risk of "contamination" of damaged tissue from previous biopsies. Pilot studies indicated that three and four biopsies from m. biceps brachii and m. vastus lateralis, respectively, were feasible.

Two subjects in the leg experiment and four subjects in the arm experiment did not go through all the scheduled biopsies due to technical circumstances or subject refusal due to discomfort from the first biopsies.

Table 3.1. Overview of the number of biopsies obtained at each time point after the leg and arm experiments (from both the exercised muscles and the control muscles)

	Bout 1								Bout 2	
Hours after exercise	*0.5*	*1*	*4*	*8*	*24*	*48*	*96*	*168*	*1*	*48*
Leg experiment	7	-	7	7	7	-	7	7	-	-
Arm experiment	-	9	-	-	-	16	10	10	9	11

3.6.1 Procedure

A 5 or 6 mm needle (Pelomi, Albertslund, Denmark) with manual suction was used to obtain tissue samples (1-3 x 30-200 mg) from the mid-section of m. vastus lateralis and m. biceps brachii. Subjects lay supine and the procedure was performed under local anaesthesia (Xylocain® adrenaline, 10 $mg{\cdot}ml^{-1}$ + 5 $\mu g{\cdot}ml^{-1}$, AstraZeneca, Södertälje, Sweden). Each needle insertion was placed approximately 3 cm proximal to the previous insertion in m. vastus lateralis and 1-2 cm medially and laterally in m. biceps brachii. The muscle samples were rinsed in isotonic physiological saline (0.9%) before visible fat and connective tissue were removed. Thereafter, the samples were divided in pieces for subsequent analyses:

1. Immunohistochemistry (light microscopy): 10-40 mg of sample was quickly frozen in isopentane on dry ice and stored at -80°C.
2. Immunoassys (western blotting and ELISA): 50-200 mg of sample was quickly frozen in isopentane on dry ice and stored at -80°C.
3. Immunocytochemistry (whole fibre preparations) and mRNA analyses: 10-30 mg of sample was immersed in RNA*later*® (cat. no. AM7020, Ambion; Applied Biosystems, Austin, Texas, USA) and stored at -20°C (not frozen).
4. Ultrastructural examination (electron microscopy) and immunogold labelling: 10-20 mg of sample was immersed in a 4% paraformaldehyde and 0.1% glutaraldehyde solution and stored at 4°C.

3.6.2 Immunohistochemistry on cross-sections

Serial cross (transverse) sections (5-10 μm) were cut with a cryostat microtome (Leica CM3050, Nussloch GmbH, Germany) at -22°C, and mounted on Superfrost Plus microscope glass slides (Menzel-Gläser, Braunschweig, Germany), air-dried and stored at -80ºC until further analysis. All samples from each subject were arranged in duplicates or triplicates on

one or two slides, and samples from the exercised muscle and the control muscle from each sampling time point were always placed on the same slide.

Optimisation for each antibody staining involved: 1) adjustments of the dilution of the antibodies, 2) adjustments of the incubation times and temperature, 3) the use of fixatives, e.g., Histofix (Histolab, Göteborg, Sweden), 4) adjustments of the blocking solution (buffer solution and serum), and 5) adjustments of the washing buffers (salt concentrations and detergents). Sections without primary antibodies were used as negative controls. The sections were stained with a panel of primary antibodies given in Table 3.2 and appropriate secondary antibodies. The staining procedure followed a traditional immunohistochemical staining procedure (details are given in each paper):

Images of the stained cross-sections were captured using an Axiocam camera (Zeiss, Oberkochen, Germany) mounted on Axioskop-2 light microscope (Zeiss, Oberkochen, Germany). Multiple images – using 20x, 40x and 100x objectives – were taken to capture the cross-sections of each sample.

3.6.3 Immunocytochemistry on whole fibre preparations

Segments of whole fibre preparations (single fibres) were analysed to investigate the staining pattern of HSP27 and αB-crystallin (Table 3.2). Fibres were teased while submerged in RNA*later* using fine tiped foreceps under dissecting microscope. Subsequent antibody staining followed principally the same steps as staining on cross-sections (paper IV). A challenge with whole fibre preparations was to achieve good penetration of the antibodies throughout the fibres. This was best achieved by using fresh tissue and overnight permeabilisation (50mM glycine, 0.04% saponin, 0.25% BSA in 0,01M PBS; pH 7.4-7.6).

Stained whole fibre segments were examined using both light (as described above) and confocal microscopy. The confocal laser-scanning microscope (TCS-SP, Leica, Mannheim, Germany) was equipped with an Ar^+/Kr^+ ion laser.

These analyses were conducted at NSSS and Department of Physiology, University of Oslo, Norway.

3.6.4 Postembedding immunogold

The tissue samples for electron microscopy were prepared following a standard method for post-embedding (32; 418). The sections were also analysed for ultrastructural changes (presented in Lauritzen *et al.* (210)). Methods were optimised for immunogold labelling of HSP27, αB-crystallin, and desmin (Table 3.2; Paper IV). The number of gold particles were counted and the surface area of each subcellular compartment were measured using analySIS Pro (Soft Imaging System, Münster, Germany).

These analyses were conducted at Department of Anatomy, University of Oslo, Norway.

Table 3.2. Primary antibodies applied.

Primary antibodies	Target protein/cell(s)	Immunoassay and dilution	Catalog no. and manufacturer	Paper/ Figure no.
αB-crystallin (monoclonal)	Small HSP	IHC/WB: 1:2000 ICC: 1:500	SPA-222, Stressgen Bioreagents	III, IV 4.14
αB-crystallin (polyclonal [rabbit])	Small HSP	IG: 1:20	Sc-22744, Santa Cruz Biotechnology	IV
Alexa Fluor 488 phalloidin	Actin	ICC: 1:50	Invitrogen	IV
CD16 (monoclonal)	Neutrophils, monocytes/macrophages/ Natural killer cells	IHC: 1:300	M7006, DAKO	I 4.3
CD66b (monoclonal)	Neutrophils (granulocytes)	IHC: 1:500	M1546, PeliCluster	II 4.5, 4.6
CD68 (monoclonal)	Monocytes/macrophages	IHC: 1:300	M0718, DAKO	I, II 4.3, 4.4, 4.5, 4.11
Collagen IV (monoclonal)	Basal membrane/ extracellular matrix	IHC: 1:500	M3F7, Hybirdoma Bank	4.9
Desmin (monoclonal)	Myofibrillar, intermediate protein	IHC: 1:100 WB: 1:500	M0724, DAKO	IV 4.8, 4.9, 4.16
Desmin (polyclonal [goat])	Myofibrillar, intermediate protein	ICC: 1:100 IG: 1:200	Sc-7559, Santa Cruz Biotechnology	IV
Dystrophin (polyclonal [rabbit])	Cytoskeleton at the sarcolemma	IHC: 1:2000	Ab15277, Abcam	II, IV 4.5, 4.8, 4.9
GAPDH (monoclonal)	Enzyme in glycolysis	WB: 1:5000	Ab9484, Abcam	III, IV 3.5
HSP27 (monoclonal)	-	IHC/ICC: 1:300 E (coating): 25 ng per well WB: 1:4000	SPA-800, Stressgen Bioreagents	III, IV 4.14, 4.15, 4.16
HSP27 (polyclonal [rabbit])	-	IHC/ICC: 1:300 E: 1:10000 (detection) IG: 1:20	SPA-803, Stressgen Bioreagents	III, IV
HSP70 (monoclonal)	-	IHC: 1:300 WB: 1:4000	SPA-810, Stressgen Bioreagents	III, IV 4.17
Ki67 (polyclonal [rabbit])	Cell proliferation marker	IHC: 1:200	CP249A, Biocare Medical	II
Laminin (polyclonal [rabbit])	Basal membrane	IHC: 1:1000	Z0097, DAKO	II 4.4, 4.5, 4.11
NCAM (CD56; monoclonal)	Satellite cells	IHC: 1:200	Ab9018, Abcam	II 4.11
SC71 (monoclonal)	Fibre type II (a + x)	IHC: 1:500	S. Schiaffino, University of Padova, Italy	IV

E: ELISA; GAPDH: Glyceraldehyde 3-phosphate dehydrogenase; HSP: Heat shock protein; IHC: Immunohistochemistry; ICC: Immunocytochemistry (on whole fibre preparations); IG: Immunogold; NCAM: Neural cell adhesion molecule; WB: western blotting

3.6.5 Protein quantification

In the leg experiment, muscle samples (50 mg) were homogenised after a modified method of Guerriero *et al.* (145). After centrifugation, we obtained a soluble fraction, i.e., a cytosolic fraction and a pellet fraction. The pellet was resuspended in Laemmli sample buffer in order to obtain a “myofibrillar fraction”.

In the arm experiment, muscle samples (50 mg) were homogenised with a subcellular extraction kit as described by the supplier (ProteoExtract® Subcellular Proteome Extraction Kit, Merckbiosciences, cat. no. 539790, Darmstadt, Germany). The extraction kit gave four different fractions containing proteins from cytosol, membranes, nuclei, and the cytoskeleton. GAPDH and desmin (Table 3.2) were used to test the specificity of the cytosolic and cytoskeletal fraction, respectively (Figure 3.5).

Protein content was determined using the RC/DC Protein Assay kit I manufactured by Bio-Rad (cat. no. 500-0121, Hercules, CA, USA) and bovine γ-globulin as standard protein, ranging from 0.125 to 1.5 $mg \cdot ml^{-1}$. Protein standard curve and samples were analysed in triplicates; standard curve: $r^2 > 0.9$.

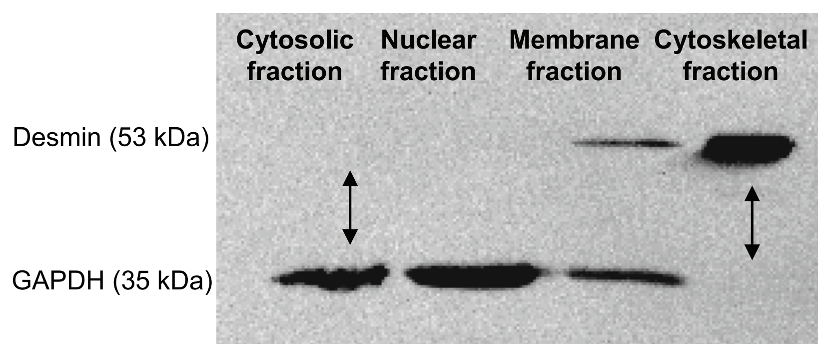

Figure 3.5. Immunoblot of desmin (intermediate filament) and GAPDH (enzyme in glycolysis) for testing the purity of the fractions obtained from the extraction kit. Desmin was found in the cytoskeletal fraction, but not in the cytosolic fraction. Oppositely, GAPDH was found in the cytosolic fraction, but not in the cytoskeletal fraction. Note that only preliminary analyses have been conducted on the nuclear and membrane fractions (paper IV), and the purity of these fractions is currently under investigation.

3.6.6 Western blotting

In order to compare protein levels between exercised and control samples equal amounts of protein were loaded and separated in SDS-PAGE gels (~15 µg per well). All samples from each time point, i.e., the cytosolic and cytoskeleton/myofibrillar fractions from both exercised and control muscles, were routinely loaded on the same gel. Blotted gels were stained with Coomassie Blue and membranes were stained with PonceauS to evaluate loading and transfer conditions. A protein ladder and recombinant HSP27 and HSP70 were used to confirm that the detected bands marked the protein of interest.

In the leg experiment, we applied the Mini-Protean® 3 Electrophoresis Cell and the Trans-Blott® Cell apparatuses from Bio-rad, while in the arm experiment we used the XCell *SureLocke*® Mini Cell apparatus and the XCell II™ Blot Module from Invitrogen. Appropriate components and buffers from each manufacturer were used. Self-made SDS-PAGE gels (4% stacking and 10% separating gels) were used together with the electrophoresis system from Bio-rad, while pre-cast NuPAGE Novex 4-12% Bis-Tris Gels were used together with the electrophoresis system from Invitrogen.

The western blotting procedure followed a traditional procedure (details are given in paper III and IV).

Digital images of the membranes were captured using a Kodak image station 2000R. The bands were analysed by selecting regions of interest (ROI; 1D Image Analysis software 3.6). ROI rectangles were positioned over the bands and values of net intensity were obtained. Net intensity is the sum of background-substracted pixel values within the ROI. The content of each analysed protein in samples from the exercised muscle was expressed in percentage of the corresponding individual control sample (from the same sampling time point). Regarding the HSPs (HSP27, αB-crystallin, and HSP70), we did not consistently obtain

bands in the cytoskeletal fraction, because the HSP levels were often under the detection limit, especially in the control samples. Consequently, we could not express ROI values from the exercised muscle samples in percentage of control cytoskeleton values (in contrast to the ELISA analyses, see below). Therefore, we expressed the cytoskeleton ROI values from both exercised and control muscle samples in percentage of control cytosol values. The control cytoskeleton values (if any) were then subtracted from the cytoskeleton values from the exercised muscle samples (i.e., we normalised for control cytoskeleton).

3.6.7 ELISA

HSP27 was analysed in the cytosolic fraction in both the leg and arm experiments, whereas the cytoskeletal fraction was only analysed in the arm experiment. The applied double antibody sandwich ELISA has been developed at NSSS by Ingrid Ugelstad (see details in paper III and IV).

HSP70 was analysed in cytosolic fraction in the leg experiment. A commercial ELISA was used according to the suppliers' protocol (EKS-700; Stressgen Bioreagents; paper III).

3.7 Microdialysis

The microdialysis procedure was applied in the arm experiment only, i.e., in m. biceps brachii of both the exercised arm and the control arm. The main aim of this procedure was to measure prostaglandins E_2 (PGE_2) in order to assess the effect of the drug (celecoxib) administration.

Two types of microdialysis probes were used in this study:

1. "Low cut-off": CMA-60 catheters (CMA/Microdialysis AB Solna, Sweden) with molecular mass cut-off at 20 kDa (length: 30 mm)

2. “High cut-off “: Custom-made microdialysis catheters with a molecular mass cut-off at 3000 kDa (length: 30 mm), constructed and sterilised as previous described (207)

After the microdialysis catheters were positioned in alignment with the muscle fibres of m. biceps brachii, the subjects rested for at least 90 minutes before starting the experiment to ensure that any reaction from the insertion trauma had minimised (207). Both types of microdialysis catheters were perfused with Ringer acetate solution containing a radioactive tracer (for recovery calculations) at a rate of 2 $\mu l \cdot min^{-1}$ for four hours. Dialysate samples were collected every 30 minutes. The first sample (0-30 minutes) was discharged also to avoid any influence from the insertion procedure. The samples were immediately frozen and stored at -80° C.

The dialysate from the CMA-60 was analysed for PGE_2, while the dialysate from the high cut-off catheters has so far been analysed for cytokines (data not shown).

3.7.1 The prostaglandin E_2 (PGE_2) analysis

PGE_2 concentration in the dialysate was analysed by a commercial competitive enzyme immunoassay (ACE™, cat. no. 514010, Cayman Chemicals Inc.). Standards and samples were analysed according to the manufacturer’s kit protocol. Samples were analysed in duplicate. Optical density (OD) was measured in a plate-reader at 405 nm (ASYS Hitech, Eugendorf, Austria).

3.8 Muscle swelling

Thigh and arm thickness were assessed by a tape measuring device with a spring mechanism applying constant force (Roche, Oslo, Norway). The thigh circumference was measured distally, just above patella and proximally, i.e., about 5 cm proximal to the most proximal biopsy (data not shown). The upper arm circumference was measured at the

thickest part of the lower half of the m. biceps brachii (and the m. brachialis underneath); approximately 5 cm above lateral epicondyl of the elbow joint. This site was chosen so that the biopsy insertions could be placed above (proximally). A waterproof pen was used to mark the sites for repeated measurements of both the exercised and control thigh/arm. The intra-individual CV for this measurement was < 1%.

3.9 Muscle soreness

Muscle soreness was rated on a visual analogue scale (VAS) where 0 represented “not sore at all” and 100 mm “extremely sore”. First, the subjects stretched and contracted their exercised muscles. Next, the muscles were relaxed and soreness was assessed during palpating (applying pressure) on predetermined areas on the muscles (see details for the leg and arm in paper I and II, respectively). These areas were marked with a waterproof pen for repeated measures. The soreness in the exercised muscles was always compared to the contralateral non-exercised, control muscles.

3.10 Resting elbow angle

Subjects stood upright, arms relaxed. The angle of the elbow joint was then measured using a goniometer. The axis of rotation on the goniometer was aligned with the flexion-extension axis of the elbow joint (indicated by the lateral epicondyle). The arms of the goniometer were aligned with the lower and upper arm, using the processus styloideus (forearm) and acromion as reference points. The intra-individual CV for this measurement was < 2%.

3.11 Blood sampling and analyses

Blood was drawn from an antecubital vein into a 10 ml serum vacutainer tube. After coagulating for 30-45 minutes at room temperature, the blood was centrifuged at 2700 *g* for 10 minutes at 4°C. Serum was then immediately pipetted into Eppendorf tubes and stored at

-80°C until analysis. In the leg experiment (paper I), creatine kinase (CK) was analysed with a Hitachi 917 automated biochemistry analyser (Roche®, Basel, Switzerland; analytic CV being < 2.8%). In the arm experiment, CK (paper II) was analysed with a Hitachi Modular P automated biochemistry analyser (Roche®, Tokyo, Japan; analytic CV being < 5 %), while myoglobin (paper IV) was analysed with a Hitachi Modular E automated biochemistry analyser (analytic CV being < 6 %).

All these analyses were conducted at Department of Medical Biochemistry, Rikshospitalet University Hospital (Oslo, Norway).

3.12 Statistics

Of the variables monitored, we had three types of data sets:

1. Muscle function, swelling and passive tension (resting elbow angle), as well as muscle soreness were assessed before and repeatedly after exercise for both the exercised leg/arm and the control (pre-post, exercised leg/arm *vs.* control).
2. Blood sampling were conducted before and repeatedly after the exercises (pre-post).
3. Scintigraphy, microdialysis and biopsies were only collected after exercise, but from both the exercised muscle and the control muscle (post, exercised leg/arm *vs.* control).

Changes in muscle function showed overall normal distribution (Gaussian distribution), whereas the other variables demonstrated a skewed distribution at certain time points, especially serum CK and myoglobin. However, when using the individual area under the curve (AUC) or peak/nadir values the data demonstrated frequently a normal distribution. Therefore, test for normality dictated the use of parametric or non-parametric statistical testes; alternatively, data with skewed distribution was log-transformed and tested with parametric tests (Table 3.3).

Because different subjects were biopsied at each sampling time point, the paired Student's t-test or the Wilcoxon signed rank test was applied to evaluate histological differences between the exercised muscle and the control muscle at each time point. The unpaired Student's t-test and the Mann-Whitney test were used to analyse histological differences between groups, i.e., celecoxib *vs*. placebo and "high" *vs.* "moderate" responders (Table 3.3; see 4.1 regarding high and moderate responders).

We used either Pearson product-moment correlation coefficient test or Spearman rank correlation test to assess numeric relationships between variables.

Data are presented as means with standard error of the mean (SEM), if not otherwise stated in the text. Two-sided p-values ≤ 0.05 were considered statistically significant.

In case of missed tests and lost/destroyed samples, values were interpolated or extrapolated if acquired, e.g., for performing statistical tests such as repeated measures ANOVA. These calculations were strictly based on individual time course trends, typically using the mean value of tests performed before and after the missed time point.

Statistics were performed with Microsoft® Excel 2003 (including statistiXL 1.8) InStat® 3.06, Prism® 5.01 and Statemate™ 2.0 (GraphPad Software Inc., San Diego, CA, USA) and SPSS 15.0 (SPSS Inc., Chicago, IL, USA).

Table 3.3. Overview of variables and statistical tests applied to analyse <u>changes over time (upper rows)</u> and <u>between groups</u>, i.e., celecoxib *vs.* placebo and high *vs.* moderate responder subjects; <u>lower rows</u>). “x” denotes that the test was applied. For variables with data from both the exercised leg/arm and the control leg/arm, we tested the exercised and control leg/arm values separately, as well as the individual difference between exercised and control values (delta [δ]-values).

		Testing changes over time				
Variable	Data set	1-way ANOVA repeated measures with Dunnett's and Tuckey's post hoc tests	The Friedman's tests for repeated measures and Dunn's post hoc test	2-way ANOVA (time x leg/arm/bout) with Bonferroni post hoc test	Peak/nadir, single time points paired T-tests	Peak/nadir, single time points Wilcoxon
Muscle function	Pre-post; ex *vs.* con	Ex and con leg/arm tested separately; ex-con (δ-value)		x	x	
Muscle soreness, swelling; resting arm angle	Pre-post; ex *vs.* con		Ex and con leg/arm tested separately; ex-con (δ-value)		x	x
CK and myoglobin	Pre-post		x			x
Scintigraphy; analyses of biopsies and microdialysate	Post		x		x	x

		Testing differences between groups					
Variable	Data set	AUC/ Peak/nadir values unpaired T-test	AUC / Peak/nadir values Mann-Whitney	GLM MANOVA	2-way ANOVA (time x group) with Bonferroni post hoc test	Single time points unpaired T-tests	Single time points Mann-Whitney
Muscle function	Pre-post; ex *vs.* con	Ex (gr.1) *vs.* ex (gr.2); Ex-con (gr. 1) *vs.* ex-con (gr.2; δ-values)		Ex (gr.1) *vs.* ex (gr.2); Ex-con (gr. 1) *vs.* ex-con (gr.2; δ-values)	Ex (gr.1) *vs.* ex (gr.2); Ex-con (gr. 1) *vs.* ex-con (gr.2; δ-values)	x	
Muscle soreness, swelling; resting arm angle	Pre-post; ex *vs.* con	Ex (gr.1) *vs.* ex (gr.2); Ex-con (gr. 1) *vs.* ex-con (gr.2; δ-values)	Ex (gr.1) *vs.* ex (gr.2); Ex-con (gr. 1) *vs.* ex-con (gr.2; δ-values)			x	x
CK and myoglobin	Pre-post		x				x
Scintigraphy; analyses of biopsies and microdialysate	Post	X	x			x	x

ANOVA: analysis of variance; AUC: area under the curve; Con: control; Ex: exercised; GLM: general linear model; Gr: group (e.g., celecoxib or placebo); MANOVA: Multivariate analysis of variance

4.0 RESULTS and DISCUSSION

In general, our findings from the leg and arm experiments were not only internally consistent, but also complementary with respect to exercise-induced changes in inflammatory markers (papers I and II) and the heat shock protein (HSP) response (papers III and IV). In both types of experiments, some subjects evidently responded more strongly than others. These “high-responder” subjects were defined as having ≥ 50% reduction in force-generating capacity, a recovery phase lasting more than one week, and a delayed peak increase of serum creatine kinase (CK; 3-7 days after exercise)[36]. The rationale behind such criteria is the association with necrosis of myofibres (9; 74; 179). In both the leg and arm experiments about ⅓ of the subjects (leg experiment: 3/11, arm experiment: 13/33) were identified as high responders. The other subjects were identified as “moderate responders”[37]. Physiologically, large individual variations might be an important aspect of the response to high-force exercise in humans, and the issue has recently been addressed by others (63; 98; 167; 292; 356; 357).

Figure 2.1 summarises (page 33) our research questions and Figure 4.1 gives a brief overview of our findings and conclusions. Notably, we recorded no effects of the COX-2 inhibitor celecoxib, except reduced muscle soreness (paper II). As a consequence, some of our research questions aiming at mechanistic explanations could not be addressed.

[36] This often implied an individual biphasic time course for changes in serum CK (paper I and II): i.e., the serum CK levels increased in the first 6-8 hours after exercise, levelled off, and then increased again after 24-48 hours.

[37] Classification of the subjects was not completely straightforward, because some subjects were borderline high responders, while others could perhaps be characterised as “low responders”. However, dividing the subjects in three subgroups would have reduced the statistical power and made comparisons more complex.

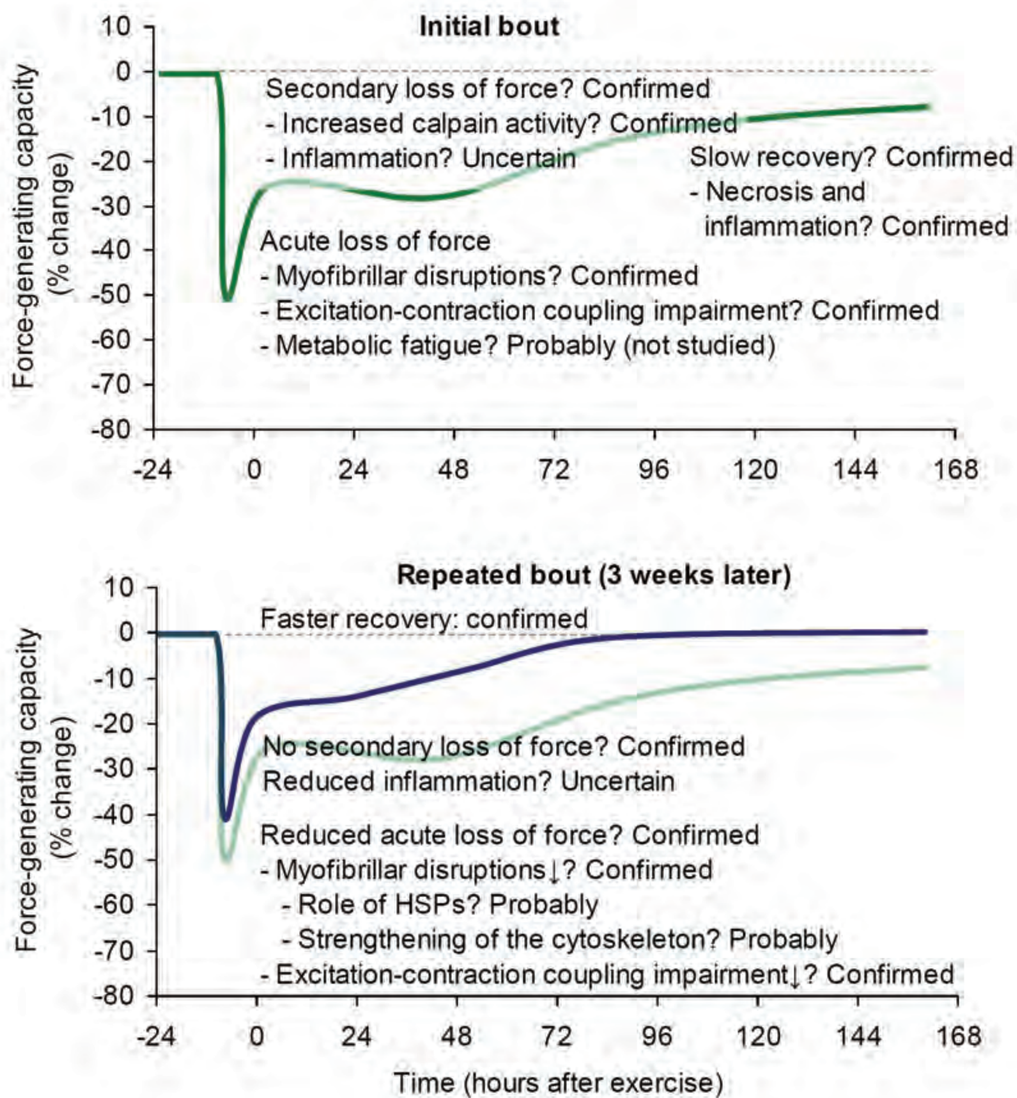

Figure 4.1. The upper graph *illustrates* recovery from an unaccustomed bout of high-force eccentric exercise; while the lower graph *illustrates* a faster recovery from a second bout (pale green denotes the initial bout). Some of the data supporting the conclusions given are presented by Raastad *et al.* (334) and Lauritzen *et al.* (210), while others are previously unpublished. Indications of excitation-contraction coupling impairment were deduced from reductions in 20:50 or 20:100 Hz ratio (i.e., low frequency fatigue; data not shown).

4.1 Leukocytes accumulate in eccentrically exercised muscles

Prior to conducting our experiments, leukocyte accumulation in muscles exposed to maximal eccentric exercise and high-force resistance exercise had been demonstrated by radionuclide imaging (scintigraphy) in three studies (239; 240; 335). Malm *et al.* (252) published a study concurrently claiming that histological signs of the inflammatory reaction after eccentric exercise were more a consequence of obtaining repeated biopsies from the same muscle than the exercise *per se*. Therefore, we included both methods in our study design in order to compare non-invasive tracking of radiolabelled leukocytes with histological analyses of tissue samples taken from both the exercised muscle and the contralateral, non-exercised muscle. For the leg experiment we adapted the exercise protocol used by MacIntyre *et al.* (240) with the intention of producing more muscle damage than what was reported by Malm *et al.* (252) after eccentric cycling exercise[38].

Our results from the leg experiment showing that accumulation of leukocytes in human exercised muscles does occur, confirmed some previous findings (26; 66; 152; 179; 389) and contradicted others (122; 130; 252; 253; 455). Both radionuclide imaging and immunohistochemical analyses of muscle samples demonstrated leukocyte accumulation after 300 voluntary maximal eccentric actions (Figure 4.2 and 4.3). The arm experiment (70 eccentric actions using the elbow flexors) verified the findings from the leg experiment (Figure 4.4 and 4.7). Nevertheless, we did encounter the issue addressed by Malm *et al.* (250; 252), namely, that the biopsy sampling itself seemed to increase the number of leukocytes in some of the samples from the control muscles (Figure 4.2 and 4.5). Not surprisingly, this was most evident in the arm experiment where the size of the m. biceps brachii necessitates quite close proximity of the repeat biopsy sites.

[38] Adapted from Fridén *et al.* (130).

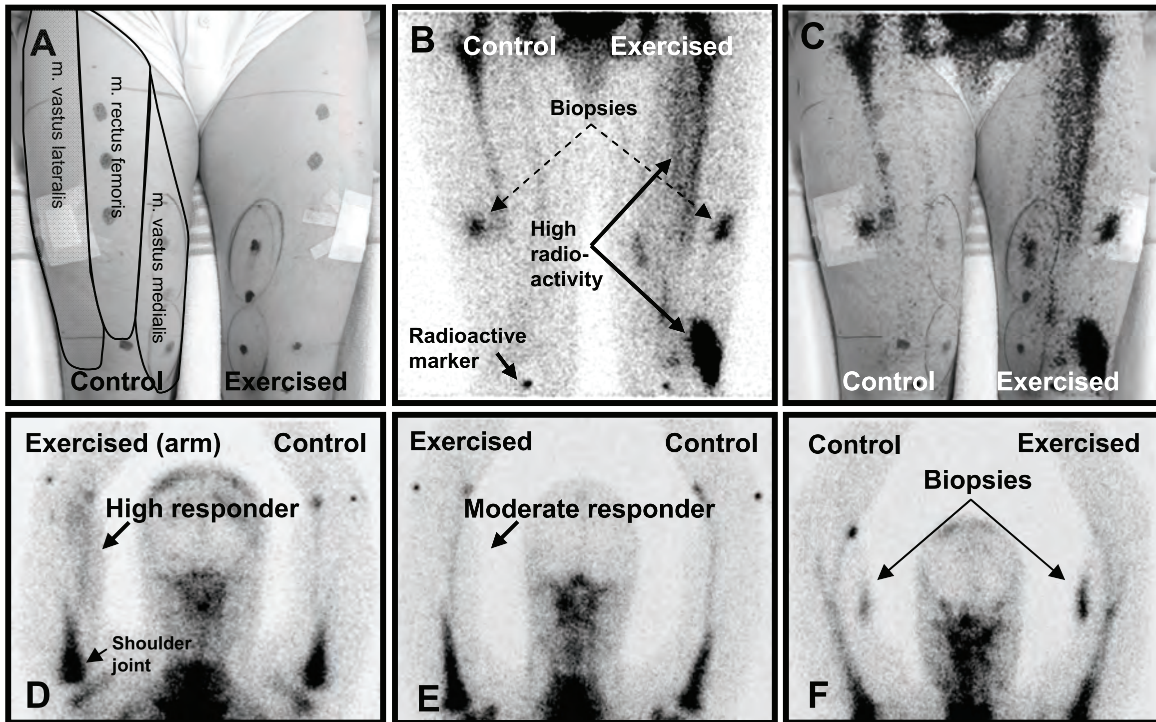

Figure 4.2. Scintigrams (radionuclide imaging) of legs and arms. The upper row of images shows how scintigrams were superpositioned on photographs (**A-C**). With this approach we could relate the radioactivity (black pixels) to reference points for each part of m. quadriceps marked on the subjects' thigh with a waterproof pen (**A**). Image **B** and **D** show a high-responder subjects with high radioactivity, i.e., large accumulation of radiolabelled leukocytes in the exercised muscles. Image **B**: This subject had especially large leukocyte accumulation in m. rectus femoris and the muscle-tendon junction (arrows). Note the difference between **D** and **E** (a high- *vs.* a moderate-responder subject). Image **B** and **F** display accumulation of leukocytes due to the biopsy procedure.

Although the biopsy procedure may have influenced our findings to some degree, two important observations suggest that the number of leukocytes found histologically in the exercised muscles were primarily due to the exercise. First, the severe loss of force-generating capacity (~ 50%) and high serum levels of CK (Figure 4.3, 4.7 and 4.13; (316; 334)) indicate that the exercise protocols caused considerable muscle damage. According to the literature (Appendix I), a study design that encompasses a large reduction in muscle function (~ 50%) combined with tissue sampling 2-14 days after exercise seems to offer the greatest likelihood for detecting increased numbers of leukocytes in exercised muscles. Our methodological approach fulfilled these criteria. Second, we observed clear associations between changes in variables for inflammation, muscle damage markers, and muscle function at the individual level (correlations). Temporal relationships between changes in the monitored variables were also found, and became even more apparent when subjects were identified as high or moderate responders. In high responders, large numbers of histologically detected leukocytes were *preceded* by a considerable accumulation of radiolabelled leukocytes and severe muscle weakness (Figure 4.3 and 4.13). Moreover, the histologically detected leukocytes were observed *concomitantly* with slow recovery of force-generating capacity, severe muscle swelling[39] and high serum levels of CK (Figure 4.7). Additionally, several signs of morphological changes at both the cellular and subcellular levels preceded the time point of peak leukocyte accumulation (Figure 4.8 and 4.10; discussed further below). Conversely, the muscle function of the control leg/arm did not change during the experiments (Figure 4.2), and the increase in serum CK levels due to multiple biopsies *per se* has been found to be very modest (252). Thus, it seems highly unlikely that these biologically reasonable relationships between variables, in terms of both amplitude and timing, would be seen if the muscle damage was not primarily exercise-induced.

[39] The increased circumference of the upper arm indicated swelling of the elbow flexors. Swelling of m. biceps brachii was also apparent when palpating this muscle, as the hardness of the muscle belly was considerably increased compared to control. Furthermore, in some high-responder subjects, several swollen myofibres could be seen in the tissue samples. These findings agree with observations of others (81; 127; 128). Muscle swelling is a consistent marker for exercise-induced muscle damage (73; 164; 300), although the aetiology is not fully understood.

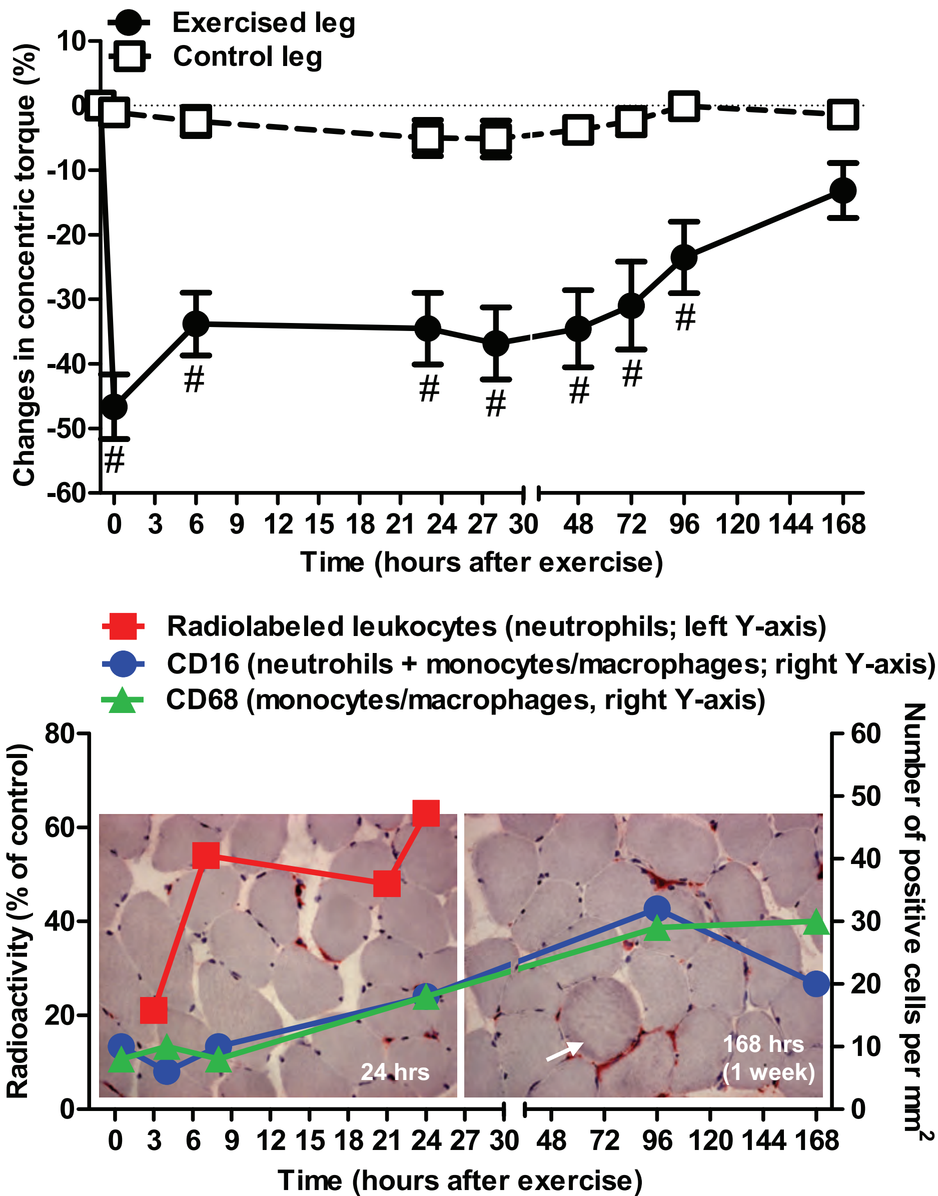

Figure 4.3. Changes in muscle function shown in temporal relation with leukocyte accumulation in the exercised muscles. Upper graph: Changes in maximal concentric torque for the exercised leg and the control leg. Error bars are ± SEM. # denotes difference from baseline and control ($p < 0.05$). Lower graph: Left Y-axis: Accumulation of radiolabelled leukocytes (primarily neutrophils). Right Y-axis: Numbers of CD16 and CD68 positive cells on cross-sections from an exercised muscle. The light micrographs of cross-sections display red staining of CD68 positive cells; the blue stain shows nuclei (hematoxylin). Note that the myofibres appear to have separated on the left image (24 hours) and the rounded and large (swollen) fibre on the right image (168 hours; arrow). Error bars are omitted for readability. The accumulation of leukocytes was higher in exercised muscles than control at all time points ($p < 0.05$). Data are from the leg experiment.

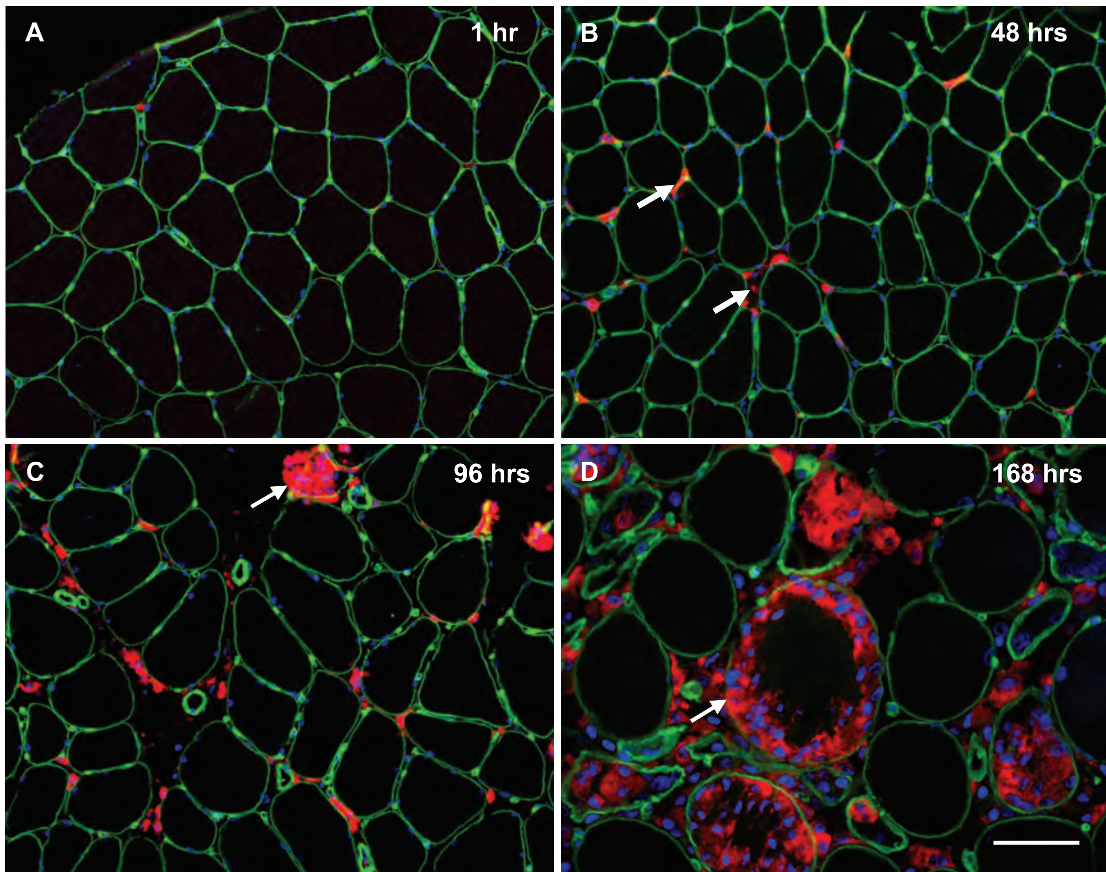

Figure 4.4. Fluorescent micrographs of cross-sections from exercised muscles stained for CD68 (red), laminin (green) and nuclei (DAPI; blue). **A**: Infrequent CD68 positive cells were observed between myofibres one hour after exercise (similar to control [not shown]). **B**: Increased numbers of CD68 positive cells were seen between myofibres (arrows, 48 hours). **C**: CD68 positive cells were observed to have infiltrated some myofibres (arrow; 96 hours). **D**: Apparently necrotic myofibres were enlarged (swollen), compared to adjacent myofibres, and frequently infiltrated by CD68 positive cells. The basal membrane appeared mostly intact (see Figure 4.9), but the laminin staining was occasionally weakened or lost (arrow; 168 hours). Note that the extent of damage shown in image **D** was merely seen in a few subjects. Data from the arm experiment (images from two subjects). Scale bar = 100 µm.

4.1.1 Methodological concerns

Large individual variations are generally a procedural disadvantage because of reduced statistical power. On the other hand, large individual variations may reflect interesting *bona fide* physiological principles that become evident when applying correlation analyses. For instance, increased blood CK levels are commonly understood to indicate muscle damage (membrane damage; (98; 379)), despite the notoriously poor correlations found between CK levels and other markers for exercise-induced muscle damage (98; 125; 196; 299; 437). However, in our experiments, when the CK levels exceeded 1000, and even 10000 $IU \cdot L^{-1}$, the muscle function was consistently and severely affected, and leukocytes were found among and within the myofibres. Correlation analyses of individual values of CK and loss of force-generating capacity four days after exercise gave $r \geq 0.7$ ($n = 11$ [leg exp.]; $n = 33$ [arm exp.]; $p < 0.01$). This relationship probably reflects the impact of myofibre necrosis on recovery of muscle function, as delayed increase in CK (4-7 days after exercise) probably signifies dying (segments of) myofibres ((9); discussed below).

Conclusion

Unaccustomed eccentric exercise can result in accumulation of leukocytes in exercised muscles. Large individual variations were seen, but the accumulation of leukocytes was associated with other markers of muscle damage, indicating that an inflammatory reaction occurs when the exercise-induced damage is severe. The discrepancy reported in the literature (Appendix I) may primarily be due to differences in exercise protocols. Since changes in muscle function were not recorded in many of the studies that have investigated the presence of leukocytes, the degree of muscle damage is uncertain and comparison between studies becomes difficult.

4.1.2 Accumulation of macrophages, but not neutrophils

In the leg experiment, antibodies against CD16 and CD68 were used. Initially, the idea was that the CD16 (Fc gamma receptor) would preferentially enumerate neutrophilic granulocytes, as well as some monocytes that were expected to enter the tissue shortly after exercise (102; 241; 339; 353; 458). Surprisingly, we found about the same number of CD16 positive cells as CD68 positive cells at all time points (Figure 4.3). CD68 is a commonly used marker for macrophages (but is also found on monocytes; (157)), and our CD68 antibody does not recognise neutrophils[40]. Moreover, histologically, there was only a moderate increase in leukocytes in the biopsies taken in the first 24 hours after exercise (compared with later time points). This finding did not fully correspond with the accumulation of radiolabelled leukocytes (Figure 4.3), which are primarily neutrophils (84; 318). Our interpretation of these data was that a significant proportion of the "stormtroopers" (neutrophils) accumulated in the microcirculation of the exercised muscle, but did not transmigrate through the vessel wall in high numbers.

That the number of mononuclear cells is increased in eccentrically exercised muscles is documented in both animal and human studies ((102; 105); Appendix I). Mononuclear cells could *inter alia* be myoblasts and fibroblasts (385; 386), but macrophages seem to be predominant – as assessed by immunohistochemistry (a procedure not without its own methodological concerns[41]; (26; 27; 319; 351; 389)). We did not apply a combination of antibodies that would have allowed us to differentiate between monocytes that transmigrated and transformed to macrophages and resident macrophages (histiocytes). Based on the very high number of monocytes/macrophages seen in some cases (Figure 4.4), it is likely that transmigration of monocytes occurred in our experiments. In contrast to mononuclear cells

[40] We tested our CD68 antibody (Table 3.2) on blood smears after isolation of leukocytes (41-43) and found that the CD68 antibody marked neither cells positive for CD66b (marker of neutrophils; discussed below) nor cells with large, circular nuclei (lymphocytes). The CD68 antibodies bound cells that were on average larger than the CD66b positive cells (neutrophils) and they displayed bean-like nuclei, indicating that the CD68 positive cells were monocytes.

[41] Antibodies against CD68 have primarily been applied for the detection of macrophages, however, CD68 antibodies might also recognise fibroblasts (204).

and macrophages, the presence of neutrophils (polymorphonuclear cells) is an equivocal finding after exercise and muscle damage in both animal and human studies (366). A methodological shortcoming in many studies appears to be the type of detection procedures applied, including type of antibodies used (366). In the human studies that claim to have detected neutrophils (e.g.: (27; 113; 247; 389)), no definite conclusions could in truth be drawn, either because specific antibodies were not used, or because the specificity of the applied antibodies was not tested[42] (366).

In the arm experiment, we wanted to further investigate the presence of neutrophils by applying an antibody for CD66b. CD66b is well recognised as a specific marker for neutrophils (granulocytes; (34; 283; 284)) and our own specificity tests confirmed this[43]. The CD66b antigen had not previously been applied to human muscle tissue exposed to exercise. CD66b positive cells were occasionally observed, but no indications of increased CD66b staining due to exercise were found (Figure 4.6). As in the leg experiment, the histological findings in the samples from the exercised muscles did not confirm the finding of accumulation of radiolabelled leukocytes (mostly neutrophils). It should be emphasised, however, that the histological detection of neutrophils could prove to be technically difficult: they may actually be present, but we cannot see them with our methodological approach.

[42] As an example, myeloperoxidase (MPO) has been used in many studies to detect neutrophils (e.g.: (247; 389)), but MPO is found in monocytes and macrophages (366). Moreover, our own experiments showed that MPO antibodies might enumerate monocytes/macrophages.
[43] This was confirmed on blood smear experiments.

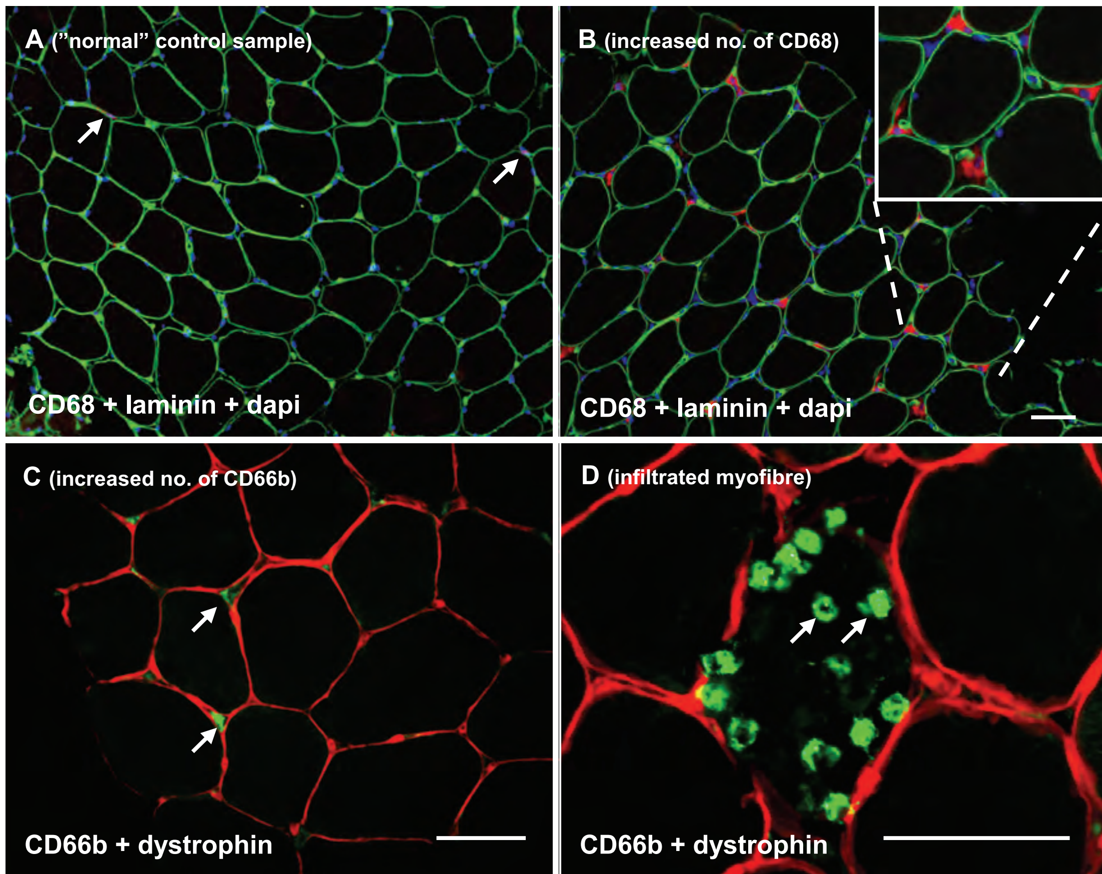

Figure 4.5. Fluorescent micrographs of cross-sections of samples from control muscles. **A** and **B**: Cross-sections of samples from control muscles stained for CD68 (red), laminin (green) and nuclei (DAPI; blue). **C** and **D**: Staining for CD66b (green) and dystrophin (red). **A**: Normal control sample with infrequent CD68 staining (arrows). **B**, **C**, and **D**: Unexpected high number of CD68 and CD66b positive cells, most likely due to previous biopsies; i.e., a repeated-biopsy effect. Image **D** shows a myofibre that is negative for dystrophin and infiltrated by neutrophils (CD66b). Note that CD66b staining was usually observed very infrequent on samples from exercised muscles (not different from control). Data from the arm experiment (images from two subjects). Scale bar = 50 µm.

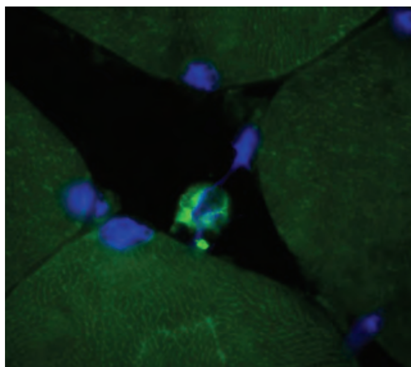

Figure 4.6. Fluorescent micrograph of a cross-section of a sample from a exercised muscle stained for CD66b (green) and nuclei (DAPI; blue). The CD66b antigen is found on neutrophils; here located in interstitial space. Note that such staining was also found in control samples (Figure 4.5). Scale bar = 25 µm.

Accumulation of radiolabelled leukocytes was clearly detected locally at the biopsy sites (Figure 4.2). In fact, the highest numbers of CD66b positive cells detected histologically were in a control sample, which indicates a response to the previous biopsy (Figure 4.5). The biopsy needle disrupts capillaries and causes some minor bleeding, allowing serum components to come into direct contact with intracellular components from damaged myofibres. This can potentially generate a sufficiently strong chemoattractive gradient for neutrophils to accumulate (353).

The apparent accumulation of neutrophils in the microcirculation of the exercised muscles indicates the generation of a chemoattractant gradient and activation of endothelial cells during or shortly after exercise. However, these changes were evidently insufficient to cause a significant transmigration of leukocytes during the first hours after exercise. Large accumulation of leukocytes in the microcirculation has been observed after closed-tissue damage (360; 363), ischemia-reperfusion (83), as well as eccentric exercise (152). But, as with our findings, these cells might not necessarily transmigrate in detectable numbers (83).

Methodological concerns

The accumulation of the radiolabelled leukocytes was frequently non-uniformly distributed in the exercised muscles, and the highest concentrations were often found in areas where no tissue samples were taken, e.g., m. rectus femoris and the muscular-tendon junction in the leg experiment (Figure 4.2). In the arm experiment, however, we apparently biopsied areas with high radioactivity (Figure 4.2), but did not histologically observe high numbers of neutrophils.

Of plausible importance, the number of neutrophils in control tissue was much lower than the number of macrophages (i.e., CD68 positive cells). Thus, expressing the accumulation of radiolabelled leukocytes (neutrophils) as a percentage of control could easily give the impression of a large relative increase, due to the very low absolute values.

Conclusion

Unaccustomed maximal eccentric exercise resulted in accumulation of neutrophils in the microcirculation. However, no accumulation around or inside the myofibres of the exercised muscles was detected. The predominant leukocyte type observed histologically was the macrophage. Our data agree with some animal studies (123; 197), but not with those that have reported early neutrophil accumulation (322; 323). Our findings are generally in agreement with previous human studies concerning the accumulation of macrophages. The role of neutrophils is scantily investigated, however, and warrants further investigation targeting the CD66b antigen.

4.1.3 Leukocyte accumulation in exercised muscles: primarily caused by myofibre necrosis?

Both animal and human studies indicate that the accumulation of macrophages after exercise-induced muscle damage is closely related to the amount of necrotic myofibres, as macrophages serve both the phagocytosis (66; 123; 179; 197; 198; 262; 265; 380; 384) and regeneration processes (50; 89; 262; 380; 407; 409). In animal studies, investigators have found that early signs of membrane disruption (reduced dystrophin staining), degradation of structural proteins (such as desmin), myofibrillar disturbances, and myofibre swelling were followed by necrosis and inflammation, and, ultimately, regeneration (e.g.: (123; 197; 235; 265)). Such findings are quite similar to our observations in the high-responder subjects, though slower in development[44]. Of the relatively few human studies that have captured exercise-induced muscle damage at the cellular and subcellular levels, most descriptions (e.g.: (66; 79; 122; 126; 154; 179; 290; 351; 383; 385; 430)) agree with our observations (outlined below). However, a rather unique aspect of our combined data from the leg and arm

[44] As expected, when comparing humans to small experimental animals (mice, rats, and rabbits) with high metabolic rate and short lifetimes.

experiments is the gathering of histological data from several stages of damage, remodelling, inflammation, and regeneration in eccentrically exercised human muscles.

Observations from immunohistochemical analyses

Using light (fluorescent) microscopy, only a modest increase in the number of interstitially located cells, such as leukocytes and myogenic precursor cells/myoblasts (CD56 positive cells)[45], was found during the first 48 hours after both the leg and arm exercises (Figure 4.3, 4.4 and 4.11). However, in some samples from the early time points, fibres tended to slide apart from one another (Figure 4.3), indicating changes in the extracellular matrix (383; 386). Furthermore, the dystrophin[46] staining was occasionally reduced, though seldom completely absent at this stage (Figure 4.7). Moderate increases in myoglobin and CK in the circulation confirmed increased permeability of the myofibre membranes during the first two days after exercise.

[45] Herein satellite cells are used as CD56 positive cells located between the plasma membrane and the basal membrane of the myofibres, whereas “myoblasts” are CD56 positive cells outside (in the extracellular space) or inside myofibres (during the regeneration; (88)). CD56 (neural cell adhesion molecule [NCAM]/Leu7) is found on Natural Killer cells (NK cells) as well, but we found no indications for the presence of this leukocyte type in the muscle tissue – in line with observations of others (351).
[46] Dystrophin is part of the sarcolemmal dystrophin-glycoprotein complex. This rod-shaped protein is essential as a membrane scaffold and connects the cytoskeleton to the extracellular matrix (via the costamere structure). Diseases affecting dystrophin, called “dystrophies”, are associated with membrane disruption and necrosis, which might cause a continuous degradation-regeneration process (70; 270).

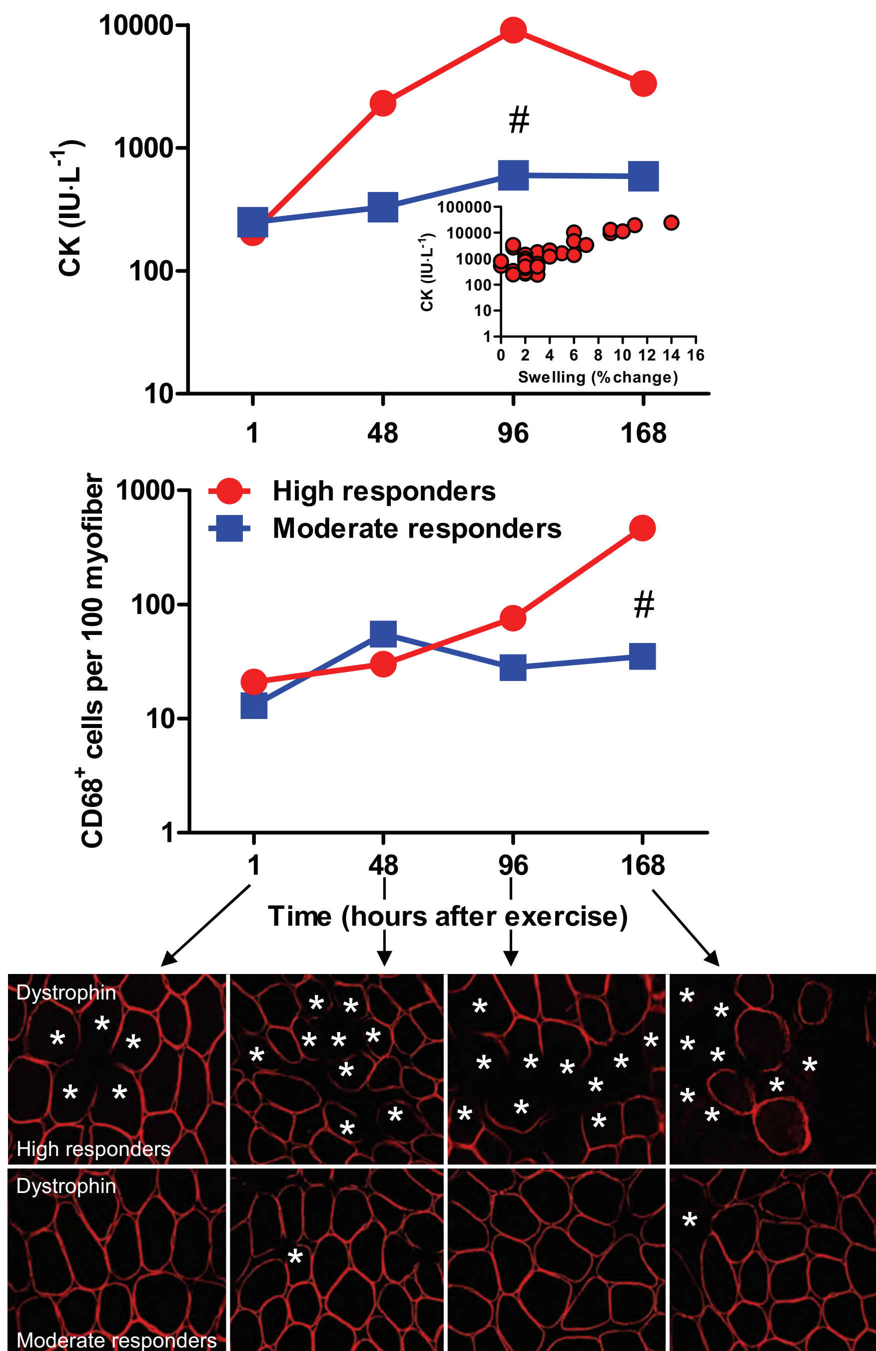

Figure 4.7. Temporal relations between changes in serum CK, accumulation of CD68 positive cells (macrophages) and changes in the scaffold structures of the sarcolemma (dystrophin). Upper graph: Serum CK levels in high- (red) and moderate- (blue) responder subjects. The inserted graph shows the relationship between swelling (increased upper arm circumference) and CK levels at 96 hours ($n = 33$; $r = 0.9$; $p < 0.01$). Lower graph: Number of CD68 positive cells in high and moderate responders. Images: Fluorescent micrographs of cross-sections stained for dystrophin; examples from high and moderate responders.
* marks myofibres with reduced or lost dystrophin staining. Note that the high responders displayed (apparently) necrotic myofibres, i.e., completely dystrophin negative fibres, which was observed concomitantly with the highest numbers of CD68 positive cells and the highest levels of serum CK. Error bars are omitted for readability. # denotes significant difference between groups ($p < 0.05$). Data from the arm experiment (images from four subjects).

Altered desmin[47] staining (observed in cross-sections) was evident inside the myofibres already one hour after the arm exercise (Figure 4.8). Especially in high responder subjects, the desmin staining intensity was generally increased at all time points, compared with control; although the intensity of staining varied within each myofibre and between adjacent fibres[48]. Yu *et al.* (455) reported a similar staining pattern. The increased staining intensity observed one and 48 hours after exercise was probably due to the myofibrillar disruptions that occurred during exercise and increased proteolysis of cytoskeletal structures following exercise (220; 223), which altogether increased the epitope availability for the desmin antibodies[49]. Indeed, we measured increased activity of calpains (~2-3 fold of control) from 0.5 hours to four days after the leg exercise (334)[50]. Similarly, immunoblotting in the arm experiment indicated increased levels of μ-calpain (calpain I) in the cytoskeletal/myofibrillar fraction of homogenated tissue samples (data not shown). Calpains are Ca^{2+}-dependent proteolytic enzymes necessary for protein turnover in the cytoskeletal structures, and both desmin and dystrophin are substrates for calpains (30; 141; 142). The increased staining intensity of desmin four and seven days after exercise implied increased expression, as confirmed by immunoblotting (paper IV). However, at these later time points, certain myofibres in samples from high responders were negative for dystrophin and displayed reduced desmin staining intensity ("desmin negative fibres"; Figure 4.8 and 4.9). Interestingly, in the same samples, we observed what looked like necrotic segments of myofibres (desmin and dystrophin negative) alongside fibres undergoing remodelling and, possibly, adaptation. With respect to remodelling, Yu *et al.* (453; 455) observed an altered staining pattern of

[47] Desmin is an intermediate filament protein, along with synemin and nestin, connecting adjacent myofibrils together at the Z-disks. Desmin is also part of the costameres and the cytoskeleton that stabilises organelles such as the mitochondria and nuclei. Desmin seems to be imperative in myofibrillogenesis. Diseases affecting desmin cause misalignment of myofibrils, abnormal mitochondria shape and position, as well as protein aggregations and necrosis (70).
[48] Staining on whole fibre preparations revealed areas of disruption in the Z-disks staining pattern (data not shown), as reported by others (122; 456). Note that these areas seemed to be stained by the small HSPs as well (Figure 4.13).
[49] We cannot exclude the possibility that the increased staining intensity was due to circumstances such as myofibre swelling, which could potentially increase the epitope availability for the antibodies, irrespective of changes in the proteolytic activity. On the other hand, swelling of the myofibre might in fact be due to increased proteolytic activity (128).
[50] Calpain activity in homogenated tissue samples was determined using a colorimetric assay as described previously by Raj *et al.* (338).

desmin on cross and longitudinal sections, and suggested that their findings indicated a central role of this protein in the remodelling and sarcomerogenesis after eccentric exercise in humans.

The apparently necrotic myofibres observed four and seven days after exercise were often rounded and swollen, and were preferentially type II fibres[51] – a finding in line with observations of others (179). Some, but not all, of these myofibres were heavily infiltrated by CD16 and CD68 positive cells (supposedly macrophages; Figure 4.4). Although the dystrophin staining was absent, staining for laminin, and collagens IV and VI demonstrated that the basal lamina was usually intact (Figure 4.9). These observations, together with the HSP staining on whole fibre preparations[52] (Figure 4.14), indicate that only segments of the myofibres were necrotic (not whole fibres; as observed in animal models (176)).

The late occurrence of myofibre necrosis probably explains the large, delayed increase of CK and myoglobin in the circulation of high-responder subjects, as soluble intracellular proteins are emptied into extracellular compartments (9; 98). The data seem to support numeric and temporal associations between loss of dystrophin, increased serum CK levels, muscle swelling, and leukocyte accumulation (Figure 4.7).

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[51] As revealed by staining for type II myosin heavy chain (SC71, Table 3.2). A confounding factor in identifying these apparently necrotic fibres was that the myosin seemed to be undergoing degradation (irregular staining).

[52] The staining of HSP27 and αB-crystallin on whole fibre preparations was non-uniform along the length of fibres obtained within 48 hours after exercise. Thus, segments with intensive HSPs staining might have visualised severely damaged segments that were probably doomed to become necrotic 4-7 days after exercise.

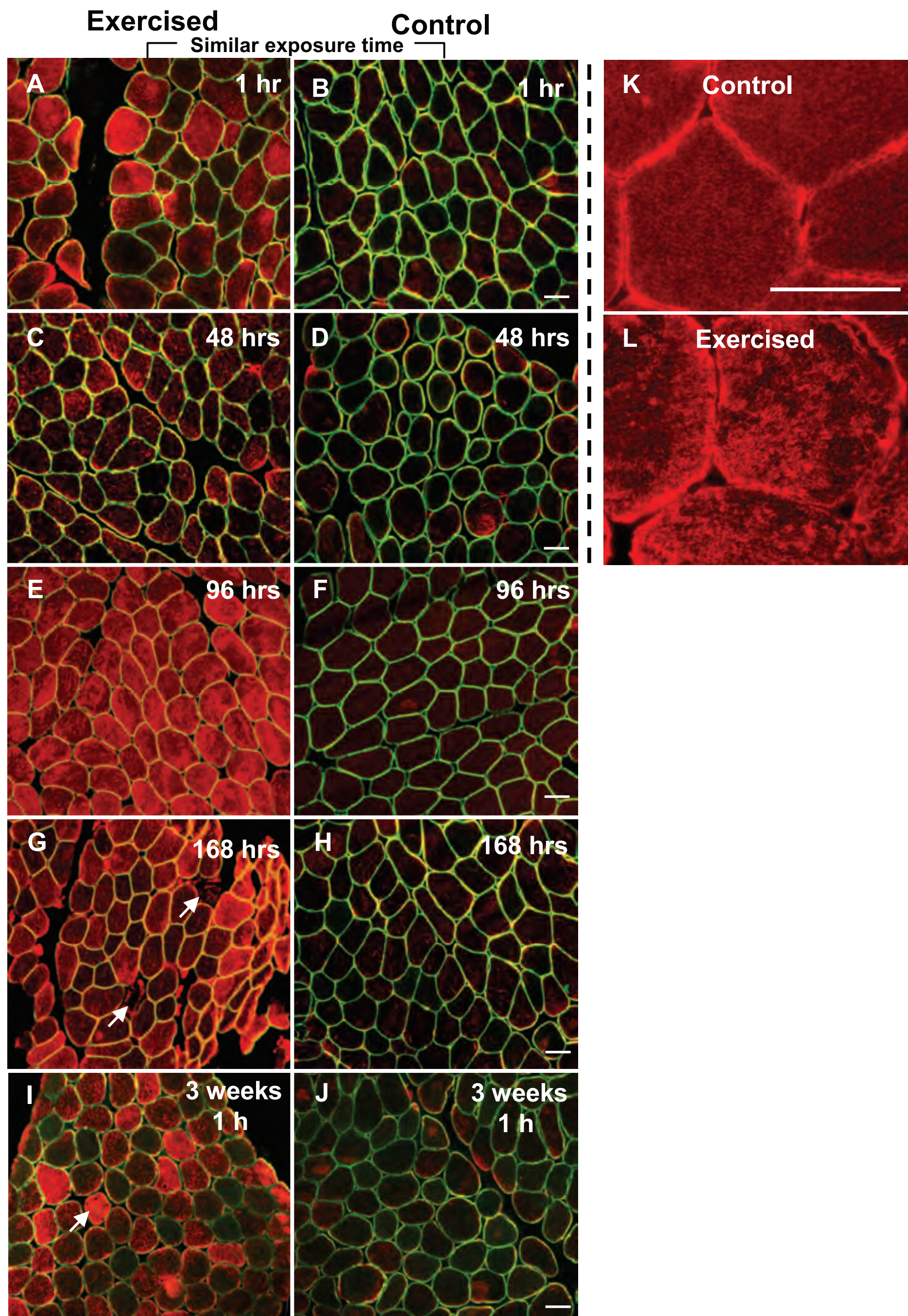

Figure 4.8. Fluorescent micrographs of cross-sections stained for desmin (red) and dystrophin (green; yellow = red + green). The staining intensity for desmin is here increased on all time points, compared to control, but the staining intensity was often uneven within each myofibre and non-uniform between fibres (**A**, **C**, **E**, **G**, **I**, and **L**). This staining pattern was most prominent in high-responder subjects. Dystrophin and desmin negative fibres were observed, here at 168 hours after exercise (**G**; arrows). After the second bout (3 weeks after the first bout), myofibres that appeared to be regenerated displayed intensive desmin staining (**I**; arrow). Note that images of samples from exercised and control muscles (**B**, **D**, **F**, **H**, and **J**) are taken with the same exposure time. The exposure time was adjusted after the exercised samples, which made the control samples appear dark ("negative"), however, all myofibres in control were positive for desmin, as show on image **K**. Data from the arm experiment (images from three subjects). Scale bars = 50 µm.

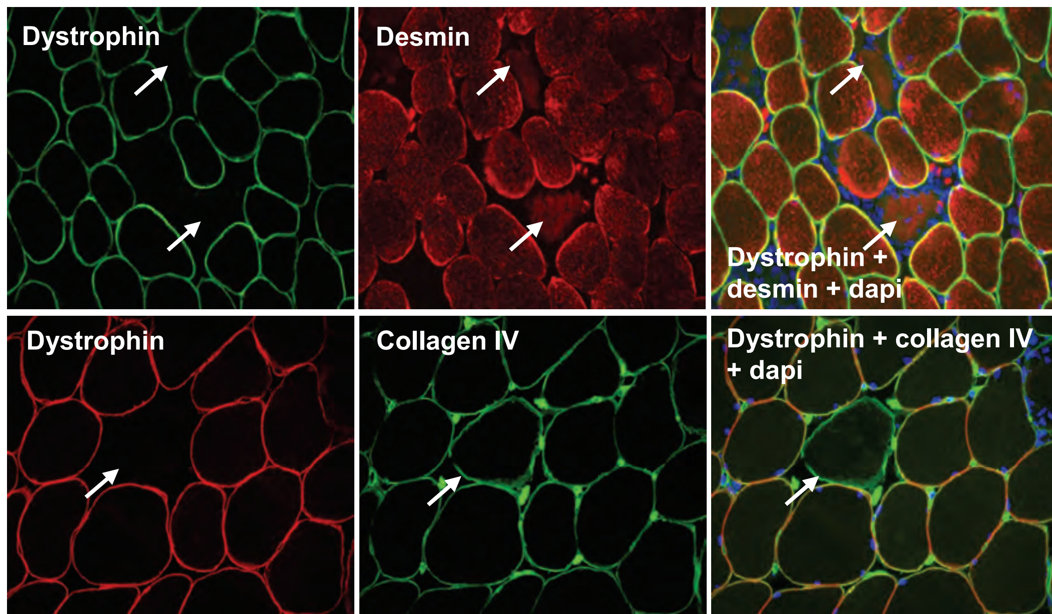

Figure 4.9. Fluorescent micrographs of cross-sections. Upper row of images: Dystrophin and desmin staining (plus nuclei [DAPI; blue stain] on the overlay). Some myofibres (arrows) displayed reduced dystrophin and desmin staining. These myofibres were probably necrotic. Lower row of images: Double-staining with dystrophin and collagen IV demonstrated that dystrophin negative myofibres was not negative for collagen IV. The same was found when double-stainings with either laminin or collagen VI (not shown here). This indicates that the scaffold of the plasma membrane (dystrophin) was degraded, whereas the basal membrane was usually intact. Data from the arm experiment.

Observations at the subcellular level (electron microscopy)

At the ultrastructural level (applying electron microscopy), disruptions of the myofibrillar structure were clearly evident 0.5-1 hour after both the leg and arm exercises (Figure 4.10; (210; 334)). These changes indicate mechanical disruptions during exercise, increased calpain activity following exercise, or both (124; 224; 244; 334)).

Two days after the arm exercise, autophagic vacuoles, which are associated with increased lysosomal degradation of cell structures (122; 126; 355), were observed together with abnormal mitochondria (electron dense, irregularly shaped, and swollen; Figure 4.10). Additionally, signs of membrane disruptions were found together with hypercontracted myofibrils (Figure 4.10). Abnormal looking mitochondria and areas with hypercontracted myofibrils[53] support other indicators of loss of Ca^{2+}-regulation, such as increased calpain activity and increased passive muscle tension (133; 134; 327; 412; 448). Increased passive tension was observed three to four days after both the leg and arm experiments[54], allegedly due to "injury contractures" from increased cytosolic resting levels of Ca^{2+} (327). Notably, Ca^{2+}-channel blockers have been shown to reduce signs of damage after eccentric muscle actions (27; 151; 378; 444).

After four and seven days, the most severe signs of damage were observed in certain myofibres of high-responder subjects. In large parts of these myofibres, the characteristic myofibrillar structure was completely absent (Figure 4.10).

[53] Hypercontracted myofibrils have been seen as an artefact of the biopsy handling (350); however, the degree of hypercontractility was clearly more frequent in samples from the exercised muscles than control muscles.
[54] In the arm experiment, the resting elbow angle was decreased by ~10° for four days after exercise (180° = extended elbow joint). In the leg experiment, passive tension was measured when subjects sat relaxed, knee joint at 90° and the lower leg connected to a force transducer (334).

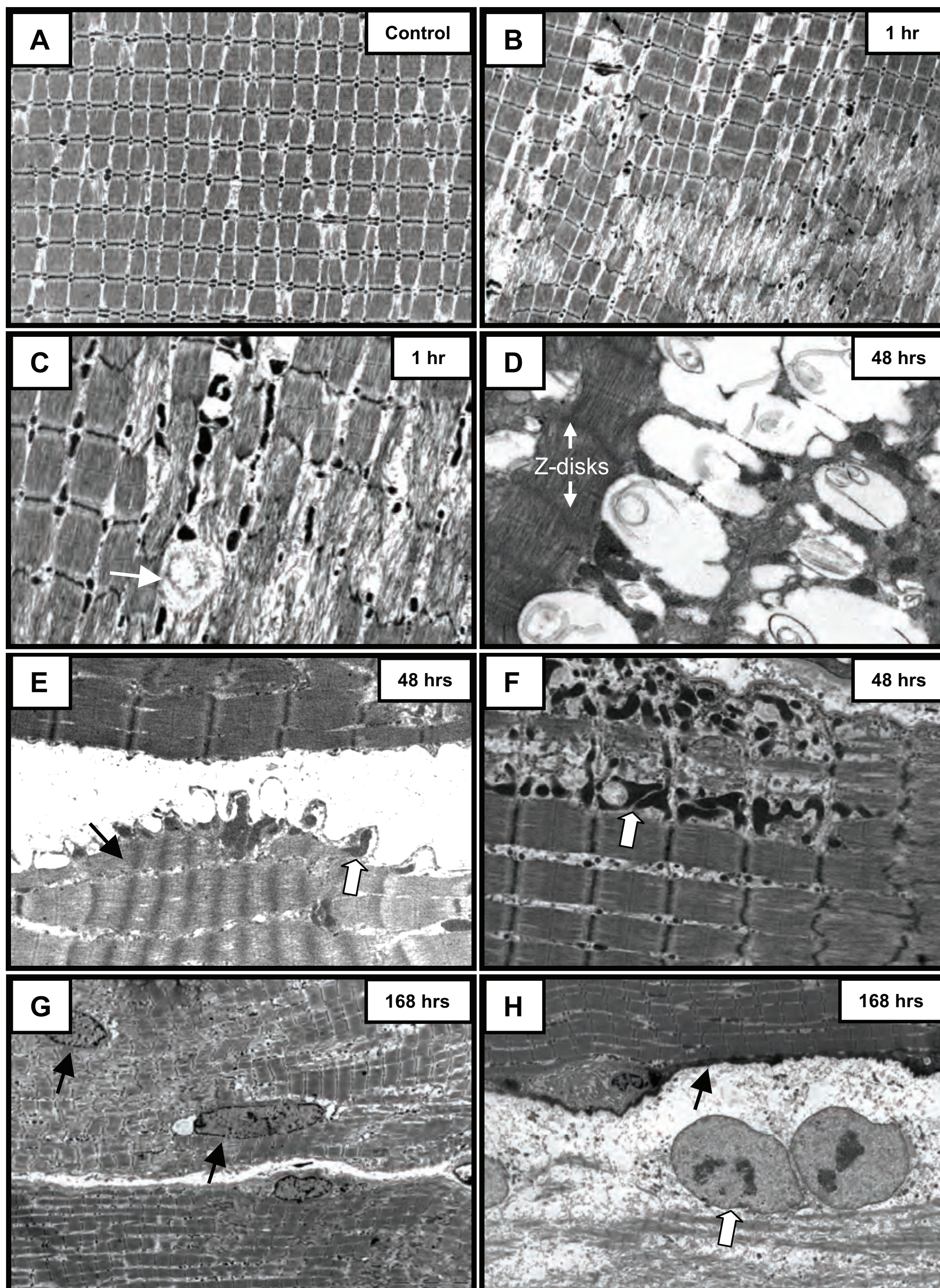

Figure 4.10. Electron micrographs of longitudinal sections. **A**: Normal structure in samples from control. **B**: Abnormal structure, i.e., myofibrillar disruptions, in a sample from an exercised muscle. **C**: Myofibrillar disruptions and apparently development of a vacuole (thin arrow). **D**: Large autophagic vacuoles (signifying lysosomal degradation). **E**: Hypercontracted myofibrils (thin arrow) and “papillary projections” of the membrane (thick arrow). **F**: Accumulation of abnormal looking mitochondria (thick arrow). **G**: Centrally located nuclei (thin arrows). **H**: An intact fibre (thin arrow) next to degraded fibre (lower part of the image); two apparently swollen nuclei are seen (thick arrow). Data from the arm experiment (images from three subjects).

Plausible sequence of events

Taken together, our observations by both light and electron microscopy suggest that segments of some myofibres go through the stages of necrosis (412), and that the observed accumulation of leukocytes is primarily a consequence of this process. The attraction of blood-borne monocytes, which differentiate to macrophages in the tissue, supposedly begins with the release of "wound hormones" (possibly bFGF[55]; (406)), or other inflammatory mediators (such as MCP-1[56]; (61; 117; 331; 365; 438)), and release of cellular constituents from damaged fibres, including immunoregulatory molecules such as HSP70 and HMGB1[57] (25; 340; 352; 416). These substances can, *inter alia*, be recognised by Toll-like receptors[58] on resident macrophages and dendritic cells (as well as fibroblasts, mast cells and endothelial cells), which are candidates for orchestrating the subsequent inflammatory reaction (59; 97; 137; 218; 271; 417; 450). The inflammatory and phagocytotic processes are followed by regeneration, which requires myogenic precursor cells recruited primarily from the satellite cell pool (150). Activation and proliferation precede myoblast migration, fusion and differentiation into new myotubes to replace the degraded segments (171; 176). It is well documented that macrophages are closely associated with proper regeneration of skeletal muscles (50; 89; 193; 391; 407; 409), and our observations concur with this (Figure 4.11). One week after exercise, large numbers of macrophages were seen within myofibres in samples from high-responder subjects. In some of these evidently necrotic myofibre segments, macrophages were observed alongside apparently fusing myoblasts. Three weeks after the first bout, the macrophages were observed only at the perimeter of regenerated fibres (Figure 4.11). Apparently, the macrophage subpopulation, or the activation profile of the macrophages, shifts during the regeneration process (61): Initially, monocytes transform

[55] Basic fibroblast growth factor
[56] Monocyte chemoattractant protein-1
[57] High-mobility group box 1 (HMGB1) is a nuclear architectural chromatin-binding factor, but it is also secreted by macrophages. HMGB1 binds a receptor called RAGE and is associated with initiation of inflammation when released by necrotic cells (in contrast to apoptotic cells; (359)).
[58] Toll-like receptors (TLRs) are a type of pattern recognition receptor. These receptors are found on immune cells (and non-immune cells) and enable them to recognise certain molecules commonly found on microbes (i.e., pathogen-associated molecular patterns), but not in the host (281). However, some TLRs (e.g., TLR3) can also function as endogenous sensors for tissue necrosis (59).

into inflammatory (M1) macrophages[59] and begin removing the debris of necrotic myofibre segments, while simultaneously stimulating the proliferation of satellite cells/myoblasts via cytokine secretion (one candidate being TNFα). Later, but still during phagocytosis, the macrophages supposedly switch to an anti-inflammatory, scavenging mode (M2)[60], which implies attraction of satellite cells/myoblasts and stimulation of differentiation and myotube formation. Finally, during the last stages of regeneration, M2 macrophages serve the regeneration process (myotube growth) from outside the basal lamina of myofibres by releasing various growth factors (e.g., TGF-β[61]; (10; 51; 61; 262; 411; 434)).

[59] Classically activated macrophages.
[60] Also called "alternatively activated macrophages".
[61] Transforming growth factor-β

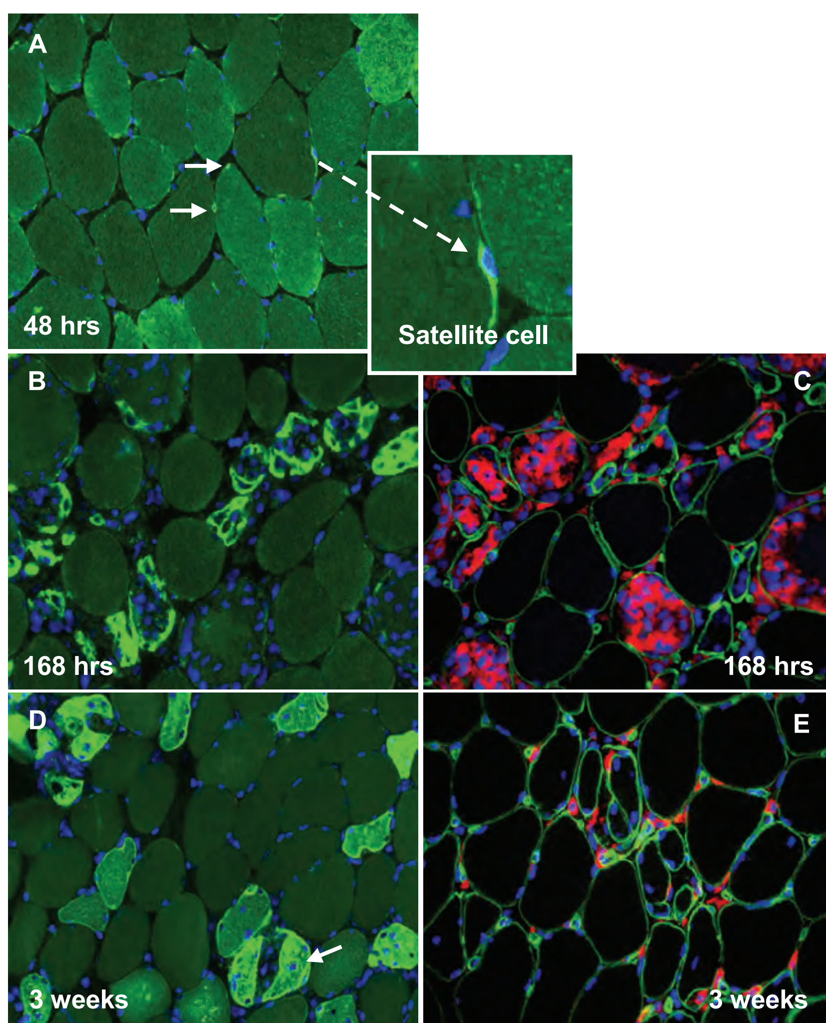

Figure 4.11. Fluorescent micrographs of cross-sections of samples from exercised muscles stained for CD56 (green) for identification of satellite cells (**A**, **B**, and **D**), and CD68 (red) for identification of macrophages (**C** and **E**; green stain shows the basal membrane [laminin]). Nuclei are blue (DAPI stain). **A**: Satellite cells observed 48 hours after exercise. **B**: At 168 hours fusion of myoblasts occurred together in the presence of high numbers of macrophages (**C**). **C**: Macrophages accumulated around and inside myofibres. **D**: Three weeks after exercise, most fibres appeared to have entered a final phase of the regeneration process. At this time point the macrophages were located outside, but near the membrane of the regenerated myofibres (**E**). Note the central locations of nuclei on image **D** (arrow). Data from the arm experiment (images from two subjects).

Necrosis vs. apoptosis

Our histological observations suggested segmental myofibre necrosis, but programmed cell death (apoptosis) may have occurred as well (183; 190; 325). Preliminary analyses (TUNEL[62] staining) indicated, however, that apoptosis was not of major importance in the observed processes[63]. Interestingly, macrophages are capable of rescuing myofibres and satellite cells from apoptosis (62; 377). Hence, the accumulation of macrophages, in addition to up-regulation of HSPs (Figure 1.2; (69)), might have saved a certain number of stressed myofibres (nuclei) from apoptosis. Nonetheless, necrosis and apoptosis are not necessarily two distinct processes (86), and further investigation is needed to fully describe the mechanisms behind certain histological findings in exercised-induced muscle damage.

Macrophages as the damaging culprit?

Our data suggest that macrophages (CD68 positive cells) serve the restoration of muscle structure and function, a finding that has been reported by others (119; 408; 409). However, a damaging effect of macrophage accumulation cannot be excluded. Thus, the macrophages might have caused the reduction or loss of dystrophin staining by attacking the myofibres from their position in the interstitium. Indeed, Lapointe *et al.* (209) reported that a decreased number of inflammatory macrophages in exercised rat muscles corresponded to attenuation of the secondary loss of force and improved early phase recovery of muscle function. On the other hand, the accumulation of macrophages appeared to peak after the period of halted recovery and secondary loss of force in our high-responder subjects. Hence, any transient, early phase deleterious effects of macrophage accumulation in humans are uncertain, and there is a need to elucidate the presence of different subpopulations and the activation mode of macrophages in human exercise-damaged muscles.

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[62] Terminal deoxynucleotidyl Transferase Biotin-dUTP Nick End Labeling; i.e., a marker for degradation (cleavage) of DNA, which in turn is related to an apoptotic process (increased endonuclease activity).
[63] No apoptotic myonuclei observed (five subjects analysed), but some extracellularly located nuclei were observed. These were plausibly leukocytes.

Conclusion

Exercise-induced accumulation of leukocytes around or within the myofibres seems to be primarily dependent on a necrotic process that is fully manifested four to seven days after eccentric exercise. The chain of events seems to begin with intracellular damage that may escalate in the days following exercise – probably due to persistently out-of-control Ca^{2+}-regulation (448); in other words, the process started inside the myofibres. A number of myofibres may "struggle" to recover from the exercise-induced perturbation of homeostasis, but in certain segments, the necrotic process may reach a point at which it becomes irreversible (412). Subsequently, the segment is probably sealed and totally degraded (digested by macrophages), before myogenic precursor cells (primarily satellite cells) develop new segments. Importantly, the necrotic process characterises high responders, but is absent in moderate (and low) responders (Figure 5.1). In this sense, our data give a more nuanced picture than previous publications on humans, and refutes claims that muscle damage does not occur after voluntary exercise. For most people, however, necrosis is probably not a regular occurrence after high-force exercise, such as resistance exercise. In fact, the events described in the high-responder subjects are presumably borderline pathological. The histological findings in samples from high responders resembled those seen in muscular dystrophies (myopathies) where structural weakness of the cytoskeleton leads to membrane damage and loss of Ca^{2+} homeostasis, followed by necrosis and inflammation (Figure 4.12).

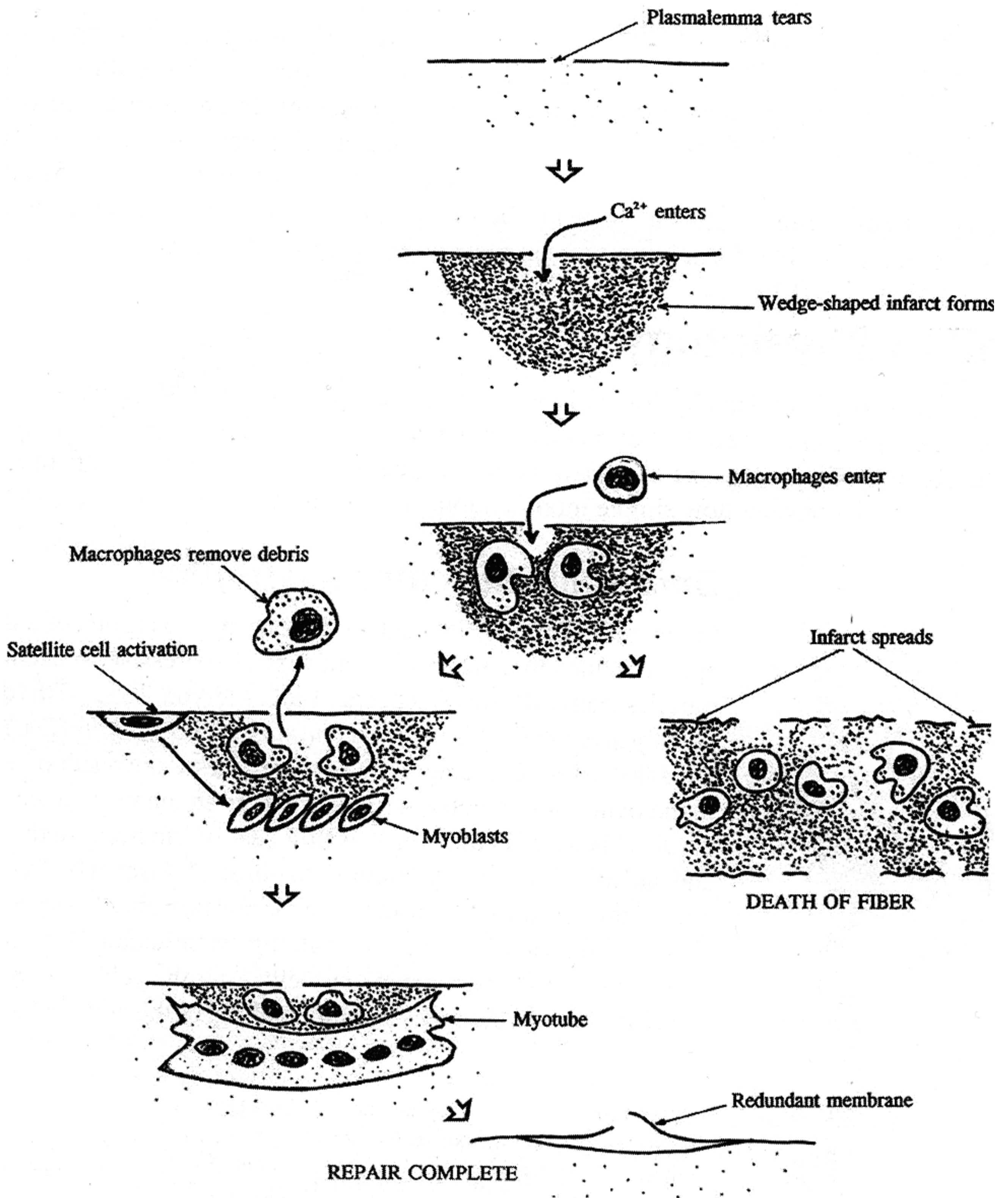

Figure 4.12. Possible sequence of events following membrane damage in muscular dystrophy. The drawing is based on the work of Mokri & Engel (270). Reprinted, with permission, from B.R. MacIntosh, P.F. Gardiner, and A.J. McComas, 2006, *Skeletal muscle: Form and function,* 2nd ed. (Champaign, IL: Human Kinetics), 20.

4.1.4 Leukocyte accumulation is related to recovery of muscle function: Do the leukocytes affect muscle function?

Early and middle phase of recovery (first four days after exercise)

In both the leg and arm experiments, a relationship between the accumulation of radiolabelled leukocytes and changes in force-generating capacity was found: subjects showing high radioactivity in the exercised muscle simultaneously experienced severe muscle weakness in the days following the exercises (Figure 4.13). To further elucidate this relationship, celecoxib (a COX-2 inhibitor NSAID) was administered during the arm experiment in order to reduce the inflammatory reaction. Unfortunately, repression of the inflammatory reaction was unsuccessful, and no drug effect on the recovery process, except muscle soreness (discussed below), was found. Although we cannot demonstrate a causal relationship with our data, animal studies provide evidence for a causal relationship between inflammation and muscle function. Reduced inflammation, achieved by the use of NSAIDs, antibodies, or genetically modified ("knockout") animals, has been reported to prevent the secondary loss of force and enhance the recovery of muscle function (45; 209; 269; 323). Contradictory results have also been reported, however (120; 236; 322). Recently, Dumont *et al.* (96) showed that accumulation of neutrophils after mechanical overloading (unloading-reloading model) did not affect recovery of muscle function. However, neutrophils stimulated by LPS[64] induced muscle damage and loss of muscle force. These findings demonstrate the tissue damaging potential of neutrophils when activated by microbial products, but cast doubt about their role in mechanically overloaded (sterile) skeletal muscles.

[64] Lipopolysaccharides, found on Gram-negative bacteria, act as endotoxins and cause a strong immune response.

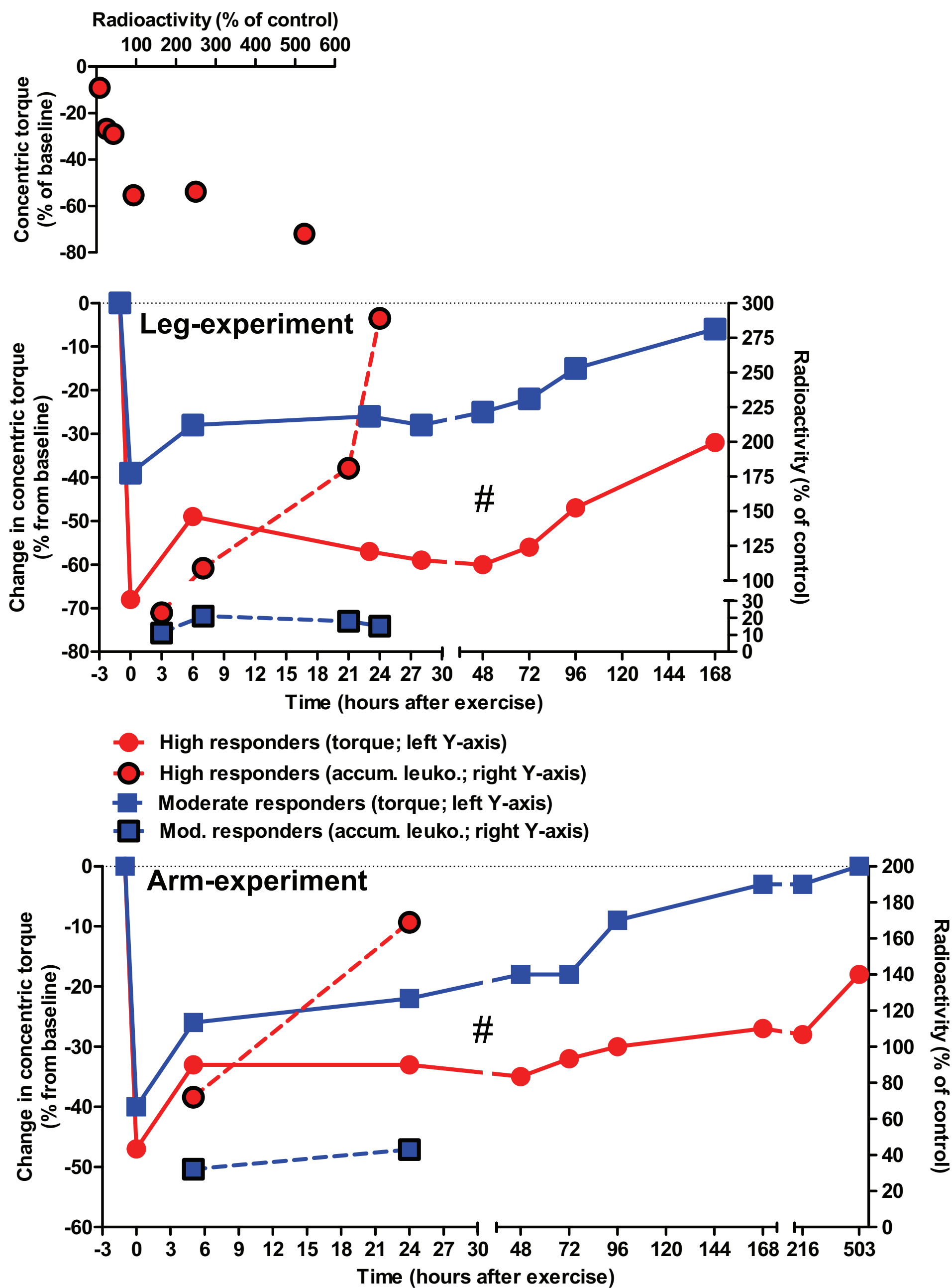

Figure 4.13. Changes in concentric force-generating capacity (left Y-axis) and accumulation of radiolabelled leukocytes (radioactivity; right Y-axis) in the exercised muscles of high- and moderate-responder subjects in the leg experiment (upper graph) and arm experiment (lower graph). Note the secondary loss of force in the high responders. The X-Y plot shows the relationship between accumulation of radiolabelled leukocytes (peak radioactivity over time) and loss of force-generating capacity (48 hours after the leg exercise; $r = 0.85$; $p < 0.01$). Error bars are omitted for readability. The groups (high and moderate responders) were statistically different with regards to both recovery of force (area under the curve; $p < 0.05$) and radioactivity (peak and mean values; $p < 0.05$).

Several human studies have tested the effect of NSAIDs on recovery of muscle function after a bout of high-force exercise. Results are equivocal, as some investigators claim no effect (90; 165; 320; 388; 410), while others report increased rate of recovery (17; 39; 95; 149; 213; 358). Unfortunately, biopsies were only collected in some of these studies. Bourgeois *et al.* (39) found that naproxen had a modest recovery enhancing effect, although leukocytes were not detected in the muscle samples obtained 24 hours after resistance exercise. Peterson *et al.* (319) did not measure muscle function, but found no effect of either acetaminophen or ibuprofen (which has been found to speed recovery (149)) on the accumulation of leukocytes 24 hours after high-force eccentric exercise. Thus, even though NSAIDs have occasionally been found to increase the rate of recovery, it is unclear whether the effect is anti-inflammatory in nature.

Importantly, our histological observations indicated that the accumulation of leukocytes was out of phase with the secondary reduction in force-generating capacity (Figure 4.3). Additionally, transmigration of neutrophils was not confirmed. The lack of neutrophils in the exercised muscle tissue casts doubt on the hypothesis that these inflammatory cells cause damage and secondary loss of force-generating capacity after eccentric exercise in humans (45; 113; 240; 411). On the other hand, based on the scintigrams it can be assumed that leukocytes accumulate in the microcirculation, as seen after contusion damage in skeletal muscle (360; 363). These leukocytes could (without transmigrating) potentially affect the myofibres through their cell-to-cell interaction with endothelial cells and by reducing or blocking the microcirculation (blood flow; (365)). Reduced delivery of oxygen and nutrients, as well as increased generation of reactive oxygen species (ROS), may cause damage *per se* and/or delay restoration the muscle structure and function (151; 184; 293; 315; 342; 360-362; 365; 394). Indeed, Hellsten *et al.* (152) found increased numbers of xanthine oxidase positive cells in tissue samples from humans after high-force eccentric exercise[65]. These cells

[65] Isometric force-generating capacity was reduced by approximately 40% from pre-value and CK levels were increased to about 13300 $U{\cdot}L^{-1}$ four days after exercise.

appeared to be primarily endothelial cells, and the authors suggested that the circulating leukocytes adhered to the endothelial cells and stimulated the expression of xanthine oxidase. Xanthine oxidase generates ROS (superoxide anions). However, in contrast to comparable studies on mice (93; 94), Hellsten *et al.* (152) could not detect any increase in malondialdehyde[66] levels in homogenised muscle tissue, indicating no major ROS attack. In addition, Child *et al.* (66) reported accumulation of leukocytes in exercised muscles in humans four and seven days after eccentric exercise, but the antioxidant status was not compromised and the malondialdehyde levels did not change. Thus, although ROS could potentially affect muscle structure and function through oxidation after high-force exercise, firm evidence is lacking (293). It should be kept in mind that investigating ROS in tissues is challenging (147) and further studies are needed.

Conclusion: The impact of the inflammatory reaction on the recovery of muscle function after exercise in humans is still unclear. Based on the time course of events, the evidence suggests that the inflammatory reaction follows the muscle damage, rather than causing it. It should be noted that these interpretations conflict with observations in some animal studies. Because the NSAID applied in the arm experiment (celecoxib) affected neither the accumulation of leukocytes nor recovery of muscle function, we were unable to explore the causality of the relationship between these events.

Late phase of recovery

Because certain aspects of the inflammatory reaction are reportedly necessary for removing debris and promoting regeneration (60; 61; 94; 168; 221), interventions that attenuate inflammation might adversely affect regeneration efficacy and full recovery. In addition, the activity of satellite cells and myoblasts – vital for regeneration – are allegedly dependent on the cyclooxygenase (COX) enzymes, which are blocked by NSAIDs (37; 369; 370).

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[66] The malondialdehyde levels are used to measure lipid peroxidase and commonly used marker of oxidative stress (426).

We did not detect any effect of the COX-2 inhibitor (celecoxib) on recovery of muscle function monitored for three weeks after exercise. Signs of necrosis and regeneration were observed in some subjects from both the celecoxib group and the placebo group. Although the number of tissue samples with necrotic and regenerating myofibres was limited (~⅓ of the subjects), we could not detect any overt drug effect. If celecoxib effectively blocked/reduced satellite cell activity, it seems improbable that the celecoxib group could recover and adapt to the exercise as well as the placebo group, since blocked satellite cell activity (by irradiation) has been shown to markedly slow the recovery process in rats after eccentric exercise (235).

Applying microdialysis, Mikkelsen *et al.* (267) and Tegeder *et al.* (401), observed no increase of the tissue fluid levels of PGE_2 in (resting)[67] muscles during the first 24 hours after eccentric exercise, thus concurring with our findings. However, Mikkelsen *et al.* (at Bispebjerg Hospital) succeeded in lowering the basal PGE_2 levels following exercise by tissue administration of indomethacin (via microdialysis), which seemed to block the exercise-induced satellite cell response (unpublished data). This finding supports those of Mackey *et al.* (243), who observed reduced satellite cell response after running exercise in subjects administered indomethacin orally four days before and eight days after the exercise. Indomethacin affects both COX-1 and COX-2. Allegedly, prostaglandins are required for a satellite cell response (326), but there might not be any need for significantly increased levels if the basal levels are sufficient. Lack of detection of any significant reduction in the PGE_2 levels 2, 24, and 48 hours after exercise could explain why the satellite cell response was apparently unaffected by our COX-2 inhibitor.

[67] Tegeder *et al.* (401) reported acutely increased PGE_2 generation in response to muscle actions (during the microdialysis procedure) 24 hours after eccentric exercise.

No adverse effects of the COX-2 inhibitor celecoxib

The benefits of NSAIDs in sport medicine have been debated for years since several studies have demonstrated that these drugs adversely affect the regeneration and healing process for injuries in the skeletal muscle system (6; 116; 326; 336; 427; 429). The basis for concern derives almost exclusively from animal studies. For humans, only circumstantial evidence of adverse effects on bone healing exists – after long-term, high dose NSAID use (344; 427). Note that NSAIDs and selective COX-2 inhibitors (celecoxib) have also been shown to improve recovery from ankle sprains and knee replacement surgery (53; 101; 282). Our findings gave no indication of adverse effects of COX-2 inhibitors, at least for short-term use of celecoxib, as in our experiment. The soreness/pain reducing effect (paper II) might explain how this drug could enhance recovery from injuries that are more painful than exercise-induced muscle soreness: by facilitating appropriate activity and rehabilitation.

Conclusion

The role of prostaglandins in the skeletal muscle cell growth and regeneration has been convincingly documented (159; 326), but the roles of the COX enzymes and prostaglandins in the satellite cell response to exercise in human muscles are unclear. Our data suggest that the COX-2 inhibitor celecoxib (at dosage used in this study: ~ 6 $mg \cdot kg^{-1}$ for nine days) leaves regeneration and adaptation processes unaffected after exercise-induced muscle damage. However, further investigations are warranted, and it should be kept in mind that celecoxib may have diverse COX-2 independent effects on a cell (e.g., influence on MAPK[68] signalling; (138; 387)).

4.1.5 The repeated-bout effect does not rely on inflammation in humans?

Lapointe *et al.* (208) concluded that the inflammatory reaction was allegedly important for the repeated-bout effect in rats. We wanted to replicate this study in a human model, but since

[68]Mitogen-activated protein kinase

the COX-2 inhibitor (celecoxib) did not effectively dampen the inflammatory reaction after the first bout of exercise, we lost the opportunity to study the impact of inflammation *per se*. Nevertheless, even though we observed a strong inflammatory reaction in some subjects and a quite small reaction in others, the repeated-bout effect was a general finding. This indicates that adaptations occur independently of inflammation. It is still possible that the inflammatory reaction influences the adaptation processes, but it does not seem to be essential. This observation concurs with studies that have documented that the repeated-bout effect can be found after just a few (2-10) maximal eccentric actions as well as after exercising with “non muscle-damaging” light loads (i.e., 10% of maximal isometric strength; (48; 211; 308)). Moreover, adaptations are found to take place before the muscle has recovered from eccentric exercise (98; 249; 303), and the remodelling processes are observed in the myofibres without inflammatory cells detected among them (107; 122; 453). On the other hand, Newham *et al.* (288) suggested that removal of certain “fragile” myofibres (by necrosis) and regeneration/development of new fibres can be a mechanism behind the repeated-bout effect. This hypothesis (“elimination of the weakest in the herd”) is hard to test experimentally in humans, but might have particular relevance for high-responder subjects.

Conclusion

Certain aspects of the inflammatory reaction seem to be fundamental for regeneration of necrotic segments of myofibres. Adaptations in humans – manifested as the repeated-bout effect – appear, however, not to be dependent on an inflammatory reaction. In other words, necrosis and regeneration are probably not prerequisites for producing the repeated-bout effect. However, the inflammatory reaction and the subsequent regeneration process might be related to adaptations in subjects stricken by large numbers of necrotic fibres. No firm conclusion can be drawn from the data presented herein.

4.2 Delayed onset muscle soreness (DOMS) is not caused by classical inflammation

DOMS commonly occurs after exercise, and especially after unaccustomed eccentric exercise (15; 161). MacIntyre *et al.* (240; 241) speculated that leukocytes and inflammation play a role in the mechanism behind DOMS, since they observed that radiolabelled leukocytes accumulated before and concomitantly with the development of soreness after eccentric exercise. Raastad *et al.* (335) investigated the same relationship after resistance exercise, but could not establish any numerical correlations between the accumulation of radiolabelled leukocytes and DOMS. Nevertheless, we were surprised to find a negative correlation between accumulation of radiolabelled leukocytes and DOMS in the leg experiment ($r = 0.88$, 24 hours after exercise; $p < 0.05$; paper I). That is, the subjects with large leukocyte accumulation experienced less DOMS than subjects with low leukocyte accumulation. At the intra-individual level, large accumulation of radiolabelled leukocytes in some parts of the quadriceps muscle, especially in m. rectus femoris, corresponded poorly with the reported DOMS in the different parts of m. quadriceps. Our histological observations indicated that the muscle soreness subsided as the number of leukocytes increased. The obvious interpretation of these findings was that the accumulation of leukocytes could not explain DOMS. Results from the arm experiment confirmed the findings from the leg experiment, although the (negative) relationship between accumulation of radiolabelled leukocytes and DOMS was less evident. Our claims against inflammation as the major culprit of DOMS are not unheard of. Newham (287) stated (page 357) that "*(…) human studies indicate that the time course of the inflammatory cell infiltration is much slower than that of the pain and occurs when the pain is either diminishing or has disappeared*".

We did observe a modest soreness reducing effect of the COX-2 inhibitor (celecoxib), which would suggest that the generation of prostaglandins is involved in DOMS. However, the pain-reducing effect of celecoxib may occur both at the site of pain signal generation in the

muscles and in the central nervous system (420). Since the tissue fluid concentration of PGE_2 remained unchanged, it seems unlikely that PGE_2 in the muscles caused DOMS in our subjects. Other studies have also cast doubt on PGE_2 as a central mechanism behind DOMS (201; 401). It appears therefore that celecoxib exerted its soreness reducing effect in the central nervous system. Unfortunately, nothing new about the mechanisms behind DOMS could be extracted from these findings.

The negative correlation between accumulation of leukocytes and DOMS might be a chance finding. If a cause-effect relationship actually exists, it signifies that the leukocytes (neutrophils) somehow *reduced* DOMS. In support of such an assumption, Rittner *et al.* (346-348) have demonstrated in animal models that neutrophils do not cause inflammatory pain, and that they contain pain-reducing opioids.

Interestingly, in some of the subjects reporting severe DOMS we found a marked increased expression of the N-terminal propeptide of procollagen type III (PIIINP; leg experiment)[69]. Because DOMS did not correlate with disruptions in the myofibrillar structure (paper I), this could imply that DOMS is primarily dependent on events in the extracellular matrix – as previously suggested by others (1; 78).

Conclusion

DOMS is a very common, but unexplained, phenomenon. Our findings suggest that DOMS is not a consequence of a classical inflammation and accumulation of leukocytes, or muscle damage (defined as myofibrillar disruptions). Based on our data and the existing literature, DOMS plausibly develops because of both increased cellular activity in the connective tissue of the exercised muscles and an as-yet elusive central mechanism (327).

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[69] Data on PIIINP and tenascin C, markers of remodelling of the extracellular matrix, are reported by Raastad *et al.* (334).

4.3 Heat shock protein response to eccentric exercise

The heat shock protein (HSP) response was investigated in both the leg and arm experiments, and our findings in m. vastus lateralis (paper III) were generally confirmed in m. biceps brachii (paper IV). As the methodological approaches in the two experiments were complementary, the results are discussed as one set of data to give insight into the HSP response to high-force exercise in humans.

4.3.1 HSP27, αB-crystallin, and HSP70 translocate to cytoskeletal and myofibrillar structures

Our first observation of an HSP response was by means of immunohistochemistry and light microscopy (leg experiment; Figure 4.14). The staining pattern of HSP27 on cross-sections displayed a peculiar, scattered, granular stain at early time points after eccentric exercise. At the individual level, it was evident that the staining intensity was markedly reduced during the first 24 hours after exercise (Figure 4.15). Interestingly, the staining intensity appeared to persist longer in high-responder subjects who recovered slowly and displayed considerable myofibrillar disturbances four and seven days after exercise. In both the leg and the arm experiments, the staining for αB-crystallin and HSP27 revealed very similar staining patterns. Prior to this study, increased staining of HSP27 and αB-crystallin in human skeletal muscle had only been observed in samples from patients with myofibrillar myopathies (20; 114).

Our observations are similar to those of Koh and Escobedo (194) who reported increased staining intensity of HSP25 (HSP27 homolog) and αB-crystallin in muscles of mice harvested immediately after exposure to eccentric actions. They also observed a translocation of HSP25 and αB-crystallin from the soluble to the insoluble fraction of homogenated muscles. This type of HSP movement between cellular compartments during cellular stress has been observed in other types of experiments as well, e.g., studies examining ischemia of cardiac and skeletal muscle (139; 140; 451).

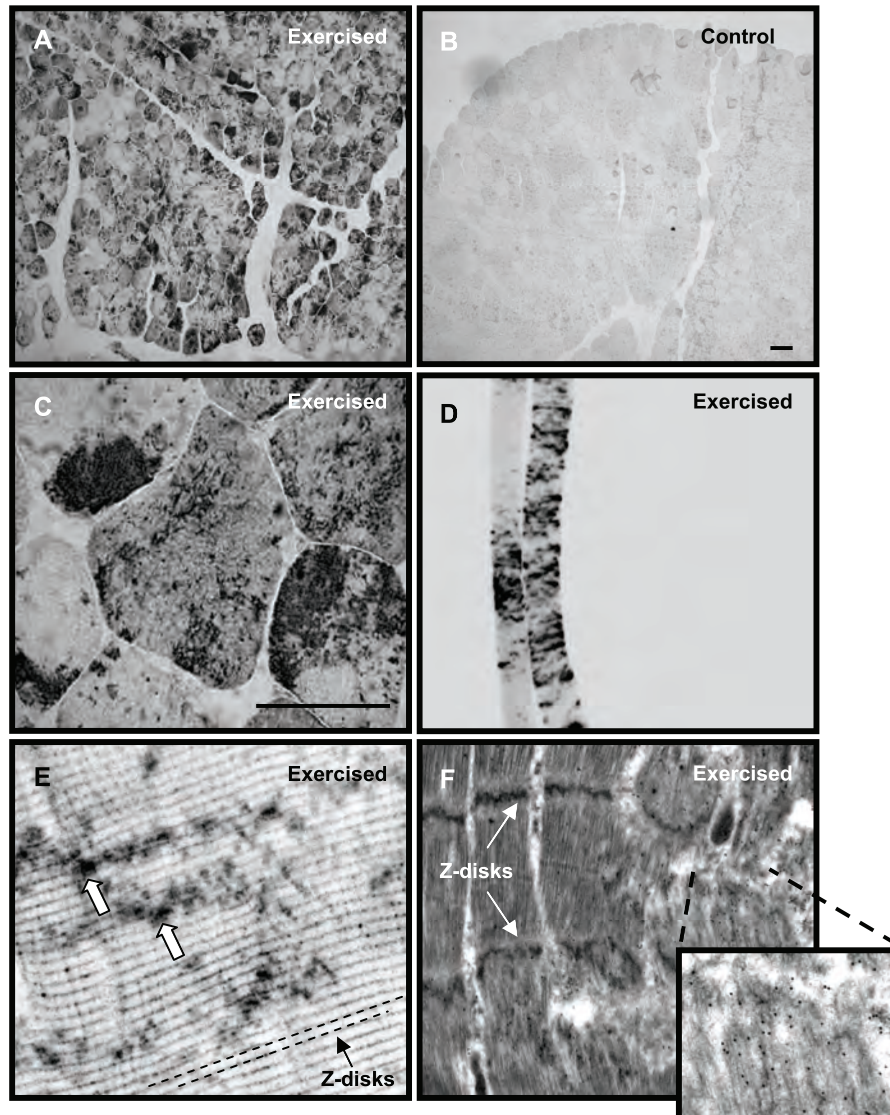

Figure 4.14. Light micrographs of HSP27 staining on cross-sections from (**A**) exercised and (**B**) control muscle obtained 0.5 hours after exercise (scale bar = 100 μm). Note that almost all the exercise strained myofibres displayed increased staining intensity (dark stain), compared to control. **C**: With higher magnification, the HSP27 staining showed a scattered, granular appearance (scale bar = 50 μm). **D**: On segments of whole fibre preparations it was demonstrated (by conventional light microscopy) that αB-crystallin (analogous to HSP27) accumulated non-uniformly along the length of the fibres. **E**: By confocal microscopy of single whole fibre preparations, the HSP27 staining displayed a fine Z-disk staining that was interrupted by areas of increased staining intensity (indicating myofibrillar disruptions; thick arrows). **F**: At the ultrastructural level (electron micrographs of longitudinal sections), areas with myofibrillar disruptions were found in samples of exercised muscles, and immunogold staining for HSP27 showed accumulation of HSP27 (black particles) in these areas, as well as in Z-disks. Data are from both the leg (**A-C**) and arm (**D-F**) experiments.

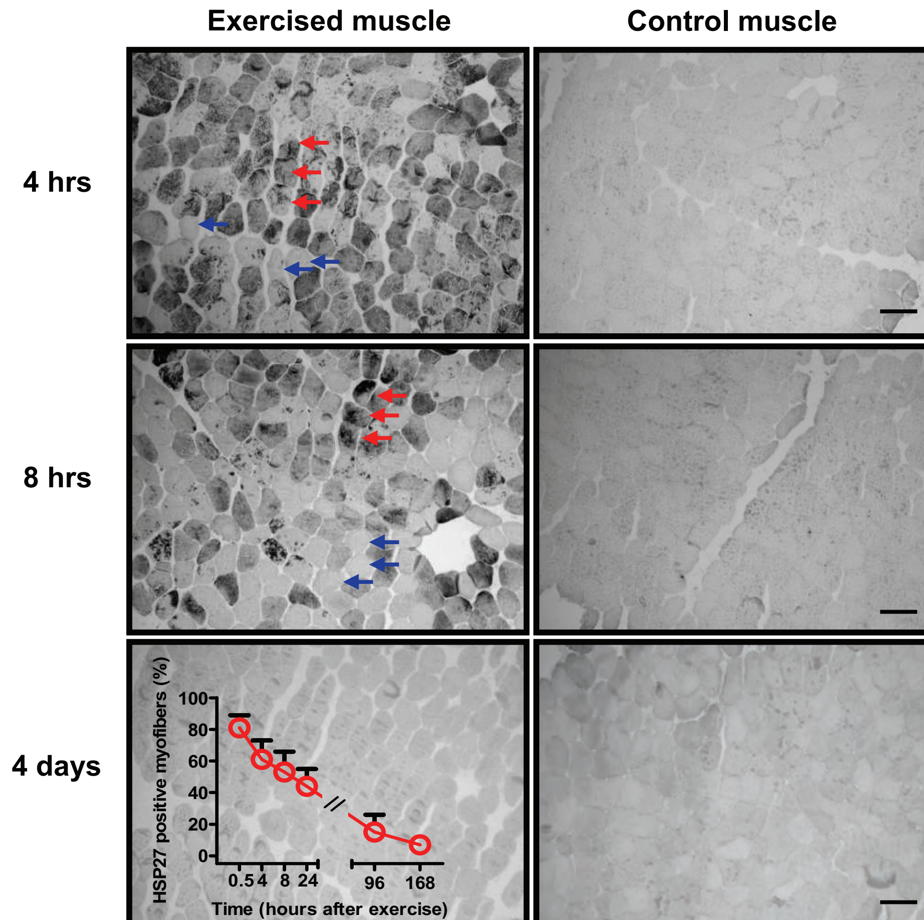

Figure 4.15. Light micrographs of HSP27 stained cross-sections from one subject (in the leg experiment) at three different time points. The red arrows point to positive myofibres, whereas the blue point to negative fibres. Note the intensive staining pattern on the four hours sample and the marked difference between the four and the eight hours samples. At four days after exercise no staining was observed in this subject (in contrast to some high-responder subjects). In the control samples only very weak background HSP27 staining was observed (no scattered, granular staining pattern). The figure inserted on the left lower image shows the data from all subjects in the leg experiment; illustrating a rapid decrement of HSP27 positive fibres with time after exercise. Scale bar = 100 μm.

Following up on our immunohistochemical observations, we homogenised and fractionated the muscle samples into cytosolic and cytoskeletal/myofibrilar fractions and applied immunoassays (western blotting and ELISA) for detecting the HSP content in each fraction. In samples obtained shortly after (0.5-1 hours) the exercises, we observed an increase of HSP27 in the cytoskeletal/myofibrillar fraction concomitantly with a reduction in the cytosolic fraction (Figure 4.16), thus confirming the findings of Koh and Escobedo (194). During the next four days, the HSP27 molecules appeared to move back to the cytosolic cell compartment, but this event seemed to proceed more slowly in the high responders than the other subjects. In short, the immunoassays supported the immunohistological observations. In the arm experiment, we found that the αB-crystallin response was similar to the HSP27 response; however, the increased amount of αB-crystallin in the cytoskeletal/myofibrillar fraction persisted longer than for HSP27, especially in high-responder subjects (paper IV).

Notably, we found statistically significant numeric relationships between the HSP27 response observed (by both immunohistochemistry and the immunoassays) and changes in muscle function (details in paper III and IV). This strongly suggests that the HSP response was dictated by the degree of muscle damage inflicted during exercise.

The immunohistochemical results demonstrated a staining pattern for HSP70 that was similar to the small HSPs, but the staining intensity observed at 0.5-1 hour after exercise was quite weak compared with later time points (four and seven days after exercise). Indeed, the later increase in staining intensity was observed simultaneously with increased protein levels of HSP70 in the cytosolic fraction, which was preceded by increased mRNA levels (Figure 4.17; see further discussion below). HSP70 did not seem to translocate to cytoskeletal/myofibrillar structures to the same degree as the small HSPs during/immediately after exercise, but increased HSP70 levels in the cytoskeletal/myofibrillar fraction were consistently found 2-7 days after exercise (paper III and IV). In other words, as the HSP27 levels decreased in the cytoskeletal/myofibrillar fraction, the HSP70 levels increased. This observation might reflect a

known interaction between HSP27 and HSP70: in the presence of damaged/denatured proteins, HSP27 is believed to "catch and hold" these proteins to prevent them from aggregating. While HSP27 holds on to the denatured proteins, HSP70 refolds them (99). Proteins that become irreversibly denatured, are selected by HSP70 and co-chaperones[70] for ubiquination and degradation in proteasomes (Figure 1.2; (156; 266)). Interestingly, proteins that are not refoldable might induce large accumulations of HSP27 molecules, forming granules (49). This might explain the especially strong HSP27 (and αB-crystallin) staining pattern in certain parts of the myofibres shortly after exercise (Figure 4.14).

[70] HSP70 works together with other HSPs, such as HSP40 and HSP90, as well as "co-chaperones", such as Hip and Hop, and Bag-1 and Chip. These co-chaperones (forming complexes with HSP40 and HSP70) seemed to somehow determine whether the protein would be a subject for the "refolding machine" or the "degrading machine" (156; 266).

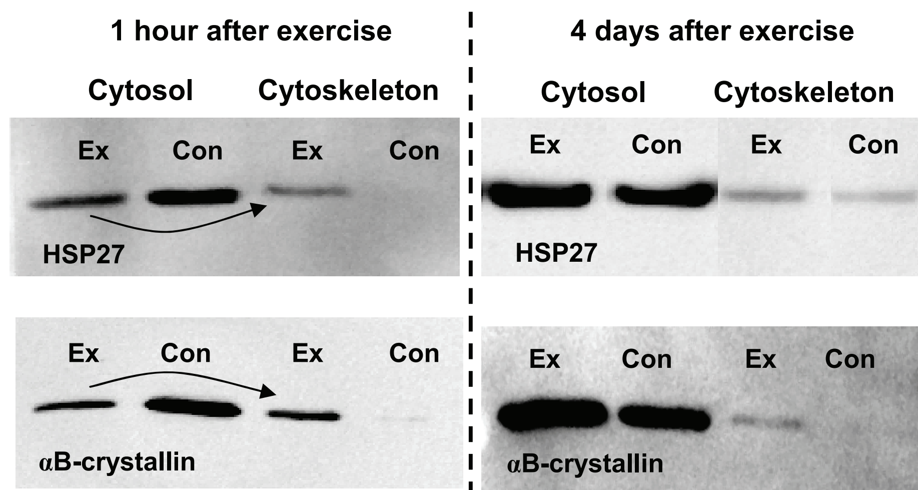

Figure 4.16. Representative immunoblots of HSP27 and αB-crystallin in tissue samples obtained one and 96 hours (four days) after exercise from both exercised (ex) and control (con) muscles. The homogenated samples were fractionated into a cytosolic and a cytoskeletal/myofibrillar fraction. The blots show reduced protein levels in the cytosolic fraction simultaneously with increased levels in the cytoskeletal/myofibrillar fraction one hour after exercise (arrows), compared to control. Thus, it appears that the small HSPs (HSP27 and αB-crystallin) move (translocate) from the cytosol to the cytoskeletal/myofibrillar structures during exercise. Furthermore, despite higher levels in the cytoskeletal/myofibrillar fraction, the protein levels in the cytosolic fraction from exercised muscles were increased compared to control four days after exercise. Data are from the arm experiment.

Localisation of the small HSPs in Z-disks

The staining pattern of HSP27 and αB-crystallin on cross-sections indicated that these small HSPs accumulated on certain “stressed” myofibrils (Figure 4.14). To further elucidate this, we applied immunocytochemistry on segments of whole fibre preparations combined with confocal imaging for examination of the myofibrillar structure in greater detail (Figure 4.14). Confocal imaging verified that the small HSPs accumulated in areas of myofibrillar disruptions – revealed by co-localisation of HSP27 and irregular phalloidin staining (paper IV). Whole fibre preparations with good antibody penetration also confirmed the scattered distribution of damaged sarcomeres throughout both the width and length of fibres.

To examine the myofibrillar structure even more closely, we used immunogold labelling and electron microscopy to examine the small HSPs at the ultrastructural level. Signs of myofibrillar disruptions seen on the whole fibre preparations were verified at the ultrastructural level (Figure 4.10 and 4.14). In samples from high-responder subjects in the arm experiment, nearly all fibres investigated from the exercised muscle displayed areas of disrupted sarcomeres in parallel and in series (210). The immunogold labelling demonstrated that both HSP27 and αB-crystallin accumulated in areas of myofibrilar disruptions and at the Z-disks in samples from the exercised muscles (Figure 4.14), confirming findings reported in animal studies (140; 194). It makes sense that the small HSPs translocate to the Z-disks, as this structure seems particularly vulnerable to both mechanical stress and increased proteolytic activity caused by high-force exercise (122; 124). αB-crystallin seemed to accumulate more than HSP27 at apparently intact Z-disks adjacent to areas of myofibrillar disruption, as well as at intermediate filaments. Desmin is an important component of intermediate filaments (70; 428), and the relationship between αB-crystallin and desmin is known from mutations in the αB-crystallin gene that cause desmin-related myopathy (aggregation of desmin; (422)).

4.3.2 Eccentric exercise induces increased HSP expression

The HSP content in skeletal muscle fibres is high (~5% of soluble proteins (231)), and the amount is regulated by the level of stress (135; 227). Indeed, increased mRNA and protein levels of HSP27, αB-crystallin and HSP70 have been convincingly documented after high-force exercise (107; 402; 403; 405) and training (135; 136; 227; 228) in humans. We also found increased HSP mRNA expression 4-24 hours after the leg exercise[71], followed by increased protein levels (predominantly HSP70) in the days after exercise (Figure 4.17). This was confirmed in the arm experiment, where increased protein levels of HSP27, αB-crystallin and HSP70 were found in the cytosolic fraction two to seven days after exercise (paper IV).

Our data do not allow us to determine why the mRNA levels increased. The increase could be due to the exercise itself, through increased temperature, oxidative stress, and mechanical strain and/or damage to the cytoskeletal and myofibrilar structures (see Figure 1.2). It is also plausible that recovery and remodelling processes are behind the increased mRNA levels via, e.g., increased generation of growth factors and inflammatory mediators, such as cytokines (Figure 1.2; (169; 170; 403)).

[71] The mean HSP70 mRNA levels were almost seven fold higher than control 0.5 hours after exercise ($p = 0.11$), but one of the seven subjects demonstrated higher levels in control than in the exercise muscle.

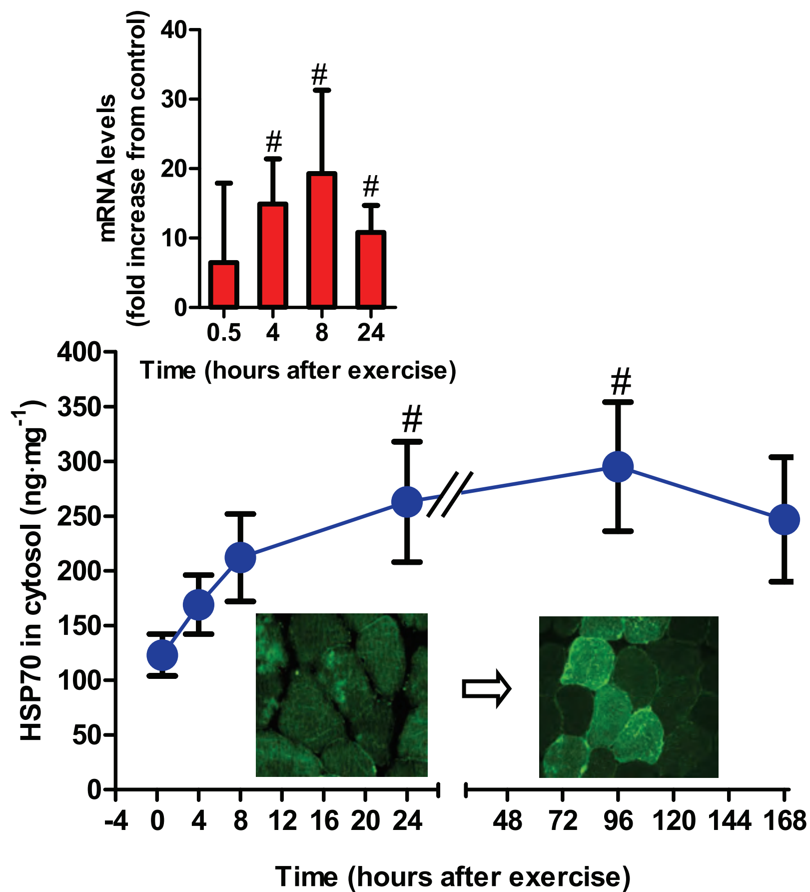

Figure 4.17. The HSP70 levels in the cytosolic fraction of tissue samples from exercised muscles showed increased levels in the days after exercise. This finding was supported by elevated mRNA levels during the first 24 hours (top figure), as well as increased staining intensity for HSP70 on cross-sections (fluorescent micrographs). # denotes significant difference from 0.5 hours ($p < 0.05$). Data from the leg experiment.

4.3.3 The HSP response and the repeated-bout effect

If the assumption about the role of HSPs in protecting the cytoskeletal/myofibrillar structure is correct (192), it seems reasonable to propose that the HSPs might be part of the mechanism behind the repeated-bout effect.

We observed that the amounts of HSP27 and αB-crystallin in the cytoskeletal/myofibrillar fraction after the first and second bouts were about the same, whereas the level of HSP70 was higher after the second bout than first bout (Figure 4.18; paper IV). This implies a "disproportionate" high amount of HSPs in the cytoskeletal/myofibrillar fraction after the second, repeated bout. This suggestion is deduced from 1) the damage inflicted appeared to be less after the second bout than the first bout, and 2) it seems to be an association between the damaged inflicted and signs HSPs of translocation from cytosol to cytoskeleton/myofibrillar structures during a initial, unaccustomed bout of exercise (Figure 4.18; paper III and IV). Although no cause-effect relationship can be established from our data, our findings indicate a role of HSPs in the protection of the cytoskeletal/myofibrillar structures during exercise. A simple explanation for the high levels of HSPs in the cytoskeletal/myofibrillar fraction after the second bout could be increased total levels of HSPs. Increased HSP levels could favour more binding between "substrates" (i.e., HSPs and hydrophobic patches on denatured proteins), according to the law of mass action. A shortcoming with our study design was that we did not obtain biopsies immediately prior to the second bout. Since we only have biopsies from after the exercise bouts, we cannot say for sure that our subjects conducted the second bout with higher HSP levels than when performing the first bout.

We applied antibodies (Table 3.2) that do not distinguish between non-phosphorylated and phosphorylated HSPs, so certain differences in the regulation of the "HSP-defence" between bouts might have gone undetected. Koh and Escobedo (194) showed that it was primarily

phosphorylated HSP25 molecules and non-phosphorylated αB-crystallin molecules that translocated to myofibrillar/cytoskeletal structures in mice (EDL) during a single bout of eccentric actions. Whether this is similar in humans awaits further investigation.

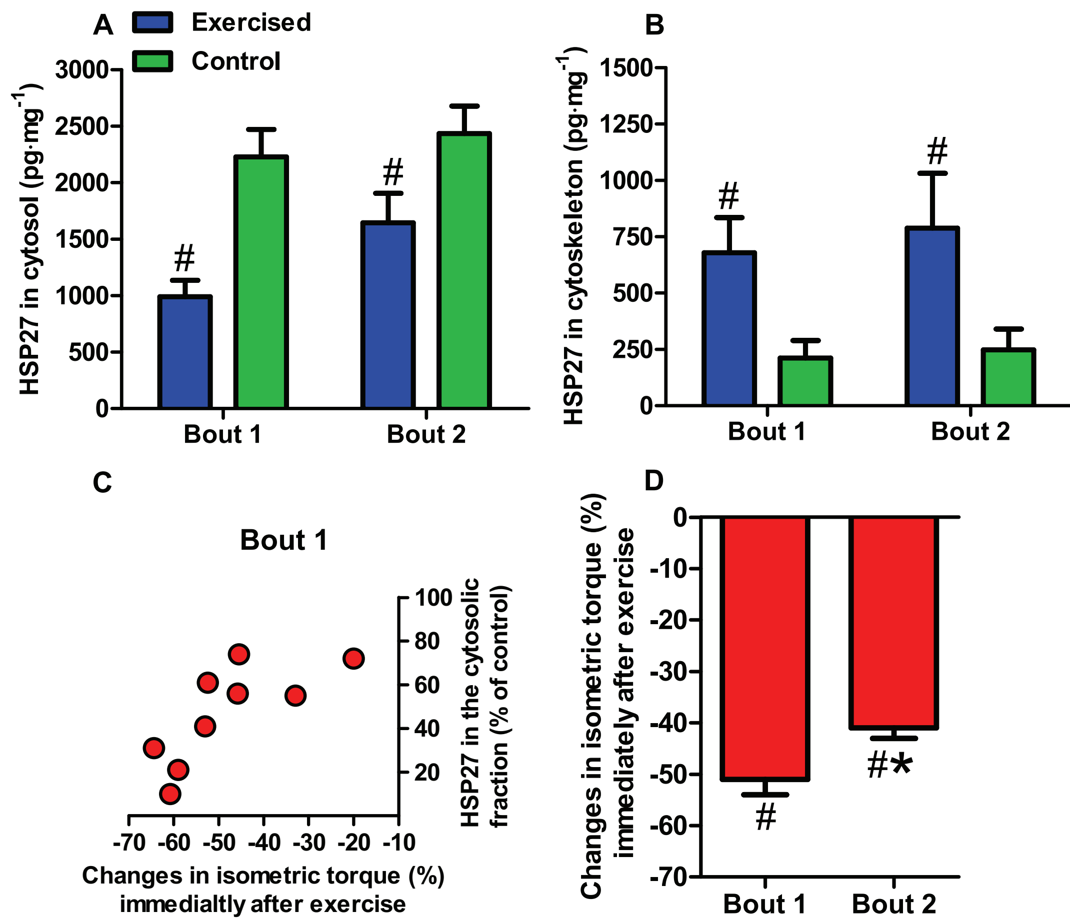

Figure 4.18. Changes in the HSP27 levels in the cytosolic and cytoskeletal (myofibrillar) fraction in relation to changes in the force-generating capacity one hour after the first and second bout (bout 1 and 2). **A** and **B**: In the exercised muscles the HSP27 levels were reduced in the cytosolic fraction and increased in the cytoskeletal fraction one hour after both bouts. Although the reduction of cytosolic HSP27 tended to be less after bout 2 than bout 1, the levels in the cytoskeletal fraction were comparable between bouts **C**: There was a correlation ($r = 0.80$; $p = 0.01$) between reduced force-generating capacity and the reduction of HSP27 in the cytosolic fraction after bout 1. **D**: The reduction in force-generating capacity was less immediately after bout 2 than bout 1. # denotes significantly different from control ($p < 0.01$). ***** denotes significant difference between bout 1 and bout 2 ($p < 0.01$). Data from the arm experiment.

Strengthening and remodelling of the myofibrilar structure

In addition to the HSP-system, it is plausible that the myofibrillar structure and surrounding cytoskeleton were reinforced between the first and the second bout. In fact, protein measurements showed increased desmin content one week after the first bout, as well as after the second bout (paper IV). This finding corresponded with our histological observations (Figure 4.8) and the results of others (21; 107; 122). A remodelling process is suggested to take place after unaccustomed high-force exercise in humans (121; 122; 453; 455), and increased number of sarcomeres in series has been suggested as one important mechanism behind the repeated-bout effect (52; 237; 238; 259; 328). Interestingly, we found two indications of a change in the optimum angle for torque generation (data not shown). First, during the second bout of eccentric actions, the subjects, on average, reached peak torque in each of the 14 sets with a slightly larger elbow angle (longer muscle length) compared to the first bout. This difference was most evident during the first three sets. Second, the angle of peak torque during maximal concentric actions tended to be greater (reached at longer muscle lengths) in tests conducted just prior to the second bout than the initial bout. Corresponding findings have recently been reported by others (46; 64; 261), and might reflect changes in the length-force relationship due to increased number of sarcomeres in series. Nonetheless, Chen *et al*. (64) gathered data indicating that such adaptation occurred only after maximal eccentric exercise, while the repeated-bout effect was achieved with submaximal eccentric exercise as well. Consequently, changes in the angle of peak torque cannot alone explain the repeated-bout effect. In line with this, we observed also increased angle of peak torque the leg experiment, but the effect was lost within four days. This suggests that a very strong stimulus is needed to initiate long-lasting changes in the angle-torque relationship (plausibly are several consecutive bouts necessary for significant sarcomerogenesis to occur).

4.3.4 Methodological concerns

Increased levels of myoglobin and CK in the circulation after exercise, especially three to seven days after exercise, indicate altered traffic over the myofibre membrane (Figure 4.7; paper I, II and IV). This was probably due to membrane damage and loss of intracellular substances to the circulation. Since the concentration of soluble HSPs in the myofibres is relatively high and because there is an efflux gradient (185), we can speculate about loss of HSPs to extracellular compartments – together with myoglobin and CK. In our assays for determination of HSP content, we analysed test samples that contained a certain amount of protein (after total protein quantification). This implies that if the HSPs are lost over the membrane in the same magnitude as (the mean of) other proteins, the analyses should fairly reflect the changes in the HSP27 protein levels. However, if the HSPs were preferentially lost (e.g., the small HSPs might escape more easily than larger proteins) or actively released (HSP70), we may have underestimated the HSP production in the exercised muscle. Consequently, we did not normalise against a control protein (e.g., GAPDH) in the western blotting analyses because we do not know how the levels of such proteins are altered over time after subcellular and membrane damage.

The protein measurements in the cytoskeletal/myofibrillar fraction frequently showed large variation when run as duplicates and triplicates (i.e., CV > 10%), which seemed to be due to the heterogeneous nature of the fraction. As for the cytosolic fraction, we did not normalise for a loading control protein. In fact, actin, a typical cytoskeletal/myofibrillar loading control protein (403; 405), was demonstrated to markedly decrease in the days after a protocol of eccentric actions in mice (~ 20% reduction after five days (172))[72]. When we analysed the HSPs together with desmin (on the same immunoblot membrane) we found that the desmin levels were stable at early time points (1 and 48 hours), but were elevated one week after the arm exercise. Thus, some proteins might decrease while others increase, and “protein

[72] The loss of force-generating capacity and recovery were comparable with some high responders in our leg and arm experiments.

normalisation" could result in under- or overestimation of protein levels. Importantly, we always compared the protein levels from the exercised muscle samples with the control samples, placing the samples side-by-side during electrophoresis.

4.3.5 Conclusion

The HSP response after the leg exercise and the arm exercise was quite similar. The first phase of the HSP response involves binding of the small HSPs (HSP27 and αB-crystallin) to cytoskeletal and myofibrillar structures. This translocation was related to the amplitude of stress and damage, as we found correlations between the reduction in force-generating capacity and the HSP response. In agreement with previous observations (e.g., (122)), we found that the Z-disks and the intermediate protein desmin appear to be especially vulnerable to exercise-induced damage, as the small HSPs preferentially accumulated at these sites. Our data support the hypothesis put forward by Koh (192), stating that the small HSPs are important in protecting and stabilising cytoskeletal and myofibrillar structures during and after the stress of high-force exercise.

The increased mRNA expression that preceded the increased protein levels, particularly HSP70, might suggest a role for the HSPs in the recovery and remodelling processes after high-force exercise. Additionally, the up-regulation of the HSPs could be part of the increased protection against exercise-induced damage from repeated bouts of exercise. The high HSP content in the cytoskeletal/myofibrillar structures after the second bout, despite less damage than after the first bout, implies that the HSPs gave enhanced protection during the second bout. Unfortunately, our experimental approach did not allow for cause and effect relationships to be established. Other mechanisms behind the repeated-bout effect could be reinforcement of the cytoskeletal/myofibrillar structure, e.g., via increased desmin content, and sarcomerogenesis (increased number of sarcomeres in series).

In light of the development of the inflammatory reaction, it seems like the HSP response is more or less independent of the accumulation of leukocytes in our experiments, at least at early time points. It is plausible, however, that the increase in HSP70 was an important event, allowing the myofibres to cope with the disturbance of homeostasis (415), and thereby avoid necrosis and inflammation. On the other hand, HSP70 might have functioned as an immune-stimulating signal when released to the extracellular environment from un-saveable myofibres that ultimately became necrotic (11). The relationship between the HSP response and the inflammatory reaction in skeletal muscle needs further investigation.

5.0 SUMMARY AND CONCLUSION

5.1 Main findings concerning the inflammatory response:

- Unaccustomed maximal eccentric exercise initiated an inflammatory reaction in the exercised muscles. In about ⅓ of the subjects the number of leukocytes increased markedly for days following exercise and a high number of accumulated leukocytes was associated with necrotic segments of myofibres.
- Increased numbers of monocytes and/or macrophages, but not neutrophils, were found histologically in the exercised muscle tissue. The neutrophils appeared to accumulate in the exercised muscle (observed by scintigraphy), but were restricted to the luminal side in the microcirculation.
- The leukocyte accumulation in the exercised muscles was related to the reduced force-generating capacity seen after exercise. However, our data did not allow us to determine whether leukocytes affect the recovery of muscle function after eccentric exercise in humans.
- A COX-2 inhibitor (celecoxib) did not influence the recovery of muscle function after muscle damaging eccentric exercise, nor did it affect the adaptation processes manifested as the repeated-bout effect.
- Delayed onset muscle soreness (DOMS) could not be explained by markers of inflammation, but was attenuated by the COX-2 inhibitor celecoxib.

5.2 Main findings concerning the heat shock proteins (HSPs):

- The small HSPs, HSP27 and αB-crystallin, translocated to cytoskeletal/myofibrillar structures (probably) during exercise, while HSP70 translocated primarily after exercise. The translocation of HSP27 was associated with the reductions in force-generating capacity (muscle damage) immediately after exercise and the detachment from the cytoskeletal/myofibrillar structures occurred concomitantly with recovery of

muscle function. At the ultrastructural level of samples from exercised muscles, the small HSPs were found to accumulate at Z-disks and disrupted areas. αB-crystallin also accumulated on desmin-like structures.

- Increases in mRNA levels of HSP27, αB-crystallin, and particularly HSP70 during the first 24 hours after exercise were followed by increases in protein levels in the days after exercise.
- The accumulation of HSPs on cytoskeletal/myofibrillar structures appeared to be equally strong or stronger after a second bout of exercise. This can be interpreted as increased protection against muscle damage.
- Increased levels of the intermediate protein desmin indicated that some of the increased resilience towards muscle damage after a repeated bout is due to reinforcement of the myofibrillar structure.

A proposed sequence of events occurring after muscle-damaging eccentric exercise is summarised in Figure 5.1.

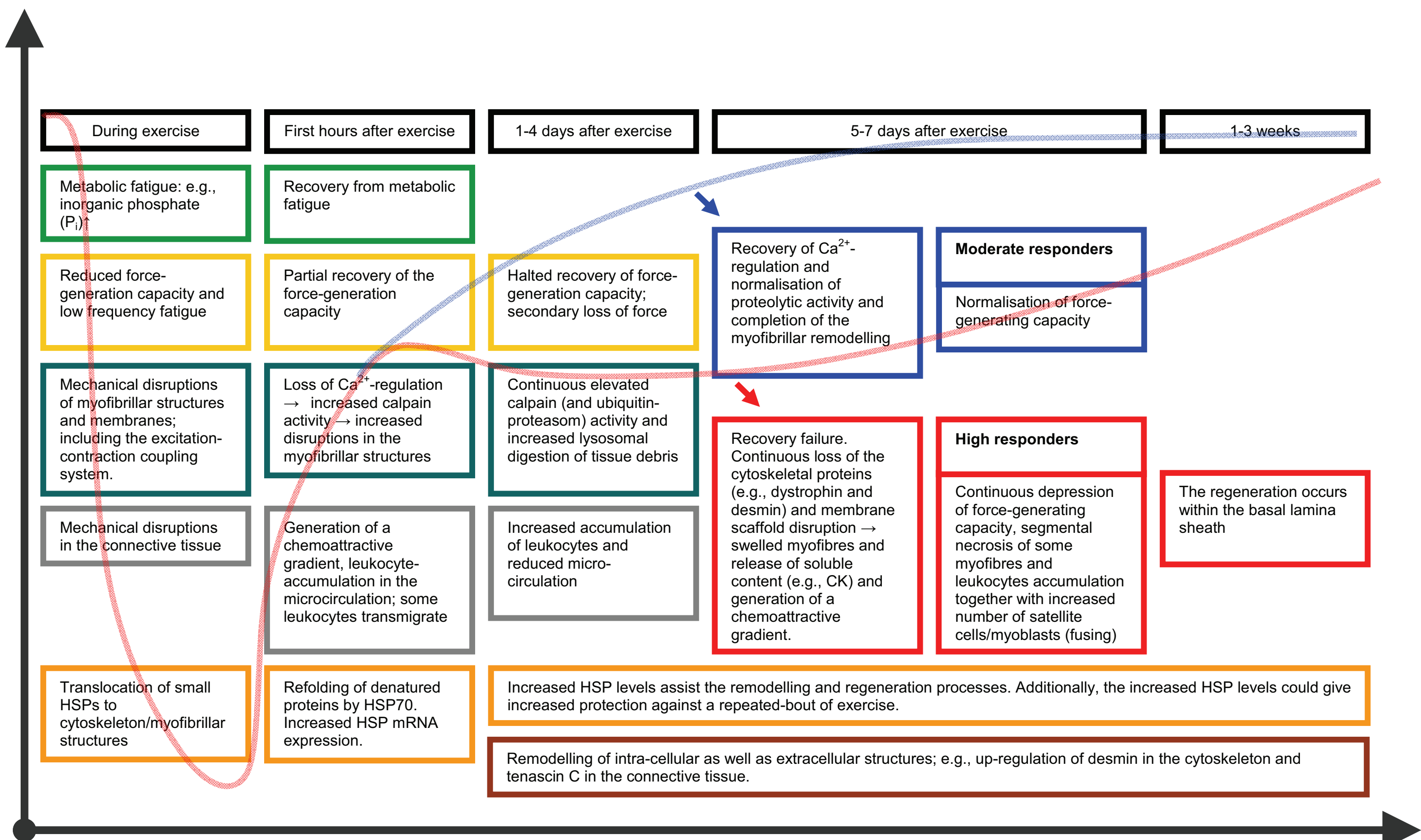

Figure 5.1. Summary of findings together with purposed events and mechanisms explaining the recovery time course after an unaccustomed bout of maximal eccentric exercise for high-responder subjects (red line and red boxes) and moderate-responder subjects (blue line and blue boxes).

6.0 PERSPECTIVIES

The impact of the inflammatory reaction in exercised muscles in humans is troubling. The literature is equivocal, both within human research and in particular between humans and other experimental species. For example, studies on animals have found that skeletal muscles are invaded by neutrophils after passive stretching of the muscles (within the physiological range (195)), and that level running protocols can result in accumulation in the exercised muscles, as well as other organs such as the heart and liver (29; 338). These findings seem to be in sharp contrast with human studies, where leukocytes often have not been observed at all in the exercised muscles (e.g.: (107; 122; 455)). Intriguingly, recent reviews in which the role of neutrophils and macrophages in skeletal muscles are described almost exclusively based on animal studies, but it is still obvious that this knowledge is intended to be of relevance for humans (e.g.: (51; 407; 411)). I claim that more investigation is needed in order to adequately describe the inflammatory reaction in humans after different types of exercises and training. An important prerequisite is the use of appropriate methods to detect the inflammatory reaction, e.g., highly specific antibodies must be applied to muscle samples in order to elucidate the players. Important players are neutrophils, different subpopulations (activation modes) of macrophages, and fibroblasts. Fibroblasts, in particular, are under-studied in skeletal muscle. Furthermore, the biopsy collection could be more effectively guided to areas of interest when used in combination with non-invasive methods (e.g., scintigraphy or magnetic resonance imaging [MRI]). With respect to the inflammatory reaction, discussions about the exercise protocols are hitherto nearly neglected in the exercise physiology literature. The impact of the exercise protocol must be evaluated by careful monitoring of changes in muscle function. As the variation in individual response to any given exercise protocol is notoriously large, there is an absolute need to assess histological findings in light of changes in muscle function.

Determining the impact of the inflammatory reaction on muscle function *per se* in humans has proven to be elusive. A challenge for future research is how to intervene with the inflammation process. NSAIDs appear not to be the best choice. One attractive approaches is infusion of antibodies to block the transmigration of leukocytes (e.g., antibodies for cell adhesion molecules (82)). However, this is obviously ethically problematic in humans, as it will reduce the capacity of the innate immune system to respond to invading microbes.

Heat shock proteins (HSPs) are certainly a “hot” topic in several fields of science as they are involved in a vast number of physiological and pathological processes. In the field of exercise physiology, we are still in the phase of merely describing the different aspects of the HSP response that can be observed after exercise and training. A number of HSPs, e.g., HSP40, HSP47, HSP90 and HSP110, have hardly been touched upon in skeletal muscle. The involvement of the HSPs in the inflammatory reactions also warrants future investigation. A feasible approach is to investigate the HSPs content in the interstitial fluid by microdialysis and to follow this with immunohistochemistry to capture HSPs, leukocytes, and potential receptors for the HSPs (e.g., TLR2, TLR4 and CD91; (12; 24)) in the tissue samples from exercised muscles.

An interesting, and highly feasible approach is to test the cytoprotective role of the HSPs in muscles exposed to high-force exercise by preconditioning of the muscles with microwave diathermy treatment. Nosaka *et al.* (301) have already demonstrated that such treatment can enhance recovery of muscle function, so the next step would be to replicate this experiment with biopsies and immunoassays to assess the HSP response.

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ERRATA

Changes made after submitting the thesis for evaluation 01.04.09:

1. Language corrections have been made throughout the thesis to improve readability, but the presented data and interpretations are not altered.
2. Figure 8. in Paper I was erroneously lacking and has now been inserted.

APPENDIX I

Appendix I: A: Human studies that have investigated the presence of leukocytes in biopsy samples obtained after types of exercise that apparently inflicted "low" degree of muscle damage, as judged from changes in muscle function.

Study	Exercise	Muscle biopsed and time points	Control biopsy?	Leuko-cytes?	Neutro-phils?	Macro-phages?	Acute reduction in force (%)	Recovery	Myofibrillar disruptions?	Necrosis?	CK-values ($IU \cdot L^{-1}$)
Bourgeois *et al.* 1999	Resistance exercise (concentric/eccentric): Leg press and knee-extension (single leg), 6 x 10 repetitions; 80-85% of 1 RM.	VL: 1d	1d (contra-lateral leg)	**No**	NA	NA	NA	~3% under pre-value 2d after exercise	NA	NA	~600-800
Féasson *et al.* 2002	30 min downhill running at 12°; 11 $km \cdot h^{-1}$; 54% of $VO2_{max}$)	VL: 0h and 1 and 14d	Pre	**No**	NA	NA	~15 (max power during cycling)	Within 7d	Yes	No	~1000
Malm *et al.* 2004	45 min downhill running at either 4° (50% of VO_{2max}) or 8° (max tolerated speed)	VL: 2d	Control group	**No**	No	No	≤15 (isometric)	Within 2d	NA	No	< 500
Crameri *et al.* 2007	210 max eccentric actions using one randomly chosen leg. The exercise had two phases: 100 actions at $30° \cdot s^{-1}$ and 110 actions at $180° \cdot s^{-1}$. ROM: 0-90°.	VL: 5h, 1, 4 and 8d	Pre	**No**	NA	No	~16% (isometric)	Within 4d	Yes	No	NA

0h: immediately after exercise
MC: Mononuclear cells
NA: Not analysed
VL: m. vastus lateralis
Sol: m. soleus
Gast: m. gastrocnemius
PL: m. peroneus longus
TA: m. tibialis anterior

Appendix I: B: Human studies that have investigated the presence of leukocytes in biopsy samples obtained after types exercise that apparently inflicted “moderate” degree of muscle damage.

Study	Exercise	Muscle biopsed and time points	Control biopsy?	Leuko-cytes?	Neutro-phils?	Macro-phages?	Acute reduction in force (%)	Recovery	Myofibrillar disruptions?	Necrosis?	CK-values ($IU \cdot L^{-1}$)
Fridén *et al.* 1983	Eccentric cycling exercise for 30 min at an intensity corresponding to 80-100% of VO_{2max} during concentric cycling	VL: 0h and 3 and 6d	Pre	**No**	NA	NA	~24% (isometric)	Within 6d	Yes	No	NA
Crenshaw *et al.* 1994, 1995	Max eccentric actions ($90° \cdot s^{-1}$) with the knee-extensors in one leg until exhaustion; the other (randomly chosen) leg performed concentric work. ROM: 30-120°.	VL: 2d	2d ("concentric leg")	**No**	NA	NA	NA	~30/36 (isometric/ concentric), 2d after exercise	Yes	No	NA
Stupka *et al.* 2001	36 eccentric actions of leg press and 100 isolated eccentric actions at 120% of max concentric strength, using the knee-extensors in one leg. ROM: 15-90° (both exercises). Two bouts separated by 5.5 wk. The weakest leg (evaluated from a pre-test) was exercised.	VL: 2d	2d	**Yes**	Yes	Yes	~31 (concentric)	Within 7d	Yes	NA	200-2100 (♀ and ♂)
Beaton *et al.* 2002	240 max eccentric actions using the knee-extensors in one leg. ROM: 50-120°.	VL: 1d	1d (contra-lateral leg)	**Yes**	No	Yes	~50 (concentric/ isometric)	Within 4d	Yes	NA	~300
Beaton *et al.* 2002	300 max eccentric actions using the knee-extensors in one randomly chosen leg. ROM: 60-120°.	VL: 4h and 1d	Pre	**Yes**	Yes	Yes	~54 (isometric)	Within 1 week	Yes	NA	~300
Hubal *et al.* 2008	300 eccentric and concentric actions. The subjects rose from a chair with one randomly chosen leg and lowered the body weight back to a seating position with the other leg (20 cm hip displacement).	VL: 6h	6h ("concentric leg")	**Yes**	NA	Yes	~32 (isometric)	~23% under pre-value 5d after exercise	NA	NA	NA

Appendix I: C: Human studies that have investigated the presence of leukocytes in biopsy samples obtained after types of exercise that apparently inflicted “large” degree of muscle damage.

Study	Exercise	Muscle biopsed and time points	Control biopsy?	Leuko-cytes?	Neutro-phils?	Macro-phages?	Acute reduction in force (%)	Recovery	Myofibrillar disruptions?	Necrosis?	CK-values ($IU \cdot L^{-1}$)
Hellsten *et al.* 1997	Max eccentric cycling; 5 x 5 min (using the knee-extensors)	VL: 45 min, 1, 2 and 4d	Pre, 45 min and 2d	**Yes**	NA	NA	~54 (isometric)	~45% under pre-value 4d after exercise	NA	Not mentioned	~13300
Jones *et al.* 1986	1) Eccentric exercise using the arm flexors until 50% loss of force 2) Backwards downhill walking (large strain on the calf muscles)	BB and Sol/Gast: 4, 5, 7, 8, 9, 10, 12, 14 and 20d	No	**Yes (MC)**	NA	NA	>50% (eccentric)	NA	NA	Yes	~750-75000 (range)
Round *et al.* 1987	1) Eccentric exercise using the arm flexors until 50% loss of force 2) Backwards downhill walking (large strain on the calf muscles)	BB and Sol/Gast: 4, 5, 7, 8, 9, 10, 12, 14 and 20d	One biopsy from one subject	**Yes**	NA	Yes	>50% (eccentric)	NA	NA	Yes	~750-75000 (range)
Child *et al.* 1999	70 max eccentric actions ($100° \cdot s^{-1}$) with the knee- extensors in one randomly chosen leg. ROM: almost full extension to almost full flexion (subjects in prone position).	VL: 4 and 7d	Pre	**Yes (MC)**	NA	NA	~50% (isometric)	NA	NA	Yes	~16000
Hikida *et al.* 1983 + Sherman *et al.* 1984	Marathon (running)	Gast: 0h and 1, 3, 5, and 7d	Pre	**Yes**	Yes	Yes	~47 (concentric)	> 7d	Yes	Yes	NA

Appendix I: D: Studies in which no leukocytes were found after voluntary exercise in humans (muscle function not assessed).

Study	Exercise	Muscle biopsed and time points	Control biopsy?	Leuko-cytes?	Neutro-phils?	Macro-phages?	Acute reduction in force (%)	Recovery	Myofibrillar disruptions?	Necrosis?	CK-values ($IU \cdot L^{-1}$)
Fridén *et al.* 1981	Running down stairs: 10 x 10th floor to ground floor.	Sol: 2 and 7d	Pre	**No**	NA	NA	NA	NA	Yes	No	NA
Kuipers *et al.* 1985	30 min of eccentric cycling exercise at 80% VO_{2max}	VL: 0h and 1d	Pre	**No**	NA	NA	NA	NA	No	NA	< 200
Warhol *et al.* 1985	Marathon (running)	Gast: Some hours, 1, 2, 3, 5, 7, and 10d and 2, 3, 4, 6, 8, 10 and 12 weeks	Control group	**No**	NA	NA	NA	NA	Yes	Yes	NA
Nurenberg *et al.* 1992	30 min downhill running at 8°; 8 $km \cdot h^{-1}$	Sol, Gast, TA, PL: 2d	No	**No**	NA	NA	NA	NA	Yes	No	~500
Malm *et al.* 2000	Eccentric cycling exercise for 30 min at an intensity corresponding to 80-100% of VO_{2max} during concentric cycling (250-300 W).	VL: 0 and 6h and 1, 2, 4 and 7d	Pre and control group	**No**	No	No	NA	NA	NA	No	~120
Yu *et al.* 2002a, b, 2003, 2004	1) Running down stairs: 15 x 10th floor to ground floor; 2) Eccentric cycling (see Malm *et al.* 2000); 3) Downhill running (8°; see Malm *et al.* 2004)	Sol: 1h, 2-3 and 7-8d; see Malm *et al.* 2000 and 2004	Control group	**No**	NA	NA	See Malm *et al.* 2004	See Malm *et al.* 2004	Yes	No	See Malm *et al.* 2000
Dennis *et al.* 2004	Resistance exercise (concentric/eccentric): Leg press and knee-extension (both legs): 4 x 8 reps (to failure in the 4th set) at 80% of 1 RM.	VL: 1 and 3d	Pre	**No**	NA	NA	NA	NA	NA	NA	NA
Przybyla *et al.* 2006	Resistance exercise (concentric/eccentric): Leg press and knee-extension (both legs): 4 x 8 reps (to failure in the 4th set) at 80% of 1 RM.	VL: 3d	Pre	**No**	No	No	NA	NA	NA	NA	NA

Appendix I: E: Studies in which leukocytes were found after voluntary exercise in humans (muscle function not assessed).

Study	Exercise	Muscle biopsed and time points	Control biopsy?	Leuko-cytes?	Neutro-phils?	Macro-phages?	Acute reduction in force (%)	Recovery	Myofibrillar disruptions?	Necrosis?	CK-values ($IU \cdot L^{-1}$)
O'Reilly *et al.* 1986	Eccentric cycling for 45 min (3 x 15 min) at 70-90% of VO_{2max} (during concentric cycling; ~200 W)	VL: 0h and 10d	Pre	Yes	NA	Yes	NA	NA	Yes	Yes	NA
Costill *et al.* 1990	Eccentric actions using the knee-extensors in one leg; 10 x 10 repetitions with 120% of 1 RM. 30 min after the eccentric exercise the subjects performed a cycling exercise to deplete their glycogen stores (both legs).	VL: 1.5h and 1 and 3d	1.5h and 1 and 3d (contra-lateral leg)	Yes	Yes	Yes	NA	NA	NA	NA	~7000
Stauber *et al.* 1990	70 max eccentric action (120°·-1) using the arm flexors in the non-dominant arm. ROM: 120°.	BB: 2d	2d (contra-lateral arm)	Yes	NA	Yes	NA	NA	NA	Yes	NA
Widrick *et al.* 1992	Eccentric actions using the knee-extensors in one leg; sets of 6 repetitions until failure with 120% of 1 RM. Subjects had the evening before performed a cycling exercise to deplete their glycogen stores (both legs).	VL: 0 and 6h and 1 and 3d	Immediately, 6h and 1 and 3d (contra-lateral leg)	Yes	NA	NA	NA	NA	NA	Yes	< 300
Crenshaw *et al.* 1993	Ultramarathon footrace (160 km)	Gast: 1d	No	Yes	NA	Yes	NA	NA	Yes	Yes	NA
Fielding *et al.* 1993	45 min downhill running at 16°; 75% max heart rate.	VL: 45 min and 5d	Pre	Yes	Yes	NA	NA	NA	Yes	NA	~300
Peterson *et al.* 2003	10-14 sets of 10 actions with knee-extensors in the non-dominant leg. Load: 120% of max concentric strength. ROM: 0-90°.	VL: 1d	Pre (contra-lateral leg)	Yes	No	Yes	NA	NA	NA	NA	~1000-5000
Mahoney *et al.* 2008	300 max eccentric actions ($120° \cdot s^{-1}$) using the knee-extensors in the non-dominant leg. ROM: 30-90°.	VL: 3h and 2d	Pre	**Yes**	Yes	Yes	NA	NA	Yes	NA	< 300

APPENDIX II

Appendix II. Summery of studies that have investigated the heat shock protein (HSP) response after different types of exercise.

Study	Exercise and intervention	Muscle biopsied, time points and analysis techniques	mRNA level/ gene expression post exercise	Protein level post exercise	Localization post exercise	Comment
Folkesson *et al.* 2008	**Resistance exercise** (RE): Totally 80 reps at 70% of 1RM **Endurance exercise** (EE): One-leg cycling either at 40% or 75% of VO_{2max} for 30 min	VL: 0h IHC			HSP27 staining immediately after RE: Muscle fibres showed granular cytoplasmic accumulations. Immediately after EE: No granular pattern	
Mahoney *et al.* 2008	Eccentric exercise; 300 repetitions (one-legged squat)	VL: 3h and 2d Microarray, RT-PCR	**DNA level 3h**: DnaJ A1↑ DnaJ B2↑ DnaJ C1↑ HSF4 ↑ HSP70↑ Protein kinase H11↑ **mRNA level 3h**: DnaJ B2↑ Protein kinase H11↑			
Vissing *et al.* 2008	Bench-stepping exercise; one leg up (concentric) and the other down (eccentric). Repeated bout after 8 weeks.	VL: 3h, 24h and 7d WB, NB	**Bout 1:** **Eccentric 3 and 24h:** HSP27↑ αB-crystallin↑ HSP70↑ **Concentric 3 and 24h::** HSP27↑ αB-crystallin↑ HSP70↑ **Bout 2:** **Eccentric 3 and 24h:** HSP27↑ αB-crystallin↑ HSP70↑	**Bout 1 and 2; 3h, 24h and 7d:** HSP27↔ αB-crystallin↔ HSP70↔	**Bout 1:** HSP27 and HSP70↑ in the cytoskeletal fraction after eccentric exercise 3h and 7d, respectively. No changes after concentric exercise. **Bout 2:** HSP27↑ in the cytoskeletal fraction after eccentric exercise 3h (bout 2 < 1)	Homogenized muscle samples were fractionated to an cytosolic and cytoskeletal fraction

Appendix II continued…

Study	Exercise and interventions	Muscle biopsied, time points and analysis techniques	mRNA level/ gene expression post exercise	Protein level post exercise	Localization post exercise	Comment
Tupling *et al.* 2007	Knee-extensor exercise at 60% of their maximal voluntary contraction with 5 sec contraction and 5 sec relaxation for 30 min	VL: 0h, 1d, 2d, 3d and 6d WB, IHC		HSP70↑ 1-6d	Staining intensity of HSP70↑ in type I fibers	Whole muscle homogenate
Watkins *et al.* 2007	Two 30 min bouts of cycling at 75% of $VO_{2\ peak}$ in hot (39°C) and cold (9°C) environment	VL: 6h ELISA		HSP70↔		
Morton *et al.* 2006	45-min “non-damaging” treadmill running protocol	VL: 0h, 1d, 2d, 3d and 7d WB		**2-7d:** HSP70 ↑ HSC 70 ↑ HSP60 ↑ HSP27 ↔ αB-crystallin ↔		Supernatant of centrifugated whole muscle homogenate
Thompson *et al.* 2003	50 maximal eccentric actions using the arm flexors and downhill running (10°) for 30 min	BB and VL: 2d WB, RT-PCR	**Arm flex. (BB):** HSP27↑ HSP70↑ **Downhill running (VL):** HSP27↔ HSP70↔	**Arm flex. (BB):** HSP27↑ HSP70↑ **Downhill running (VL):** HSP27↔ HSP70↔		Whole muscle homogenate. Increased expression and phosphorylation of JNK and ERK (MAPK pathways) were more evident in BB than VL.
Khassaf *et al.* 2003	One-legged cycling with 70% of $VO_{2\ max}$ for 45 min. Subjects divided into two groups: 1: “Vit-C”: received 0.5 g vitamin C per day for 8 weeks prior to exercise 2: “Placebo”	VL: 2d WB		**Placebo:** HSP60↑ (tendency) HSP70↑ **Vit-C:** HSP60↔ HSP70↔		Supernatant of centrifugated whole muscle homogenate

Appendix II continued…

Study	Exercise and interventions	Muscle biopsied, time points and analysis techniques	mRNA level/ gene expression post exercise	Protein level post exercise	Localization post exercise	Comment
Féasson *et al.* 2002	30 min treadmill downhill running (12°) at 54% of VO_{2max}	VL: 0h, 1d and 14d WB		1d and 14d: HSP27↑ αB-crystallin↑		Whole muscle homogenate
Thompson *et al.* 2002	50 maximal eccentric actions using the arm flexors Repeated bout 4 weeks after first exercise protocol	BB: 2d WB		**Bout 1:** HSP27↑ HSP70↑ **Bout 2:** HSP27↑ HSP70↑		Whole muscle homogenate. The HSP27 and HSP70 response were relatively similar between bouts; but the control (basal) values appeared to be lower after bout 2 than bout 1.
Thompson *et al.* 2001	50 maximal eccentric actions using the arm flexors	BB: 2d WB		HSP27↑ HSC/HSP70↑		Whole muscle homogenate
Khassaf *et al.* 2001	Unilateral cycling at 70% VO_{2max} for 45min	VL: 1d, 2d, 3d, and 6d WB		HSP60↑ 3d HSP70↑ 6d		
Febbraio and Koukoulas 2000	Cycling until exhaustion at workload corresponding to 63% peak O_2 uptake	VL: 10 min after start of exercise, 40 min prior to fatigue and at fatigu; RT-PCR	HSP72↑ (during exercise)			
Puntschart *et al.* 1996	30 min “non-damaging” treadmill running protocol	VL: 0h, 0.5h and 3h RT-PCR, WB	**0-3h:** HSP70↑	HSP70↔		Whole muscle homogenate

↑ Increase
↔ No change
↓ Decrease

BB: m. biceps brachii
VL: m. vastus lateralis

ELISA: Enzyme-linked ImmunoSorbent Assay (protein)
IHC: Immunohistochemistry (protein localisation)
NB: Northern blotting (mRNA)
RT-PCR: Real-time PCR (mRNA)
WB: Western Blotting (protein)

HSF: Heat shock factor
MAPK: Mitogen activated protein kinase
RM: Repetition Maximum
h/d: Hours/days
$VO_{2\ max/peak}$ Maximal/peak oxygen uptake

PAPERS I-IV

PAPER I

TITLE:

Time course of leukocyte accumulation in human muscle after eccentric exercise

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Running head:

Inflammation, recovery, and muscle soreness

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ABSTRACT

PURPOSE: To investigate the time course of leukocyte accumulation in eccentric exercised human muscles and its relation to recovery of muscle function and soreness. METHODS: Eleven young males performed 300 unilateral, maximal voluntary, eccentric actions with m. quadriceps femoris ($30°\cdot s^{-1}$). Before and at regular intervals for seven days after exercise, force-generating capacity was measured with maximal concentric knee-extensions ($60°\cdot s^{-1}$). Accumulation of radiolabeled (autologous) leukocytes was measured with scintigraphy. Biopsies from m. vastus lateralis were obtained 0.5, 4, 8, 24, 96, and 168 hours after exercise from both the exercised and control leg. Muscle cross-sections were stained with antibodies against leukocytes (CD16 and CD68). Muscle soreness was rated on a visual analogue scale. RESULTS: Immediately after exercise the subjects' ability to generate force was reduced by 47±5%. Muscle function recovered slowly, and was not fully restored after one week. Radiolabeled leukocytes accumulated in the muscles during the first (3-24) hours after exercise, and leukocytes were at the same time observed histologically, primarily in the endo- and perimysium. A part of the accumulated radiolabeled leukocytes appeared to be located within local blood vessels. The highest numbers of $CD16^+$ and $CD68^+$ cells were found four and seven days after exercise. There was a positive correlation between accumulation of radiolabeled leukocytes and muscle weakness measured 1-3 days after exercise ($r = 0.8$, $p < 0.05$), and, surprisingly, a negative correlation between radiolabeled leukocyte accumulation and muscle soreness ($r = -0.96$, $p < 0.01$). CONCLUSION: Exercise-induced muscle damage initiated a rapid local inflammatory response that gradually increased over the next days. Halted recovery of muscle function was associated with local accumulation of leukocytes, whereas muscle soreness could not be explained by the presence of leukocytes.

INTRODUCTION

Paragraph 1.

Unaccustomed high-force eccentric exercise and extreme forms of exercise performed by athletes, such as marathon running, have been reported to cause muscle damage and accumulation of inflammatory cells (15;36). An acute, local inflammation is initiated by a rapid extravasation of fluid and blood-borne neutrophilic granulocytes into the damaged tissue (21). Peak accumulation of neutrophils occurs after 1-2 days, overlapped in time by accumulation of monocytes/macrophages, which peaks after several days (21). However, because of the methodological limitations of repeated biopsies and radionuclide imaging (radiolabeling of leukocytes), a detailed time course of the local inflammatory response to exercise in humans has so far not been established (23;29;37). In particular, the first 24 hours after exercise have scarcely been investigated, and no study has hitherto aimed to explore development of the inflammatory reaction after exercise-induced muscle damage, using repeated biopsies during this time interval. Labeling and tracking of leukocytes (primarily neutrophils) with radionuclide imaging techniques have indicated that blood borne leukocytes begin to accumulate in the exercised muscles immediately after exercise (20;33). However, such techniques give no information about the diapedesis of leukocytes. Thus, it is not possible to distinguish between the radiolabeled leukocytes that accumulate in the blood vessels, attach the endothelial wall, and those that actually have migrated into the tissue. Furthermore, it has recently been pointed out that published claims concerning animal and human studies are inconsistent, in respect to the accumulation of neutrophilic granulocytes in exercise-injured muscles: Neutrophils, in contrast to macrophages, have in some experiments not been observed at all (37).

Paragraph 2.

From animal studies it appears that inflammatory events can explain certain changes in muscle function (9). Interruption of recovery and a secondary loss of force, seen 1-3 days after exercise, have repeatedly been observed together with marked tissue damage and an

inflammatory reaction (9). Furthermore, pharmacological, antibody-mediated and genetic (knockout) inhibition of the inflammatory reaction have reduced signs of muscle damage and the secondary loss of muscle force (5;19;30). Altogether, these findings suggest a cause-effect relationship between the inflammatory response and the secondary loss of force. As the exercise protocols used in animal studies in some extent are meant to model hard, human exercise, it is not unreasonable to assume that the inflammatory process may affect the recovery of muscle function in humans as well (9;21). In fact, biphasic or bimodal recovery of muscle function has been observed in human subjects (20;31;33), and accumulation of radiolabeled leukocytes in the exercised muscles have been found to forerun the secondary loss of force-generating capacity (20;33). Nevertheless, this has not been convincingly supported by biopsy methods documenting and enumerating leukocytes in the muscle tissue (23).

Paragraph 3.

The time course of leukocyte accumulation after eccentric exercise seems to mirror not only changes in muscle function, but also that of delayed onset muscle soreness (DOMS; (9;21;38)). The mechanistic link might be a leukocyte-mediated release of cytotoxic and algetic substances (6;9). In particular, reactive oxygen species (ROS) from neutrophils and macrophages seem to increase the muscle damage (41), whereas eicosanoids may be mediators of DOMS (1;6;38). However, the relationship between these events has not been explored thoroughly in humans. Noticeably, some investigators have even questioned the view that eccentric exercise can initiate an inflammatory response in the exercised muscles and/or that DOMS can be a consequence of this inflammation (see e.g. (23;26;44)).

Paragraph 4.

The purpose of this study was to explore the time course and location of leukocyte accumulation after unaccustomed eccentric exercise, with emphasis on the first 24 hours. This is the first study in which analyses of multiple biopsies from exercised and a non-exercised,

control muscles have been combined with scintigraphic imaging of transfused, autologous, radiolabeled leukocytes. These combined procedures make it possible to determine both exact tissue localization and overall distribution of leukocytes in whole muscle. Our main hypothesis has been that leukocytes begin infiltrating the exercised muscle tissue shortly after a bout of hard exercise, accumulating rapidly during the first 24 hours, so that the accumulation of leukocytes would be closely related in time – and possibly also causally – to both the recovery of force-generating capacity and the development of muscle soreness.

MATERIALS AND METHODS

Subjects

Paragraph 5.

Eleven healthy male students (28±4 years, 1.80±0.1 m, 83±6 kg; mean ± SD) gave written, informed consent to participate in the study. The subjects' level of fitness varied due to a variable level of daily physical activity; three subjects were sedentary (subject 4, 6 and 8), whereas the other eight ranged from 'physically active' to 'very active' (exercising 4-7 days a week), but none of the subjects were engaged in heavy strength training. The study complied with the standards set by the Declaration of Helsinki and was approved by the Regional Ethics Committee of Southern Norway.

Experimental design

Paragraph 6.

Changes in muscle function were monitored for one week after a bout of one-legged, maximal voluntary eccentric exercise (300 repetitions) with the knee-extensors (m. quadriceps femoris). The other leg (randomly chosen dominant/non-dominant) functioned as control for all tests and measurements. The recovery of muscle function was assessed with repeated tests of voluntary maximal, isokinetic knee-extensions. The first test was performed before the workout to establish baseline values, the second test started approximately three minutes after exercise, and tests were thereafter repeated 6, 23, 28 hours and 2, 3, 4, and 7 days after exercise. Blood sampling for creatine kinase measurements and evaluation of muscle soreness were scheduled just before assessment of muscle function.

Paragraph 7.

Distribution of ^{99m}Technetium (radio-)labeled leukocytes was followed for 24 hours – the duration was limited by the six-hour half-life of ^{99m}Technetium. Radionuclide images (scintigrams) of both thighs (anterior and lateral views) were taken at 3, 7, 21, and 24 hours after exercise. Blood for

radionuclide-labeling of leukocytes was obtained by venipuncture at 7:30 am and the labeled leukocytes were reintroduced intravenously two hours later. This was followed by the exercise protocol, which started 11:20 am and lasted 40 minutes. Six subjects underwent radiolabeling of leukocytes and scintigraphy.

Paragraph 8.

Biopsies were collected from both exercised and control muscle 0.5, 4, 8, 24, 96, and 168 hours after exercise. With the frequent biopsy sampling during the first day we wanted to obtain a detailed time course for the initial events, including tissue accumulation of leukocytes. The later biopsy times (four and seven days after exercise) were chosen to study the relation between the prolonged reduction in force-generating capacity and muscle soreness and the prolonged presence of leukocytes. To reduce the stress on the subjects and to reduce risk of contamination of tissue damage from previous biopsies, biopsies were collected from each subject at four of the six scheduled time points (from both the exercised and control muscle). This means that biopsies from a total of seven subjects could be analyzed at each time point.

One-legged eccentric exercise

Paragraph 9.

The subjects performed 300 unilateral, voluntary maximal, isokinetic, eccentric actions ($30^{\circ}\cdot s^{-1}$) with the m. quadriceps femoris on a Cybex[6000] (Lumex, Ronkonkoma, NY, USA). The subjects sat with ~90˚ in the hip joints, fastened with seat belts and arms held crossed over the chest. The range of motion was 35-105˚ (0˚ equals full extension in the knee joint) and the workout consisted of 30 sets of 10 repetitions with 30 seconds rest in-between sets. The subjects were instructed to resist the movement maximally through the full range of motion in every repetition and received real time visual feedback on their performance on a computer screen. They were also verbally encouraged during the exercise to ensure maximal effort.

Muscle function

Paragraph 10.

Maximal force-generating capacity was measured as voluntary maximal, isokinetic, concentric knee-extension peak torque at $60^{\circ} \cdot s^{-1}$ (on the Cybex^{6000}). All subjects participated in four familiarization tests on separate days, before they entered the study. A warm-up of five minutes cycling at 100-150 W always preceded the concentric knee-extension test, in addition to four warm-up contractions on the dynamometer. The intra-individual coefficient of variation (CV) of this test was < 5%.

Radionuclide imaging: scintigraphic monitoring of leukocyte accumulation

Paragraph 11.

The method has been described in by Raastad *et al.* (33). In brief, 50 ml blood was drawn and leukocytes (mainly neutrophilic granulocytes) were isolated and labeled with ^{99m}Technetium, before re-infused. Accumulation of radiolabeled leukocytes in the subjects' thighs was quantified scintigraphically (with a gamma camera) on anterior and lateral view images (Figure 1).

<Figure 1>

Paragraph 12.

The radioactivity in different parts of the m. quadriceps and hamstrings was calculated with custom made software (GE healthcare, Oslo, Norway). Regions of interest were divided into thirteen 16 cm^2 squares (Figure 1). For each square, the number of counts corrected for background radioactivity was calculated. One large square was positioned over the whole thigh to quantify total radioactivity in m. quadriceps, and the smaller squares were used to quantify the radioactivity in different parts of the muscle. The biopsied areas in the m. vastus lateralis were omitted. The accumulation of radiolabeled leukocytes in the exercised leg was expressed as the percentage (%) difference in radioactivity from the control leg.

Paragraph 13.

Prior to exercise, marks with a waterproof pen were placed on m. vastus lateralis, m. vastus medialis, m. rectus femoris, and the muscle-tendon junction of each subject. The subjects' thighs were then photographed (Figure 1). The pictures were adjusted in size, using Adobe PhotoDeluxe (Home Edition 3.1), to fit the scintigrams. The scintigrams, which were printed on transparent paper, could be super-positioned on the photos to visualize quantification of accumulated radiolabeled leukocytes in the different parts of m. quadriceps.

Muscle biopsies

Paragraph 14.

A 5 mm Pelomi-needle (Albertslund, Denmark) with manual suction was used to obtain tissue samples (usually 3 x 50-100 mg) from the mid-section of m. vastus lateralis. Subjects were in a supine position, and the procedure was performed under local anaesthesia (Xylocain® adrenaline, 10 mg·ml^{-1} + 5 µg·ml^{-1}, AstraZeneca, Sweden). Each needle insertion was placed approximately 3 cm proximal to the last insertion to avoid affected tissue from previous biopsies. The muscle samples were rinsed in saline before visible fat and connective tissue were removed, and subsequently frozen in isopentane on dry ice and stored at -80°C until analysis.

Immunohistochemistry

Paragraph 15.

Five micrometer thick, serial transverse sections were cut with a cryostat microtome (Microm, Walldorf, Germany) at -22°C and mounted on glass slides, air-dried and stored at -80ºC until further analysis. Serial sections were immunohistochemically stained for CD16 (M7006, DAKO, Copenhagen, Denmark) and CD68 (M0718, DAKO). Dilution used for both primary antibodies was 1:200. Biotin conjugated goat anti-mouse (E0433, DAKO) was used as the secondary antibody, followed by complexes of avidin and biotinylated enzymes (ABComplexes, DAKO). Specific antibodies were visualized with the Fuchsin Substrate-

Chromogen System (K0624, DAKO). The sections were finally counter-stained with hematoxylin and mounted with coverslips. Negative and positive controls for both antibodies were always included. During quantification of cells positive for CD16 and CD68, sample identity was concealed. Four microscopic fields (20x) were randomly chosen, and the number of positive cells per mm^2 was manually counted. The area of muscle section assessed was measured by imaging software TEMA (CheckVision, Hadsund, Denmark).

Paragraph 16.

The anti-CD16 antibody (against the Fc gamma receptor III found on granulocytes, monocytes/macrophages, NK-cells and reactive T-cells) was chosen because we wanted to be able to detect most blood borne leukocytes that might infiltrate the muscle due to exercise-induced muscle damage. The anti-CD68 antibody (associated with lysosomal granules) was chosen in order to more specifically detect monocytes and macrophages.

Blood sampling

Paragraph 17.

Blood was drawn from an antecubital vein into a 10 ml serum vacutainer tube. After coagulating for 30-45 minutes at room temperature (~20°C), the blood was centrifuged at 2700 *g* for 10 minutes at 4°C. Serum was then immediately pipetted into Eppendorf tubes and stored at -80°C until analysis. Creatine kinase (CK) was analyzed with the Hitachi 917 Automated Biochemistry Analyzer (Roche®, Basel, Switzerland); analytic CV being < 2.8%.

Muscle soreness in m. quadriceps femoris

Paragraph 18.

Muscle soreness was rated on a visual analogue scale where 0 represented “not sore at all” and 100 mm “extremely sore”. The subjects stretched, contracted (isolated contractions and squats) and palpated the quadriceps muscle to assess general soreness in the whole m. quadriceps and local soreness in m. vastus medialis, m. vastus lateralis and centrally

(including m. rectus femoris and m. vastus intermedius), as well as the muscle-tendon junction just above patella (Figure 1). During palpation the subjects pressed two fingers against each region of interest, applying enough force to cause light discomfort in the control muscle. The subjects were instructed to evaluate the soreness in the exercised muscle in comparison with the corresponding site in the control muscle. Two evaluations of soreness were thus obtained, during stretching and active muscle use and during palpation of relaxed muscles.

Statistics

Paragraph 19.

For muscle function, a one-way repeated measures ANOVA with Dunnett's and Tukey post-hoc tests were performed to identify statistically significant changes from baseline and between time points. We assessed the differences from baseline for both exercised and control legs, as well as the differences between the legs. The Friedman's test with Dunn's post-hoc test was applied for analyzing the scintigraphy, DOMS and CK data. Because different subjects were biopsied at each time point, a Student's paired t-test or a Wilcoxon signed rank test (choice dependent on a normality test for Gaussian distribution) was used to analyze differences detected histologically (exercised *vs*. control). The Mann-Whitney test was applied for testing differences between subgroups of subjects. Selected bivariate relationships were examined with the Pearson product-moment correlation coefficient test or Spearman rank correlation test (choice dependent on a normality test for Gaussian distribution). Due to the fact that scintigrams were obtained from six subjects and biopsies from seven of the eleven subjects at each biopsy time point, the number of subjects will vary from six to eleven in the different analyses. $P \leq 0.05$ was used for establishing statistical significance. Data are presented as means with standard error of the mean (SEM), if not otherwise stated in the text. The statistics were performed with Microsoft® Excel 2003 and InStat® 3.06 (GraphPad Software Inc, San Diego, CA, USA).

RESULTS

Muscle function

Paragraph 20.

Three hundred maximal voluntary, isokinetic, eccentric actions (a total work of -50 ± 4 kJ) resulted in a 47 ± 5% reduction in maximal voluntary, concentric torque ($p < 0.01$; $n = 11$). The recovery of the force-generating capacity was biphasic: During the first six hours there was a fast recovery, whereas between six and 95 hours (four days) after exercise there was no significant recovery. Thereafter, the force-generating capacity recovered slowly, and after one week (168 hours) the subjects were 13 ± 4% below baseline values ($p < 0.01$). The force-generating capacity of the control leg was statistically unaffected during the week of experiment.

Paragraph 21.

Three subjects (4, 6 and 8) had an extraordinarily large acute loss of force-generating capacity (64, 66 and 73%) and suffered a secondary loss of force (Figure 2). In addition, these three "high responders" had the greatest accumulation of radiolabeled leukocytes in the exercised muscles (see below). They recovered to 49 ± 2% under baseline values during the first six hours after exercise, but then their force-generating capacity declined again to a 60 ± 6% reduction 47 hours after exercise. One week after exercise, they were still 32 ± 6% weaker than before exercise. We continued to test these subjects every week, and they did not fully recover before one to two months after exercise. It is, however, important to state that the halted recovery 1-3 days after exercise was a general trend for all subjects, not just the high responders (Figure 2).

<Figure 2>

Muscle soreness in m. quadriceps femoris

Paragraph 22.

Perceived muscle soreness during stretching, unloaded contractions and squats with body weight, increased after exercise and peaked with a value of 53 ± 9 mm in m. vastus medialis 47 hours after exercise ($p < 0.01$; $n = 11$; Figure 3). Muscle soreness evaluated by palpation followed the same time course as the soreness during stretching and contractions and peaked at 47 hours after exercise: 31 ± 5, 25 ± 5 and 24 ± 4 mm for m. vastus medialis, m. vastus lateralis and m. rectus femoris, respectively. Individual peak soreness (over time) during palpation was significantly higher in m. vastus medialis than m. vastus lateralis and m. rectus femoris ($p < 0.05$). Only minor (not significant) soreness was reported for the muscle-tendon junction (just above patella).

<Figure 3>

Accumulation of ^{99m}Tc-labeled (radiolabeled) leukocytes

Paragraph 23.

The number of ^{99m}Tc-leukocytes (measured as radioactivity) was higher in the exercised muscles than in the control muscles at all time points, but large individual differences were observed (Figure 4). The radioactivity increased in m. vastus medialis and m. vastus lateralis between three and eight hours after exercise ($p < 0.05$; $n = 6$; Figure 5). In the three high responders (subject no. 4, 6 and 8), there was a steady increase of accumulated leukocytes until 24 hours after exercise and massive accumulation was observed in m. rectus femoris (Figure 4 and 5). The muscle-tendon junction was the other location where the accumulation of leukocytes was large – four of the six subjects had the largest leukocyte accumulation in this region. The peak difference between the muscle-tendon junction in the exercised and control muscle ranged from 27-764% (mean value of 223%). We found positive correlations between peak accumulation of radiolabeled leukocytes (over time) in the whole thigh and muscle weakness, at all time points from 23 to 71 hours after exercise ($r = 0.81$-0.85; p <

0.05). A negative correlation was observed between peak accumulation of radiolabeled leukocytes in m. quadriceps and muscle soreness ($r = -0.96$, $p < 0.01$); the correlation between accumulation of radiolabeled leukocytes and muscle soreness at 24 hours after exercise, was $r = -0.88$. There was no consistent relationship between soreness and accumulation of leukocytes in different regions of the exercised muscle at the intra-individual level (e.g., the degree of DOMS was only moderate in m. rectus femoris in the high responder subjects).

Paragraph 24.

There was no detectable accumulation of radiolabeled leukocytes in the hamstrings muscle of the exercised leg. The difference between exercised and control leg being -4±4% at the time point when individual leukocyte accumulation peaked in m. quadriceps.

<Figures 4 and 5>

Immunohistochemistry

Paragraph 25.

We observed an increased number of $CD16^+$ and/or $CD68^+$ cells in the exercised muscle, compared with control muscle, at all time points (0.5, 4, 8, 24, 96, and 168 hours after exercise; $p < 0.05$; n = 7; Figures 6). The highest individual numbers of $CD16^+$ and $CD68^+$ cells were observed four and seven days (96 or 168 hours) after exercise. The peak values in the exercised leg were 30 ± 5 and 34 ± 6 cells per mm^2, for CD16 and CD68, respectively, whereas the corresponding values for the control muscle were 8 ± 1 and 13 ± 3 cells per mm^2. $CD16^+$ and $CD68^+$ cells were mainly observed in the endo- and perimysium (Figure 7). Intracellular infiltration was seen only occasionally: In four of the eleven subjects 0.2-1.35% of the myofibers of the exercised muscle were heavily infiltrated by $CD16^+$ and/or $CD68^+$ cells (Figure 7 – inserted picture). There was a positive correlation between the number of $CD16^+$ cells and reduced force-generating capacity four days (96 hours) after exercise ($r = 0.69$; n = 7; $p < 0.05$).

Paragraph 26.

The muscle sections were also stained with an antibody against CD56 (data not presented), which showed no clear signs of NK-cells. As the number of activated T-cells in response to sterile muscle-damage in healthy subjects was expected to be rather low (22;24;36) and the number of basofilic and eosinofilic granulocytes in the blood stream is very and relatively low, respectively, we assumed that the great majority of $CD16^+$ cells (especially at early time points) should be either neutrophilic granulocytes or monocytes/macrophages. Consequently, any difference between the CD16 and CD68 staining presumably gave us a fair estimate of the presence of neutrophilic granulocytes.

Paragraph 27.

In the non-exercised, control muscle there was a trend to moderately increased numbers of $CD68^+$ cells over time. The mean values ranged from 2-5 cells per mm^2 at 0.5-24 hours after exercise, compared to 11 and 9 per mm^2 at 96 and 168 hours after exercise, respectively (0.5 *vs*. 168 hours gave $p = 0.1$; unpaired t-test).

<Figures 6 and 7>

Creatine kinase

Paragraph 28.

Serum CK levels were increased six hours after exercise and remained high throughout the experimental week; the median value was 457 $U \cdot L^{-1}$ (range: 210 - 25 000 $U \cdot L^{-1}$) 95 hours after exercise ($p < 0.01$; $n = 11$; Figure 8). The three subjects (no. 4, 6 and 8) with the largest loss of force and leukocyte accumulation had the highest peak values, ranging between 13 000 and 25 000 $U \cdot L^{-1}$ 95 hours after exercise, and their peak values were significantly higher than in the other eight subjects ($p < 0.01$; Figure 8). The values of the high responders followed a biphasic pattern, i.e., the CK levels increased during the first 23 hours after exercise, declined

slightly (not significantly) at 47 hours, then increased again and peaked 95 hours after exercise.

<Figure 8>

Individual responses and high *vs*. moderate responders

Paragraph 29.

In general, large individual differences were seen in the different variables and this is outlined in Table 1. Subject 4, 6 and 8 diverged markedly from the other eight subjects with greater acute loss of force-generating capacity and slower recovery, as well as larger accumulation of radiolabeled leukocytes (of the whole thigh) and higher serum CK levels. In addition, they did less work during the exercise protocol, because of greater reduction in work capacity during exercise. However, soreness and the accumulation of leukocytes in m. vastus lateralis did not differ detectably between high and moderate responders; still, the soreness tended to be lower, whereas the number of $CD16^+$ cells and radiolabeled leukocytes tended to be higher in the high responders. Intracellular infiltration of $CD16^+$ and/or $CD68^+$ cells was seen in the three responders, but in only one of the other eight subjects. Moreover, the three high responders acquired more ultrastructural (myofibrillar) disruptions than the others, but exhibited low/moderate expression of N-terminal propeptide of procollagen type III – compared to some of the other subjects that experienced intense DOMS. These latter data are presented in Raastad *et al.* (32). In terms of body mass, height and age, subject 4, 6 and 8 did not differ significantly from the other eight subjects, but the high responders were the least physically trained, based on the self report of regular physical activity.

<Table 1>

DISCUSSION

Paragraph 30.

The main finding of this study was that the fast accumulation of radiolabeled leukocytes (assessed by scintigraphy) in the exercised muscles was observed concomitantly with histologically detection of CD16 and CD68 positive cells. Together, these findings demonstrated a local inflammatory cell response to muscle-damaging eccentric exercise in humans. Furthermore, a positive correlation was observed between accumulation of radiolabeled leukocytes and muscle weakness the first three days after exercise and between accumulation of CD16 positive cells and reduced force-generating capacity four days after exercise. During this period (1-4 days after exercise) there was no detectable recovery of force-generating capacity. Surprisingly, there was a strong, negative correlation between accumulation of radiolabeled leukocytes and sensation of delayed onset muscle soreness (DOMS). Hence, large accumulation of radiolabeled leukocytes was correlated with low degree of DOMS (Table 2).

<Table 2>

Time course and types of leukocytes accumulated locally

Paragraph 31.

This study focused on the early local inflammatory response to exercised-induced muscle damage. As anticipated (20;33), the radionuclide imaging technique (scintigraphy) showed an early accumulation of radiolabeled leukocytes in the exercised muscles (3-24 hours after exercise). The histology data verified increased numbers of both CD16 and CD68 positive cells in the endo- and perimysium at the early time points (0.5-24 hours). The radiolabeled leukocytes are primarily neutrophils (8), but significant accumulation of neutrophils in the exercised muscle tissue was, however, not supported by the histological data. Although we could not distinguish explicitly between neutrophils and monocytes/macrophages with our immunohistochemical approach the dominating leukocyte subtype detected appeared to be monocytes/macrophages. This assumption is based on the fact that the mean numeric values of CD16 and CD68 positive cells were very

similar at all time points, and that CD16 is found on both neutrophilic granulocytes and monocytes/macrophages, whereas CD68 is primarily found on monocytes/macrophages.

Paragraph 32.

Thus, our observations indicate that circulating neutrophilic granulocytes are captured in the micro-vessels in the exercised m. vastus lateralis during or shortly after exercise, but apparently few cells cross the endothelial lining. However, based on the scintigraphy, it is plausible that higher numbers of neutrophils infiltrated the tissue in the m. rectus femoris and the muscle-tendon junction. More evident than neutrophils, monocytes (i.e. CD68 positive cells) appeared to transmigrate and accumulate gradually in the m. vastus lateralis during the days following exercise. Our findings are in line with those of previous human studies. Among eleven publications (4;7;10;12;14;16;22;24;25;28;44) reporting on biopsies obtained in the first hours after muscle-damaging exercise (0-6 hours), a significant increase in the numbers of leukocytes was only observed in two (10;16). Of these, only Fielding *et al.* (10) reported on the presence of neutrophils. At later time points (from 48 hours to ≥7 days), increased numbers of macrophages (or mononuclear cells) have been a more consistent histological finding (e.g. (17;22;39)).

Paragraph 33. Histological observations of macrophages and neutrophils might be biased, because macrophages are presumably more easily detectable than neutrophils, due to size differences and a longer life span in inflamed tissue. Furthermore, neutrophils could be difficult to detect histologically in human muscles if substantial tissue accumulation only occurs in some parts of the exercised muscle; e.g. m. rectus femoris and the muscle-tendon junction, which are difficult to obtain tissue samples from. We do believe that the high levels of radioactivity registered in these regions reflected the magnitude of initial tissue damage. The anatomical structure of the m. rectus femoris and muscle-tendon junction seems to predispose these sites to both exercise-induced muscle damage and sport injuries (2;10;18).

Accumulation of leukocytes and recovery of muscle function

Paragraph 34.

In accordance with previous studies (20;33), accumulation of radiolabeled leukocytes preceded the halted recovery of muscle function – and a secondary force loss in the high responders. We now report for the first time a strong correlation between individual changes in muscle function and accumulation of radiolabeled leukocytes in humans. In addition, we observed increased numbers of CD16 and CD68 positive cells present in the muscle tissue during the whole period of halted recovery (1-4 days after exercise). This supports the hypothesis that local inflammation in the exercised muscles can affect the force-generating capacity of the myofibers during the days after unaccustomed eccentric exercise (9;21). Even though a portion of the leukocytes are located on the luminal side of the endothelial blood vessel wall, it is still plausible that they may affect the myofibers through their interaction with endothelial cells (14). However, it could also be that the changes in force-generating capacity are primarily due to the initial muscle damage, the severity of which is reflected by the accumulation of radiolabeled leukocytes. Our experiment does not allow us to establish whether leukocyte accumulation is a cause or effect of exercise-induced muscle damage and loss of force-generating capacity. It has, however, been shown that leukocytes can damage cells in inflamed tissues (41). A cause-effect relationship has been indicated by Pizza *et al.* (30), who showed that the secondary damage after eccentric "exercise" (*in situ* lengthening contractions) was reduced in mice deficient in CD18, which hinders local accumulation of neutrophils. Similarly, Lapointe *et al.* (19) observed a reduced secondary loss of force-generating capacity after lengthening contractions in rats, when the accumulation of $ED1^+$ (phagocytic) macrophages was reduced by NSAID-treatment. Observations from other animal studies have shown no association between changes in muscle function and inflammatory cell accumulation (e.g. (11)). The discrepancy is hard to explain, but could be due to differences between species (experimental animals), the number of infiltrated leukocytes and their activation state, in addition to the ability of the muscle cells to buffer leukocyte-derived damaging agents and oxidative stress.

Biphasic CK response

Paragraph 35.

The serum CK levels increased in a biphasic way in our three high responders. The first peak, observed one day after exercise, may reflect increased permeability of the sarcolemma, caused by the exercise *per se* and/or accumulation of leukocytes. However, the peak after four days was more likely due to myofiber necrosis (2). In support of this assumption, we observed areas with necrotic fibers (heavily infiltrated by leukocytes) in the exercised m. vastus lateralis samples from the high responders. This makes it very likely that their m. rectus femoris suffered even more necrosis, considering the large accumulation of radiolabeled leukocytes in this muscle. Moreover, necrosis has been observed in human studies, and it seems that the large increase in circulating CK levels (i.e., above ~ 10000 $U \cdot L^{-1}$) precedes the end stage of this process, which is not histologically apparent until several days after exercise (17). Finally, it seems reasonable to propose that long lasting depression in force-generating capacity (weeks) in the high responders was a result of such necrosis.

Accumulation of leukocytes and delayed onset muscle soreness (DOMS)

Paragraph 36.

Variations were seen in all variables measured, but there was a general association between the individual changes in force-generating capacity, ultrastructural damage, accumulation of radiolabeled leukocytes and CK-activity (moderate *vs.* high responders). However, there was no such association between DOMS and the other indicators of muscle damage (see Table 1). Interesting, the generally small intra-subject variability in DOMS between different parts of m. quadriceps was in sharp contrast to the large differences in the accumulation of radiolabeled leukocytes. This was especially evident for the m. rectus femoris and the muscle-tendon junction where high numbers of radiolabeled leukocytes were found together with low degree of DOMS. In m. vastus lateralis, where the biopsies were obtained, there seems to be no support for the assumption that leukocyte accumulation and inflammation *per se* cause DOMS (6). Furthermore, the accumulation of CD16 and CD68 positive cells among the myofibers

seemed to increase from day 2 (24 hours after exercise) to day 5, whereas the degree of DOMS was higher on day 2 than on day 5. Others have also questioned the relationship between inflammation and DOMS, since DOMS is present on time points when no accumulation of leukocytes could be detected among the myofibers (13;24-26;44). Malm *et al*. (25) did, however, suggest that activation of leukocytes present in the epimysium already before exercise could be involved in DOMS. The idea that DOMS is related to activity in the connective tissue was suggested many years ago (3), and it is still an attractive hypothesis. We did indeed find increased expression of tenacin C and N-terminal propeptide of procollagen type III (PIIINP; see Table 1; and Raastad *et al.* (32)), which might suggest that damage and remodeling of the extracellular matrix somehow are related to DOMS (7). If leukocytes do not cause DOMS, the activity of fibroblasts and mast cells could potentially play a role in DOMS (39). Interestingly, the high responder subjects demonstrated severe muscle weakness, but reported quite low DOMS, and correspondingly, they clearly exhibited most ultrastructural myofibrillar damage, but only low/moderate tenacin C and PIIINP responses (see Table 1; Raastad *et al.* (32)). In support of our findings, ultrastructural disruptions are related to changes in force-generating capacity (42), but changes in muscle function are not related to DOMS (27). Swelling and increased intramuscular pressure may also play a part in DOMS (13), but contradictory results have been reported (26). Finally, DOMS could also have mechanistic components in the central nervous system (43).

Paragraph 37.

The strong negative correlation between overall accumulation of radiolabeled leukocytes and DOMS was unexpected. It suggests the unorthodox idea that neutrophilic granulocytes somehow reduce or counteract pain-provoking substances and/or reduce the sensitivity of the pain receptors. Corroborating our finding, Rittner *et al.* (35) observed that selective accumulation of neutrophilic granulocytes in rat paws did not cause pain (measured by paw withdrawal). Neutrophils have the potential to reduce pain due to their content of opioid peptides, which act as anti-nociceptive mediators (34). Moreover, administration of a peripheral acting opioid (morphine-6-β-glucuronide)

has been demonstrated to effectively reduce DOMS (40) – indicating that activation of opioid receptors in the exercised muscle reduces muscle soreness.

CONCLUSION

Paragraph 38.

We have documented the inflammatory response in muscles exposed to maximal eccentric exercise in humans, by means of repeated biopsies from both exercised and non-exercised control muscles combined with non-invasive radionuclide imaging. The local inflammatory response was observed immediately after exercise, but leukocyte accumulation appeared predominantly on the luminal side of the blood vessels, at least during the first hours. During the following days, mostly macrophages accumulated in the extravascular space, and they remained there for at least one week. Three subjects were classified as high responders, with severe muscle weakness and heavily accumulation of radiolabeled leukocytes in m. rectus femoris. The magnitude of the inflammatory cell accumulation in the exercised muscles was correlated to the halted recovery of force-generating capacity that occurred 1-4 days after exercise. Accordingly, the accumulation of leukocytes can possibly have contributed to the muscle weakness during this period, or alternatively, leukocyte accumulation may simply reflect the degree of muscle damage and the need for regeneration. Based on the serum CK levels, substantial necrosis of myofibers occurred, which certainly could have contributed to the long-term suppression of muscle function, especially in the high responders. We found no evidence for the notion that accumulation of blood-borne leukocytes causes DOMS. On the contrary, our data indicate that DOMS may be relieved by local accumulation of neutrophils.

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TABLE AND FIGURE LEGENDS

Table 1. Individual data for main variables. The subjects are ranked after the acute loss in force-generating capacity. The subjects are also grouped into "moderate responders" and "high responders". The mean value of each group are given and "*" indicates significant difference between groups ($p < 0.05$). In order to compare events locally in the biopsied m. vastus lateralis ([VL] as a part of m. quadriceps femoris [Q]), specific values are given for: Muscle soreness (DOMS), accumulation of radiolabeled leukocytes, and histological data (CD16 and CD68 staining), in addition to data on ultrastructural changes and expression of N-terminal propeptide of procollagen type III (from Raastad *et al.* (32)). Values are only from the exercised leg.

1: See Figure 2

2: See Figure 8

3: DOMS assessed during stretching and contraction of m. quadriceps

4: See Figure 4 and 5; and note that it was especially the accumulation in m. rectus femoris that separated the subgroups

5: Mean values for repeated biopsies; these data are presented in Raastad *et al.* (32)

6: These data are presented in Raastad *et al.* (32); AU: arbitrary unit

Table 2. Overview of the changes of measured variables during the experimental week (hours after exercise).

NA: Not analysed

*: M. rectus femoris in the high responder subjects

$^{\$}$: This marked increase was only seen in the high responder subjects

Figure 1

Analysis of accumulation of ^{99m}Technetium (radio-)labeled leukocytes: A) photo of the thighs of a subject with marks for regions of interest. B) and C) scintigrams obtained from anterior and lateral views, respectively. Note that dark areas reflect high radioactivity. The large gray square was used to analyze the radioactivity of the whole thigh. The smaller squares (1-5) were used to analyze the radioactivity in m. rectus femoris, m. vastus lateralis, m. vastus

medialis, the muscle-tendon junction, and hamstrings. Recordings were always performed on exactly corresponding locations on both sides – the exercised and the control thigh. The regions on picture A) were also assessed (palpated) for muscle soreness.

Figure 2

Changes in maximal voluntary knee-extension torque ($60°·s^{-1}$) in high (n = 3) and moderate (n = 8) responders. Values are means + SEM. The force-generating capacity of the control leg was not affected. #: Significantly different from baseline ($p < 0.05$).*: Significant difference between high and moderate responders ($p < 0.05$).

Figure 3

Time course of muscle soreness (VAS scale) in different parts of m. quadriceps femoris. Values are means + SEM, n = 11. #: Significantly different from control muscle ($p < 0.05$).

Figure 4

Whole thigh scintigrams of six subjects obtained seven hours after exercise. The graphs show changes in the accumulation of radiolabeled, autologous leukocytes (radioactivity) in the exercised muscles (m. quadriceps; % of control) at 3, 7, 21, and 24 hours after exercise. Note the different Y-axis scale for subject 4 and the massive accumulation of radiolabeled leukocytes in the m. rectus femoris of this subject. Subject 1, 2 and 10 exercised with their right leg, whereas subject 4, 6 and 8 exercised with their left leg. HR = high responders.

Figure 5

Radioactivity in m. vastus lateralis and medialis (left panel) and m. rectus femoris (right panel). The values are percentage difference between the exercised muscle and the corresponding control muscle. In the graph to the right the high and moderate responders are separated in order to illustrate the large difference between subgroups in m. rectus femoris; note the logarithmic scale on the Y-axis. Values are means + or - SEM, n = 6. #: Significantly different

from control muscle ($p < 0.05$). *: Significantly different from the previous time point ($p < 0.05$).

Figure 6

Changes in the number of $CD16^+$ cells (left panel) and $CD68^+$ cells (right panel). Values are means + or - SEM, n = 7 at each time point. #: Significantly different from control muscle ($p < 0.05$). *: Significant difference between the numbers of $CD16^+$ and $CD68^+$ cells found in samples from the exercised muscles obtained 0.5-24 hours after exercise and those obtained at 96 and 168 hours after exercise ($p < 0.01$).

Figure 7

A) $CD16^+$ cells (red stain) were observed in the interstitial spaces of the exercising leg (m. vastus lateralis) shown here at 96 hours (four days) after exercise; the inserted picture shows $CD68^+$ cells (red) inside a muscle cell (scale bar = 50 μm). B) A small number of $CD16^+$ cells were noted in the control leg. Blue stain (hematoxylin) shows nuclei. Scale bar = 100 μm.

Figure 8

Changes in serum creatine kinase (CK). The figure shows values for the high (n = 3) and moderate responders (n = 8); note the logarithmic scale on the Y-axis. Values are means + SEM. #: Significantly different from baseline ($p < 0.05$). *: Significant difference between high and moderate responders ($p < 0.05$).

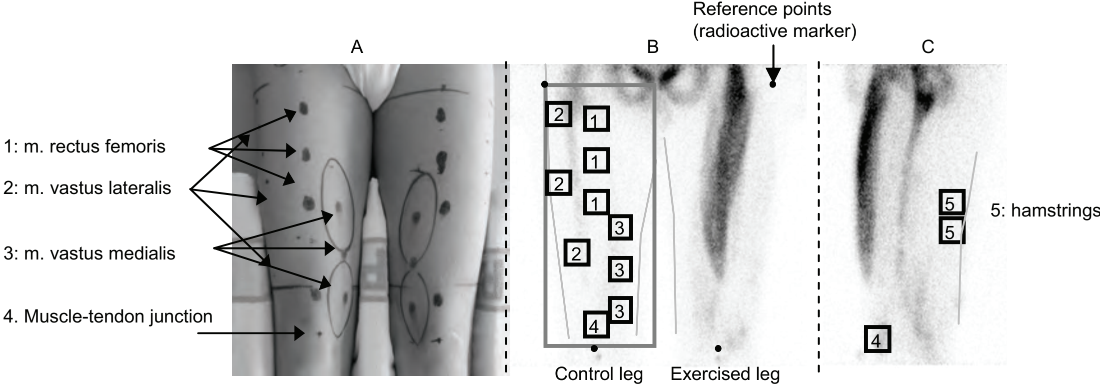
Table 1

	Subject	Acute reduction of force-gen. capacity (% change from baseline)[1]	Force-gen. capacity at 1 week (% change from baseline)[1]	Total work during exercise (J)	Peak CK-activity ($U \cdot L^{-1}$)[2]	Peak DOMS (mm)[3]		Peak accumulation of radiolabeled leukocytes (% of control)[4]		Peak number of leukocytes (VL)		Fibers with myofibrillar disruptions (% of analysed fibers from VL)[5]	Peak staining intensity for PIIINP (AU; VL)[6]
						Q	VL	Q	VL	CD16	CD68		
Moderate responders	5	-19	4	56686	695	27	13	-	-	20	12	0	14
	10	-31	-2	55630	1087	65	75	8	35	16	26	10	23
	7	-32	0	76734	1244	72	77	-	-	14	20	31	23
	11	-35	-7	60993	711	36	17	-	-	19	23	-	12
	1	-44	-4	40570	503	82	82	25	69	28	60	13	37
	2	-47	-7	39775	616	49	43	43	51	32	14	29	16
	9	-50	-17	45979	2046	80	81	-	-	58	65	40	18
	3	-53	-16	68110	821	81	84	-	-	16	55	54	11
High responders	6	-64	-30	37356	13241	48	44	93	55	56	32	44	9
	4	-66	-22	32522	18551	9	10	523	122	25	25	63	-
	8	-73	-42	34266	25022	35	23	250	60	46	47	61	13
Mod resp. (mean value)		-39	-6	55560	965	62	59	25	52	25	34	25	19
High resp. (mean value)		-68*	-32*	34715*	18938*	31	26	289*	79	42	35	56*	11

Table 2

	0-1/2 hr	3-8 hrs	24 hrs	48 hrs	72 hrs	96 hrs	168 hrs
Muscle function	↓↓↓↓	↓↓	↓↓↓	↓↓↓	↓↓↓	↓↓↓	↓
DOMS	-	-	↑↑↑	↑↑↑↑	↑↑↑	↑	-
Accumulation of radiolabeled leukocytes in exercised muscles	NA	↑↑↑	↑↑↑(↑)*	NA	NA	NA	NA
Accumulation of $CD16^+$ and $CD68^+$ cells in exercised muscles	↑	↑	↑↑↑	NA	NA	↑↑↑↑	↑↑↑↑
Serum CK levels	NA	↑	↑↑	↑(↑)$^{\$}$	↑(↑↑)$^{\$}$	↑(↑↑↑)$^{\$}$	↑

Figure 1



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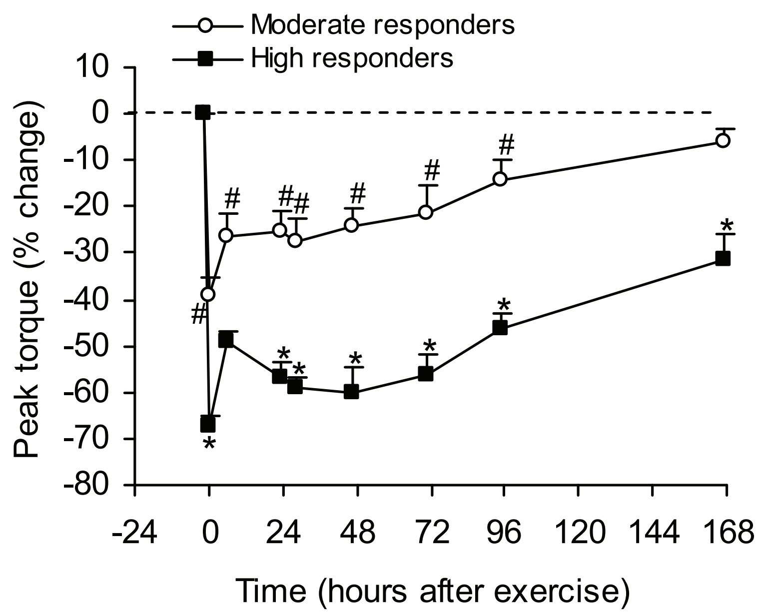

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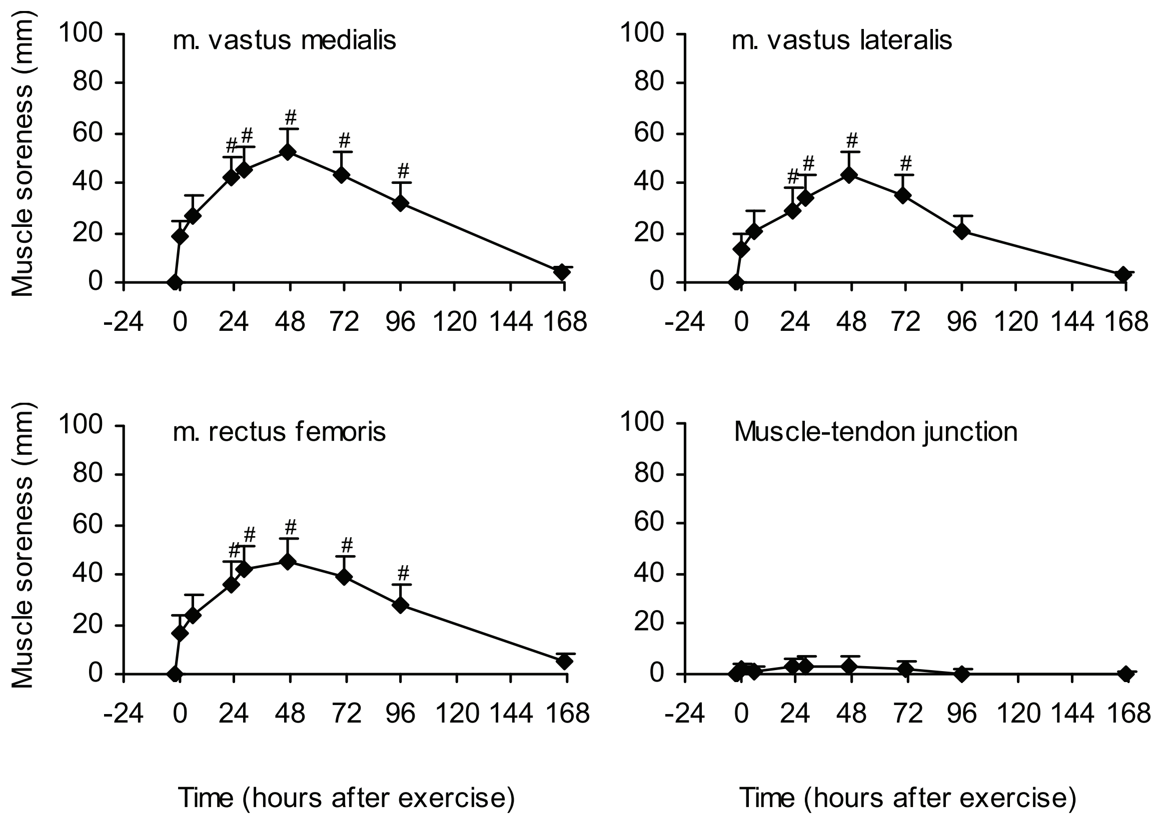

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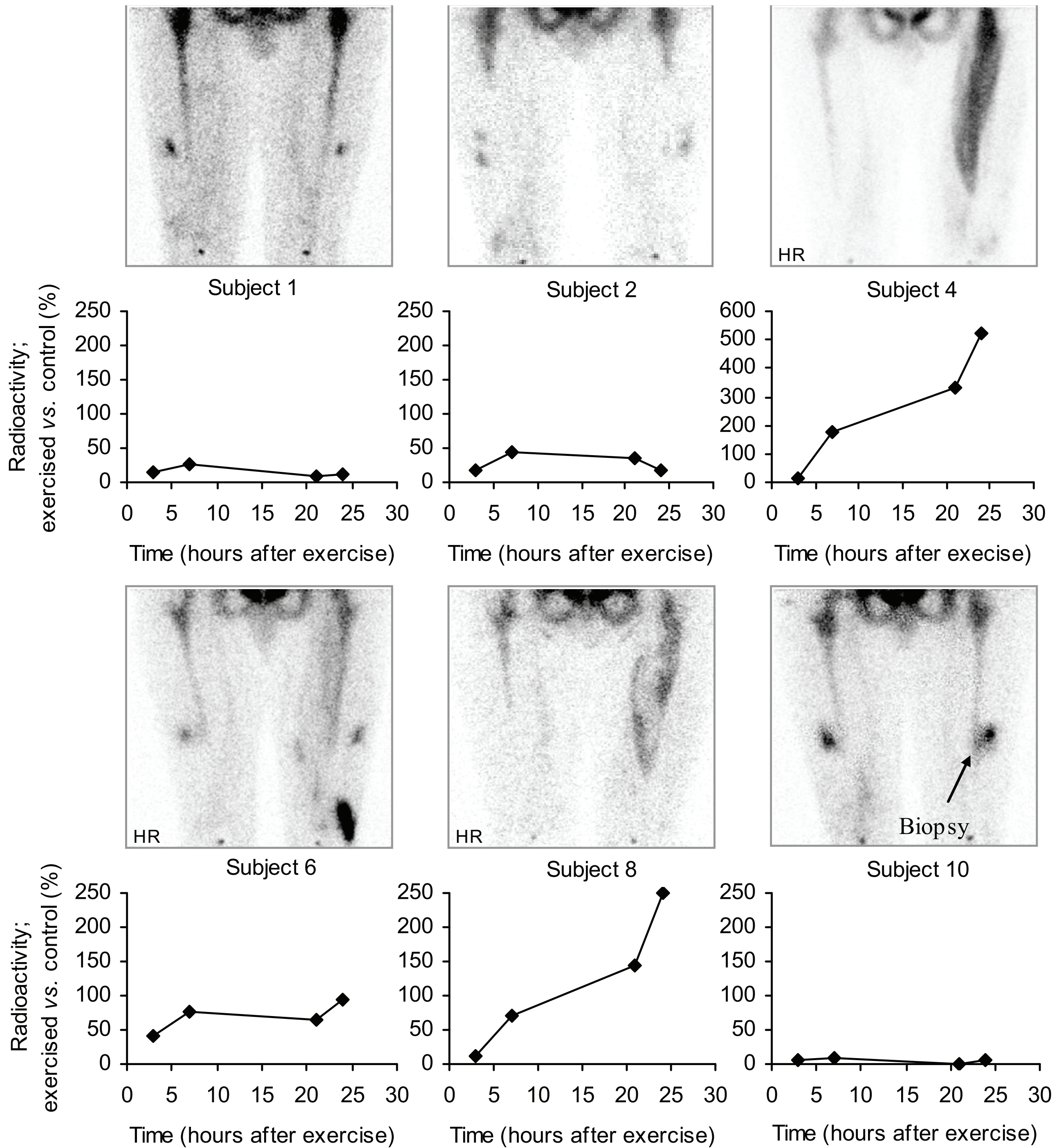
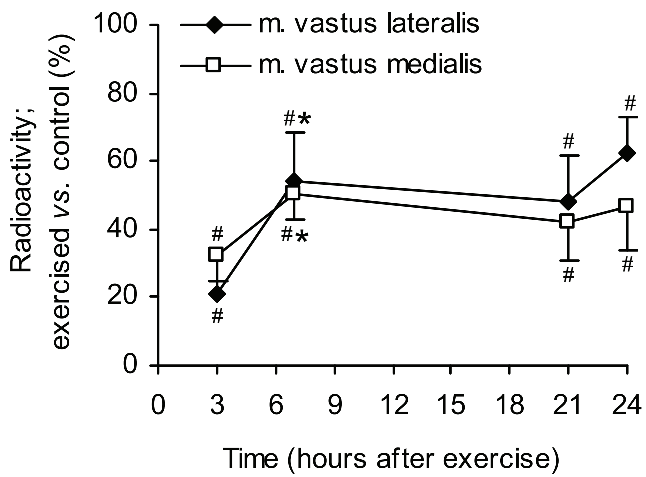


Figure 5



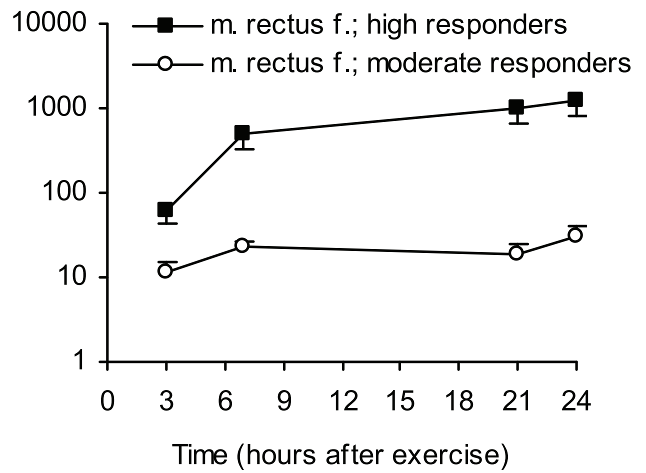


Figure 6

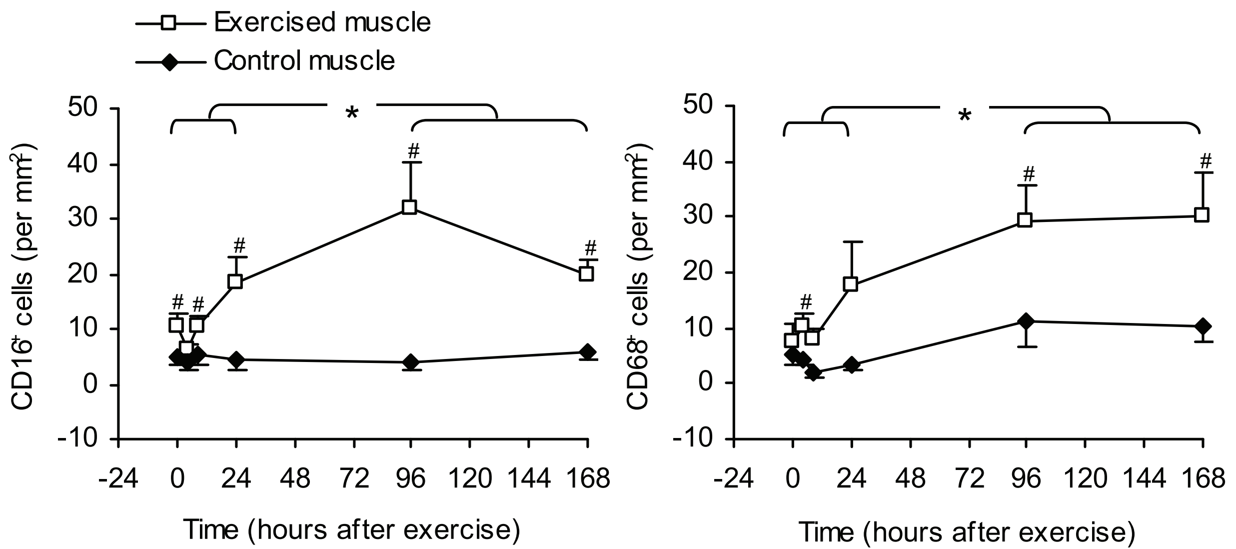


Figure 7

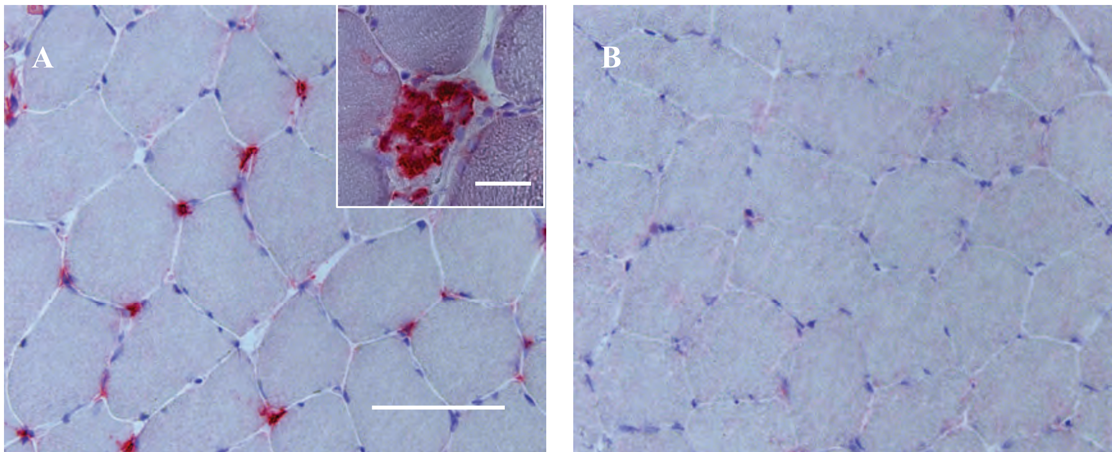
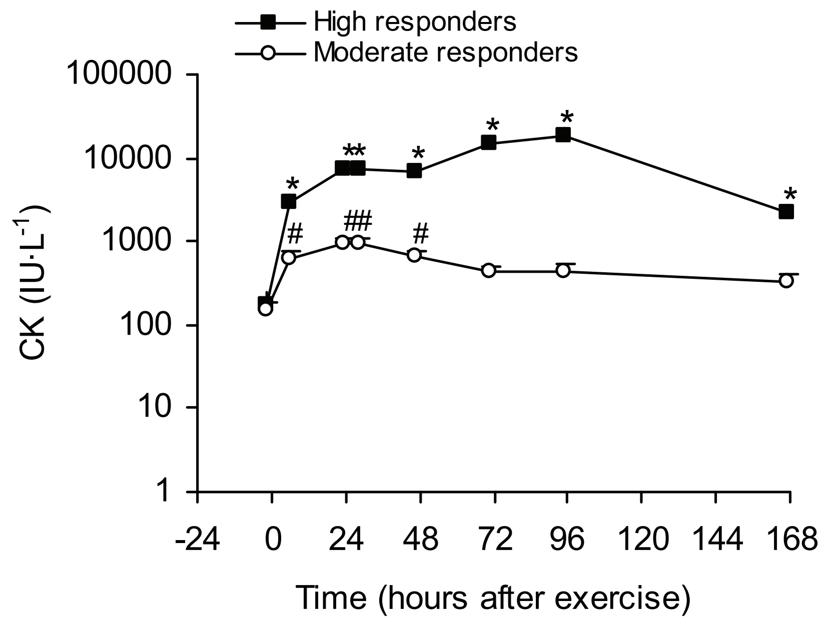


Figure 8



PAPER II

The paper was published online in its final form in June 2009 (Scand.J Med.Sci.Sports).

TITLE:

A COX-2 inhibitor reduces muscle soreness, but does not influence recovery and adaptation after eccentric exercise

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RUNNING TITLE:

COX-2 inhibition after eccentric exercise

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KEYWORDS: Muscle damage, non-steroidal anti-inflammatory drugs, leukocytes, satellite cells

ABSTRACT

Aim: To investigate the effect of a COX-2 inhibitor on the recovery of muscle function, inflammation, regeneration after, and adaptation to, unaccustomed eccentric exercise.

Methods: Thirty-three young males and females participated in a double-blind, placebo-controlled experiment. Seventy unilateral, voluntary, maximal eccentric actions with the elbow flexors were performed twice (bout 1 and 2) with the same arm, separated by three weeks. The test group participants were administered 400 mg·day^{-1} of celecoxib for nine days after bout 1.

Results: After both bout 1 and 2, concentric and isometric force-generating capacity was immediately reduced (~ 40-50%), followed by the later appearance of muscle soreness and increased serum CK levels. Radiolabelled autologous leukocytes (detected by scintigraphy) and monocytes/macrophages (histology) accumulated in the exercised muscles, simultaneously with increased satellite cell activity. These responses were reduced and recovery was faster after bout 2 than 1, demonstrating a repeated-bout effect. No differences between the celecoxib and placebo groups were detected, except for muscle soreness, which was attenuated by celecoxib.

Conclusion: Celecoxib, a COX-2 inhibitor, did not detectably affect recovery of muscle function or markers of inflammation and regeneration after unaccustomed eccentric exercise, nor did the drug influence the repeated-bout effect. However, it alleviated muscle soreness.

INTRODUCTION

According to some reports, non-steroidal anti-inflammatory drugs (NSAIDs) may have detrimental effects on regeneration and healing processes after injuries to the different components of the muscle-skeletal system (Almekinders 1999). Therefore, warnings have been voiced against the wide use of NSAIDs in exercise and sports medicine (Almekinders 1999; Warden 2005; Paoloni & Orchard 2005; Mehallo et al. 2006).

The original NSAIDs (e.g. ibuprofen) inhibit both the constitutive cyclooxygenase (COX)-1 and the more inducible COX-2 enzyme, which both catalyze the generation of prostanoids (prostaglandins [PGE_2 and $PGF_{2\alpha}$], prostacyclins and thromboxanes) that are involved in numerous physiological processes (Miller 2006). The more recently developed selective COX-2 inhibitors are in theory more appropriate and specific to dampen excessive inflammatory reactions than the non-selective (COX- 1 and COX-2) inhibitors (Warden 2005). However, it has been demonstrated that these new drugs can also have negative long-term effects on regeneration and healing in animals (Warden 2005; Buvanendran & Reuben 2006) – especially through inhibition of satellite cell activity in skeletal muscle (Bondesen et al. 2004; Shen et al. 2006). But there is presently no clear evidence for detrimental effects of COX-2 inhibitors on regeneration and healing of injuries in humans (Warden 2005; Reuben & Ekman 2005; Mehallo et al. 2006; Reuben & Ekman 2007; Vuolteenaho et al. 2008). Furthermore, neither clinical nor experimental exercise-induced muscle damage studies have to our knowledge been conducted in order to evaluate the effect of selective COX-2 inhibitors on recovery from skeletal muscle injuries in humans.

In humans, some reports have demonstrated that NSAIDs, such as naproxen and ibuprofen, can reduce eccentric exercised-induced muscle damage and hasten the recovery process (Cheung et al. 2003; Connolly et al. 2003; Baldwin 2003). The improved recovery might stem from the anti-inflammatory effect of these drugs (Sayers et al. 2001), but this assumption has never been properly documented. Howell *et al.* (1998a; 1998b) detected no effect of ibuprofen, but a slightly retarded recovery of maximal isometric torque after high-force eccentric exercise was observed in

subjects administered flurbiprofen. Other negative effects of NSAIDs, such as blunted increase in the rate of protein synthesis after high-force eccentric exercise (Trappe et al. 2001; Trappe et al. 2002), and reduced satellite cell response to long distance running (Mackey et al. 2007), have been reported as well. In contrast to these studies, ibuprofen and acetaminophen may actually enhance muscle growth in response to resistance training in elderly subjects (Carroll et al. 2008), and celecoxib (a COX-2 inhibitor) can allegedly boost the increased rate of protein synthesis after a bout of high-force resistance exercise (Burd et al. 2008). Thus, it seems fair to conclude that the jury is still out concerning the effects of NSAIDs on human skeletal muscle – especially regarding the COX-2 inhibitors (Warden 2005).

“The repeated-bout effect” comprises the physiological adaptation that takes place after a single bout of unaccustomed high-force exercise (Clarkson et al. 1987). Basically, the exercised muscles become significantly more resistant toward damage from a repeated bout, preformed days or weeks after the first one. However, the mechanism behind this adaptation is still not clear (McHugh 2003). A local inflammatory reaction in the exercised muscle may be involved in the strengthening of the tissue after unaccustomed exercise, because when perturbed, a reduced repeated-bout effect has been reported (Lapointe et al. 2002a). In the study by Lapointe et al. (2002a), rats were exposed two times to a muscle-damaging exercise protocol. An NSAID was given to one group after the first exercise bout to reduce the post-exercise inflammation. These rats showed clearly a smaller repeated-bout effect than the controls, with larger force deficits and larger accumulation of leukocytes in the muscle tissue after the second bout. This study design has previously not been tested with human subjects, and it is suited to clarify both early and late effects of NSAIDs, including the mechanisms behind the repeated-bout effect.

Therefore, the aim of the present study was to investigate whether administration of a COX-2 inhibitor would 1) enhance the recovery after maximal eccentric exercise, by reducing the inflammatory reaction during the first days after exercise, 2) slow down the recovery in the final stage (1-3 weeks after exercise), and 3) reduce the repeated-bout effect.

MATERIALS AND METHOD

Subjects

Thirty-three healthy students and employees at the Norwegian School of Sport Sciences gave written, informed consent to participate in the study (Table 1). The subjects were physically active and involved in different activities, such as running and cycling, and team sports, such as basketball and soccer. None of the subjects were familiar with maximal eccentric exercise with the elbow flexors. No exercise was performed for three days prior to the experiment. The subjects ate a light breakfast at home and were offered a mixed meal shortly after exercise. Water was available *ad libitum* during exercise. The subjects were asked to continue their habitual diet and not to take any form of medications or prescription-free supplements (such as antioxidants) and avoid exercise, stretching and massage therapy during the experimental periods. The study complied with the standards set by the Declaration of Helsinki and was approved by the Regional Ethics Committee of Southern Norway.

<Table 1>

Study design

This investigation was carried out with a double blinded design. The subjects were randomized to a celecoxib group and a placebo group. There were no group differences in anthropometric values (Table 1). The celecoxib group was administrated 400 mg (Celebra [Pfizer]; 200 mg morning and evening) for nine days, with the first dose approximately 45 minutes before the exercise (bout 1; Figure 1). This dosage is the highest recommended dosage in the Norwegian Pharmaceutical Product Compendium (NPPC). Maximal blood concentration of the drug is reached after 2-3 hours and the half-life of the drug is 8-12 hours (NPPC). The placebo group received similar looking lactose pills. The subjects were contacted every morning and evening to ascertain that the pills were taken. Three weeks after bout 1, the exercise was repeated (bout 2), but without drug administration (Figure 1).

The exercise was performed with the same arm, randomly chosen, on both occasions (bout 1 and 2). The other arm served as a non-exercised control. Before and for nine days after the exercise bouts, tests of muscle function (force-generation capacity), muscle soreness (pain) and swelling, as well as resting elbow angle were performed and blood was drawn, daily (Figure 1). Assessments of muscle function were always performed after blood sampling and the other measurements, except immediately after exercise when muscle function was tested first.

<Figure 1>

Radionuclide imaging was used to monitor the muscle accumulation of leukocytes 6 and 20 hours after bout 1 and bout 2. Microdialysis was performed 2, 24, and 48 hours after bout 1 for measurement of prostaglandins (PGE_2). Biopsies from m. biceps brachii were collected from both exercised and control muscles 1, 48, 96, and 168 hours after bout 1, and 1 and 48 hours after bout 2. The different time points after bout 1 were chosen in order to evaluate early (1 and 48 hours) and late (96 and 168 hours) inflammatory reactions and regenerating processes.

No subjects withdrew during the study. However, four subjects did not provide all the scheduled biopsies due to technical problems or the discomfort experienced. Six subjects (three in the placebo and three in the celecoxib group) performed only the first of the two bouts of exercise, because they were specially recruited for the microdialysis experiment (see below).

The double-blind experimental design implied that the subjects were unaware of which group – test or control – they belonged to, and during all analyses the subjects' group affiliation was concealed for the recorders.

Medication side-effect registration

The subjects were asked to inform the test leader and write down on a form any symptom they thought could be related to the pills.

Unilateral arm exercise

For the eccentric exercise the subjects were positioned in a chair (Technogym, REV 9000, Gambettola, Italy) and fastened with belts over the hip, chest and shoulder, and the upper arm was supported by a cushion. Thus, the shoulder joint was kept in a slightly flexed position (30-35° from the vertical axis) and fixed during the elbow exercise. The subjects gripped a handle connected to the lever arm of the dynamometer. Since the handle could be rotated about the longitudinal axis, the subjects were instructed to supinate their forearm (elbow joint) for maximal activation of m. biceps brachii. The exercise protocol consisted of 14 x 5 repetitions of maximal voluntary, eccentric actions using the elbow flexors, with 30-35 seconds rest in-between sets. The lever arm of the dynamometer was automatically returned to the starting point; no muscle force being necessary for the elbow flexion phase. The range of motion (ROM) in the elbow joint was 40-175° (180° = full extension) and the velocity was $30°\cdot sec^{-1}$. The subjects were verbally motivated to resist maximally through the whole ROM and they received real time visual feedback on their performance on a computer screen.

The total work of each eccentric action was registered and work per set and total work per exercise were calculated. All values registered during exercise and tests (see below) were corrected for gravity and passive tension throughout the full ROM.

Muscle function

In the same position as during the exercise, maximal force-generating capacity was measured as peak torque during two consecutive maximal, isokinetic, concentric elbow flexions at $60°\cdot s^{-1}$ (ROM: 175-40˚) and as peak torque during isometric actions at 90˚ in the elbow joint (five second actions; two attempts; Technogym, REV 9000). All subjects participated in 1-2 familiarization tests on separate days within one week before they entered the study. Two pre-tests were performed on day 1 of the experiment (with 30-60 minutes rest in-between) and the mean of these tests were used for further analysis. Subjects did always warm up by three minutes arm cranking (30-

50 W) and four submaximal, concentric, isokinetic actions in the dynamometer. The intra-individual coefficient of variation (CV) for the force-generating capacity measurements were < 5%.

Scintigraphic monitoring of leukocyte accumulation

The method has been described by Raastad *et al*. (2003). In brief, 50 ml blood was drawn, and leukocytes (mainly neutrophilic granulocytes) were isolated and labelled with ^{99m}Technetium (^{99m}Tc), before being re-infused. Accumulation of ^{99m}Tc-leukocytes (radiolabelled leukocytes) in the subjects' arms was quantified scintigraphically (with a gamma camera) on anterior view images (Figure 4). The radioactivity in upper arm elbow flexors was calculated with a custom-made software (from GE healthcare, Oslo, Norway). The accumulation of radioactivity within a region of interest (ROI) in the exercised arm was related to radioactivity in the same ROI in the control arm, corrected for background radiation. This procedure was performed on 22 subjects (celecoxib: n=9, placebo: n=13).

Muscle biopsies

A 5 or 6 mm Pelomi-needle (Albertslund, Denmark) with manual suction was used to obtain tissue samples (2-3 x 30-100 mg) from the mid-section of m. biceps brachii. Subjects were in supine position, and the procedure was performed under local anesthesia (Xylocain® adrenaline, 10 $mg \cdot ml^{-1}$ + 5 $\mu g \cdot ml^{-1}$, AstraZeneca, Södertälje, Sweden). Each needle incision was placed approximately 1-2 cm medially and laterally to the first incision and care was taken to avoid tissue affected by earlier biopsies. The muscle samples were rinsed in saline before visible fat and connective tissue were removed, and subsequently frozen in isopentane on dry ice and stored at -80°C until analysis.

Twenty-four subjects delivered biopsies from the m. biceps brachii of both the exercised arm and the control arm. To reduce stress on the subjects and to reduce risk of contamination from previous biopsies, each subject was scheduled for biopsy at three out of the six biopsy time points: 1 hour, 2, 4 and 7 days after bout 1, and 1 hour and 2 days after bout 2 (Figure 1).

Immunohistochemistry

Serial cross-sections (7 μm) were incubated with antibodies (ab) against leukocytes: CD66b (neutrophilic granulocytes, monoclonal ab, M1546, PeliCluster, Sanquin, Amsterdam, The Netherlands; 1:500), and CD68 (monocytes/macrophages; monoclonal ab, M0718, DAKO, Copenhagen, Denmark; 1:300), together with antibodies against laminin (polyclonal ab, Z0097, DAKO; 1:1000) or dystrophin (polyclonal ab, ab15277, Abcam, Cambridge, UK; 1:2000). The two latter antibodies were used to visualize the sarcolemma. In order to visualize satellite cells/myoblasts, sections were analysed for immunoreactivity against CD56/NCAM (monoclonal ab, ab9018, Abcam; 1:200). Sections with overt satellite cell/myoblast activity were double stained with CD56 and Ki67 (a marker of proliferation; polyclonal ab, CP249A, Biocare Medical, Concord, CA, USA; 1:200). Alexa-488 (FITC) and -594 (goat anti-rabbit or goat anti-mouse; Invitrogen-Molecular Probes, Eugene, OR, USA) were used as secondary antibodies. The sections were finally counter-stained with DAPI (for nuclear staining) and mounted under coverslips (ProLong Gold Antifade Reagent with DAPI, P36935, Invitrogen).

Images of the stained cross-sections were captured using an Axiocam camera (Zeiss, Oberkochen, Germany) mounted on a Axioskop-2 light microscope (Zeiss, Oberkochen, Germany). Multiple images (20x, 40x and 100x objectives) were taken so that the whole muscle biopsy cross-section was captured. To quantify the number of cells positive for a leukocyte associated antigen, a cell that contained both DAPI and antibody staining was considered as positive, independent of the staining intensity. A satellite cell was identified as CD56 staining within the basal lamina of the myofibres; most often the staining appeared as a rim around a nucleus. A myoblast was defined as CD56 staining and a nucleus outside/inside the sarcolemma. Data are presented as number of positive cells per 100 myofibres.

Microdialysis and Prostagandin E_2

Tissue fluid (dialysate) from both the exercised arm and the control arm of twelve subjects (six administrated the COX-2 inhibitor and six placebo) were collected at three time points after bout 1. All twelve subjects underwent the microdialysis procedure 2-6 hours after exercise and then again starting at either 24 (n = 5) or 48 hr (n = 5) after exercise. A CMA-60 microdialysis probe (20 kDa molecular cut-off; length 30 mm; CMA/Microdialysis AB, Solna, Sweden) was used for collection of tissue fluid for later measurements of prostaglandins E_2 (PGE_2; see below). Before the insertion of the CMA-60 catheter, positioned 2 cm medial to the centerline of the m. biceps brachii muscle, the skin was locally anaesthetized with 0.3 ml of Lidocain (10 $mg \cdot ml^{-1}$; SAD, Copenhagen, Denmark). The CMA-60 catheters were perfused with a Ringer acetate solution containing 5 nM ^3H-PGE_2 (specific activity, 3.7GBq $mmol^{-1}$; NEN, Boston, USA) to determine the recovery of PGE_2. The subjects rested for at least 90 minutes before starting the experiment to ensure that any reaction from the insertion trauma had minimized. Dialysate samples were collected every 30 minutes. The samples were immediately frozen (-80°C) until analysis.

The prostaglandin E_2 (PGE_2) concentration in the dialysate was analysed by a commercial competitive enzyme immunoassay (ACE™, catalog no 514010, Cayman Chemicals Inc., Ann Arbor, MI, USA). Standards and samples were analysed in accordance with the protocol of the kit. Samples were analysed in duplicate. Optical density (OD) was measured in a plate-reader (ASYS Hitech, Eugendorf, Austria) at 405 nm.

Muscle soreness

Muscle soreness was rated on a visual analogue scale where 0 represented "not sore at all" and 100 mm "extremely sore". The subjects stretched and contracted their elbow flexors (both arms) to assess soreness in m. biceps brachii and brachialis. In order to assess soreness/tenderness at the lower (distal) and upper (proximal) part of the upper arm elbow flexors, the subjects sat with their arms extended, but resting, on a bench (90° flexed and lateral rotated shoulder joints). The upper arm muscles were then palpated manually by the test leader. In addition, a probe (3 cm^2

cross-sectional area) giving 10 N·cm^{-2} of pressure, was applied perpendicularly to the elbow flexor muscles of the upper arm at predetermined locations, i.e., a distal and a proximal spot (marked with a waterproof pen). The middle part of the upper arm elbow flexors was discarded because of location of the biopsy incisions.

Muscle swelling and resting arm angle

Swelling was assessed by measuring the arm circumference with a tape measuring device, utilizing a spring mechanism to apply constant force (Roche, Oslo, Norway). The circumference was measured at the person's thickest part of the lower half of the m. biceps brachii (and the m. brachialis underneath), below the biopsy incisions. A waterproof pen was used to mark the site for repeated measurements. Resting arm angle was measured with a goniometer. The intra-individual CV of these measurements was < 1.8%.

Blood sampling and creatine kinase (CK)

Blood was drawn from an antecubital vein into a 10 ml serum vacutainer tube. After coagulating for 30-45 minutes at room temperature (~ 20°C), the blood was centrifuged at 2700 *g* for 10 minutes at 4°C. Serum was immediately pipetted into Eppendorf tubes and stored at -80°C until analysis. Creatine kinase (CK) was analysed with an automated chemistry analyzer (Modular P, Hitachi High- Technologies Corporation, Tokyo, Japan); analytic CV being < 5 %.

Statistics

All parameters were measured repeatedly after exercise and for each variable the area under the curve (AUC) was calculated for each subject. The AUC values, in addition to peak/nadir values, for the celecoxib and placebo group were compared with the unpaired Student's t-test or the Mann-Whitney test. A multivariate analysis of variance (MANOVA) with repeated measures and a two-way ANOVA (time x group and time x arm) with Bonferroni post-hoc test were also applied. For the different variables, changes over time within each group were followed with a one-way ANOVA or Friedman's test with repeated measures and Dunnett's and Tukey's or Dunn's post-

hoc tests. We evaluated the differences from baseline and between selected time points for the exercised arm and the non-exercised (control) arm separately, as well as the difference between arms. Due to the fact that different subjects were biopsied at each sampling time point, the unpaired Student's t-test and the Mann-Whitney test were used to analyze group differences in histological data. The paired Student's t-test or the Wilcoxon signed rank test was applied to evaluate differences between the exercised arm and the control arm. Data are presented as means with standard error of the mean (SEM), if not otherwise stated in the text. $P \leq 0.05$ was considered statistically significant. Cohen's effect size (difference between group means divided by the pooled SD) was calculated with bias correction for unequal numbers of subjects in the two groups (Hedges). Testes for normality (Gaussian distribution) dictated the choice of parametric or non-parametric tests. The statistics were performed with Microsoft® Excel 2003 (including statistiXL 1.8) InStat® 3.06, Prism® 5.01 and Statemate™ 2.0 (GraphPad Software Inc., San Diego, CA, USA) and SPSS 15.0 (SPSS Inc., Chicago, IL, USA).

RESULTS

Exercise and muscle fatigue

The total work (sum of 14 x 5 repetitions) performed by each experimental subject during bout 1 and 2 was similar within and between groups: celecoxib: ~ -4500 ± 500 kJ (bout 1 and 2) and placebo: ~ -4800 ± 300 kJ (bout 1 and 2). Total work during the last (14th) set was reduced by 45 ± 4% and 47 ± 4% of the first set values for the celecoxib and placebo group, respectively, during bout 1 ($p < 0.01$). This reduction was smaller in bout 2 than bout 1 for both groups (celecoxib: 34 ± 3% and placebo: 35 ± 3%; $p < 0.01$).

Muscle function

The force-generating capacity measured as isometric and isokinetic concentric torque was markedly reduced (~ 40-50%) after both exercise bouts, but there were no significant differences between groups at any time point (Figure 2). The force-generating capacity was not recovered nine days after bout 1, but was in both groups not detectably different from baseline values before bout 2, three weeks after bout 1. Of note, about half of the subjects in each group were more than 5% (CV for the test) below their baseline values three weeks after bout 1. After bout 2 the acute reduction in force-generating capacity was only slightly attenuated compared to after bout 1 (calculating with the pre-values of bout 1; $p < 0.05$; Figure 2). However, the recovery of both isometric and concentric force-generating capacity was faster after bout 2 than bout 1 (comparing AUC values; $p < 0.01$). The force-generating capacity of the non-exercised, control arm did not change from baseline in either group during the experimental periods (Figure 2).

<Figure 2>

Muscle soreness

Muscle soreness of the upper arm, as assessed during contractions and stretching, was less in the celecoxib group than in the placebo group, after both bouts (Figure 3, AUC: bout 1: $p = 0.04$, and bout 2 $p = 0.06$; effect size: 0.75 and 0.86 for bout 1 and bout 2, respectively). For both

groups, less soreness was reported after bout 2 than after bout 1 ($p < 0.01$). The peak soreness values after bout 1 tended to be higher in the placebo group (6.7 ± 0.6 mm) than in the celecoxib group (5.1 ± 0.7 mm; $p = 0.08$). This was less evident after bout 2 (peak values: 2.5 ± 0.4 mm [celecoxib] vs. 3.6 ± 0.6 mm [placebo], $p = 0.14$). The placebo group tended to report more soreness in the control arm than the celecoxib group after bout 1 (AUC; $p = 0.1$). This soreness/pain seemed to be due to the biopsy procedure.

<Figure 3>

For soreness as assessed with palpation, lower peak values were reported by the celecoxib group than the placebo group, but only in the proximal part of elbow flexors (3.4 ± 0.8 vs. 6.5 ± 0.7; $p < 0.05$). After bout 2, peak soreness values during palpation were reduced in both groups ($p < 0.01$).

Scintigraphy

The radioactivity, reflecting autologous radiolabelled leukocytes, was in both groups higher in the exercised arm than in the control arm six and 20 hours after bout 1, and the difference between arms was larger at 20 than six hours after exercise ($p < 0.05$; Figure 4). After bout 2 the radioactivity at six hours was similar to that found in bout 1; however, no further increase at 20 hours was detected after bout 2 (Figure 4). There were no significant differences between groups.

<Figure 4>

The scintigrams confirmed that m. biceps brachii was affected by the exercise protocol. We could not precisely differentiate between the elbow flexors (due to low image resolution), but, in addition to m. biceps brachii, both m. brachialis and m. brachioradialis appeared to be affected. There was a trend towards higher radioactivity in the lower and middle part than the proximal part of the upper arm (Figure 4). To exemplify, three subjects demonstrated ~ 1000% difference between

middle part of the exercised muscles and control muscles 20 hours after exercise. In five subjects, high radioactivity (> 100% higher in the exercised muscles compared with control) was found over a localized area that appeared to correspond to the proximal muscle-tendon junction.

Microdialysis (PGE_2)

The PGE_2 concentration in the interstitial fluid (dialysate) of the exercised muscle was not detectably different from control muscle at any time point (2, 24 and 48 hours after bout 1), and there were no group differences. The mean values (combined over groups and time points) were 880±134 and 900±135 $pg \cdot ml^{-1}$ for the exercised and control muscle, respectively. However, the PGE_2 concentration tended to decrease in both the exercised muscle and the control muscle during the first days after exercise in the celecoxib group (mean of both arms: 1000 ± 167 vs. 635 ± 187; $p = 0.16$), but not in the placebo group (mean of both arms: 988 ± 412 vs. 1151 ± 296). This suggests that celecoxib generally lowered the PGE_2 levels in the interstitial fluid over time.

Immunohistochemistry

Leukocytes

$CD68^+$ cells, primarily monocytes/macrophages, were observed in the samples from both exercised and control muscles, but higher numbers were counted in the exercised muscle after both bouts (74 ± 21 vs. 22 ± 3 $CD68^+$ cells per 100 myofibre; data combined over groups and time; $p < 0.01$). In the exercised muscles the number of $CD68^+$ cells increased from 1 to 48 hours after exercise and from early (1 and 48 hours) to later time points (four and seven days) after exercise ($p < 0.05$). No differences between groups were observed (Table 3), however, at later time points the five highest values registered were all found in samples from subjects in the placebo group. $CD68^+$ cells seemed preferentially related to necrotic and regenerated myofibres (Figure 5). Necrotic myofibres (probably segments) were identified as dystrophin negative fibres (no staining); 8 of 23 subjects displayed ≥ 2% dystrophin negative myofibres on samples from the exercised muscle:15% ± 19 (standard deviation [SD]) of the analysed fibres. This was only observed in samples obtained four and seven days after exercise. Dystrophin negative fibres

were only seen in four of all samples from control. Staining for laminin indicated that the basal membrane (with few exceptions) stayed intact – despite loss of dystrophin staining, and signs of the regeneration process occurred within the basal lamina sheath.

CD66b$^+$ cells (neutrophils) were found in very low numbers and no significant differences between exercised and control muscle, or between groups, could be detected (Table 3).

<Figure 5>

Satellite cells

No differences between the groups were detected in the number of CD56$^+$ cells, i.e., satellite cells (located underneath basal lamina, but outside the plasma membrane) and myoblasts (CD56$^+$ cells primarily inside necrotic myofibres; Table 3). Myoblasts were observed only in some subjects and preferentially clustered together (see next paragraph). For the groups combined, the number of satellite cells/myoblasts was higher in exercised than in control muscles at both early (1 and 48 hours) and late time points (four and seven days after exercise; $p < 0.05$), but there was an increase from early to late time points after bout 1 ($p = 0.01$). The number of satellite cells/myoblasts per myofibre was 0.11 ± 0.04 (SD) in samples from the non-exercised, control arm, and 0.19 ± 0.18 (SD) in the exercise samples (all combined). Expressed as the proportion of the nuclei underneath basal lamina, 4.8% ±1.5 (SD) were defined as satellite cells in control samples and 5.7% ± 1.5 (SD) in the exercise samples. Hence, these latter numbers do not include myoblasts.

Intense satellite cell activity, including increased number and cell volume, long cytoplasmatic extensions, cell division activity (Ki67 positive nuclei) and fusion of myoblasts were seen in five subjects one week after exercise (Figure 6). CD68$^+$ cells occasionally were positive for Ki67 as well. Thus, in areas with large number of cells (nuclei), differentiation of the cells could be very difficult. No detectable differences between the celecoxib and placebo subjects could be found.

In biopsies obtained after bout 2, relatively high numbers of $CD56^+$ myofibres with centrally located nuclei and often small diameters were observed (≥ 10% of the myofibres in six subjects; three from each group; Figure 6). A great majority of these $CD56^+$ myofibres were also verified to contain embryonic myosin heavy chain (F1.652, Hybridoma Bank; data not shown). Although this was observed after bout 2, the regeneration processes were very likely to be related to bout 1. This implies that complete regeneration after severe exercised-induced muscle damage takes more than three weeks. There were no signs of failed regeneration in biopsies after bout 2, in either group.

<Table 3 and Figure 6>

Muscle swelling and resting arm angle

The upper arm circumference increased equally between groups and peaked four days after bout 1, 4.2 ± 0.9% vs. 4.6 ± 1.0% for the celecoxib and placebo group, respectively ($p < 0.01$ from baseline). The resting arm angle decreased (180° = extended arm) equally between groups by 10 ± 2° and 11 ± 2° 24 hours after bout 1 for the celecoxib and placebo group, respectively ($p < 0.01$). Both the circumference and the resting arm angle changed less after bout 2 than bout 1 ($p < 0.01$), and no group difference were detected.

Creatine kinase (CK)

The serum CK levels were markedly elevated after bout 1, but not after bout 2 (AUC; $p < 0.01$; Figure 7). There were no differences between groups.

Of note, for about half of the subjects the increase in serum CK levels followed a biphasic time course. Thus, there was an early increase during the first eight hours, followed by a more or less steady phase from 8-24 hours, before the values increased markedly until peak values were reached typically at 96 hours (four days) after exercise (Figure 7).

<Figure 7>

Gender

There were no gender differences in relative changes of muscle function, in muscle soreness or in the histological data, neither within groups nor across groups. However, the serum CK pre-values (before bout 1) were lower in the females than in the males ($p = 0.05$), and the females had a lower exercise-response to bout 1 (area under the curve; $p < 0.05$). The three highest values for accumulation of radiolabelled leukocytes (scintigraphy data) were observed in female subjects, but we found no significant differences between the sexes. Exclusion of the females from these analyses did not change the group comparisons appreciably.

Adverse effect of the pill administration

Three subjects mentioned slight nausea, but one of these subjects was in the placebo group. One subject from the celecoxib group experienced an unexpected swelling of the forearm of the exercised arm; the excess fluid appeared to be in the subcutaneous tissue.

DISCUSSION

The main finding in this study was that celecoxib, a selective cyclooxygenase-2 (COX-2) inhibitor, did not affect recovery of muscle function after a bout of unaccustomed, muscle-damaging, eccentric exercise in human subjects. The COX-2 inhibitor did, however, reduce the delayed onset muscle soreness.

A repeated-bout effect was clearly demonstrated by the faster recovery of muscle function and markedly blunted DOMS and serum CK response after the second bout of exercise. Nine days of celecoxib administration during the recovery period after bout 1 did not have any detectably adverse effect on the adaptation processes and recovery after the second bout, three weeks after the first. The exercise protocol clearly initiated an inflammatory reaction with accumulation of leukocytes in the exercised muscles (markedly in some “high-responder subjects”), and stimulated increased satellite cell activity as well. There were no statistically significant differences between the celecoxib and placebo group concerning indicators of inflammation. Nevertheless, the highest numbers of accumulated leukocytes at late time points (four and seven days after bout 1) were found in the placebo group.

This study is to our knowledge, the first to investigate the effect of a COX-2 inhibitor on recovery after exercise-induced muscle damage in humans. Furthermore, the necrosis and regeneration processes observed in this study are rarely reported for humans; especially in combination with measurements of muscle function.

NSAIDs, changes in muscle function and inflammation

Administration of non-selective NSAIDs in the recovery phase after high-force, muscle-damaging exercise in human studies has resulted in equivocal findings (Cheung et al. 2003; Connolly et al. 2003; Baldwin 2003). Some researchers have observed enhanced recovery of muscle function during the first days after exercise (e.g. (Dudley et al. 1997)), while others observed no such effect in comparison with placebo medication (e.g. (Howell et al. 1998a)). The most obvious

mechanism behind any effect of NSAIDs on muscle restitution, on which our working hypothesis was founded, is reduced inflammation (Lapointe et al. 2002a; Lapointe et al. 2002b; Shen et al. 2005).

Accumulation of radiolabelled leukocytes in the exercised muscles six and 20 hours after exercise indicates an early inflammatory reaction, but no effect of celecoxib was detected. The blood-borne leukocytes (primarily neutrophils) detected by scintigraphy were probably either infiltrating the interstitial space of the muscle tissue or merely adhering to the luminal side of the local micro-vessels. Neutrophils were, however, histologically not found to have accumulated in the muscle tissue two days after exercise. Conversely, the numbers of monocytes and/or macrophages increased in the muscle tissue, and the highest values were found four and seven days after exercise, in both the celecoxib and placebo group. Again, there were no significant differences between groups, but there was a tendency towards higher leukocyte numbers in “high-responder” subjects from the placebo group. This could mean that celecoxib had some dampening effect when the inflammatory reaction exceeded a certain intensity. The effect size between groups was 0.6, but our statistical power to detect such a difference was very low (26%).

Experimental evidence indicates that high-force, eccentric exercise protocols can cause significant muscle damage and inflammation in humans, but the peak accumulation of leukocytes (primarily macrophages) among the myofibres, seemed to occur several days after exercise (Jones et al. 1986; Hellsten et al. 1997). Thus, scant evidence of a “classical” local inflammatory reaction after exercise-induced muscle damage exists for humans (in contrasts to rats, rabbits and mice; (Schneider & Tiidus 2007)). In accordance with our data, the most severe muscle weakness has been seen well before the cell damage and inflammation are fully manifested (Jones et al. 1986; Newham et al. 1987; Jones et al. 1989; Hellsten et al. 1997; Child et al. 1999). Consequently, if there is no real inflammation during the first days after exercise, it seems reasonable to deduce that possible effects of anti-inflammatory drugs could not be mediated through reduced inflammation at the early stage. In line with this, Bourgeois et al. (1999) reported

enhanced recovery of muscle function after the use of a NSAID (naproxen), but no inflammatory cells could be detected histologically in muscle samples obtained 24 hours after resistance exercise. Peterson et al. (2003) found accumulation of monocytes/macrophages (CD68), but not neutrophils (CD15) 24 hours after eccentric exercise, and no effect of acetaminophen and ibuprofen was detected. This was observed despite the fact that the NSAIDs blunted the exercise-induced increases in the muscle levels of PGE_2 and $PGF_{2\alpha}$ (Trappe et al. 2001). Therefore, the lack of effect (as in the present study) could be due to the absence of a sufficiently strong and early inflammatory reaction, in response to the exercise. Alternatively, the generation of prostaglandins are not of major importance for the leukocyte accumulation in exercised muscles. We could not detect any increase of PGE_2 in the exercised muscles.

NSAIDs and delayed onset muscle soreness (DOMS)

The celecoxib group reported less muscle soreness and pain than the control group after both bouts of exercise. The DOMS reducing effect of celecoxib after bout 1 may be due to the drug's known analgesic effect (Ekman et al. 2002; Reuben & Ekman 2005), in the muscle tissue, in the central nervous system or both (Veiga et al. 2004).

We obtained circumstantial evidence for a drug effect on the PGE_2 generation in the muscle, but no evidence for an exercise effect on PGE_2. The role of local PGE_2 in DOMS has been questioned by others, who like us analysed microdialysis fluid (Tegeder et al. 2002), and by investigators who found no NSAID effect on DOMS (e.g. (Kuipers et al. 1985)). Our findings are also supported by previous reports (Paulsen et al. 2009), indicating that accumulation of leukocytes (as assessed by use of radiolabelled leukocytes and immunohistochemistry) is both spatially and temporally out of phase with development of muscle soreness. To exemplify, subjects with the largest accumulation of radiolabelled leukocytes did not report more intense soreness than others. Furthermore, large accumulations of leukocytes among the myofibres were seen one week after exercise, but at that time the soreness was almost gone. Thus, our data

(PGE_2 and accumulation of leukocytes) indicate that inflammation, in the classical sense, is not a main mechanism behind DOMS.

NSAIDs and satellite cells

A remarkable finding was myofibre necrosis (degradation) and regeneration observed in as much as ~1/3 of our subjects. The regeneration process was heralded by cell proliferation leading to increased numbers of satellite cells/myoblasts (CD56 positive cells), as well as their migration, fusion and development of new myotubes to replace degraded segments of damaged myofibres. This has rarely been reported in otherwise healthy human muscles after exercise. Indeed, degenerative and regenerative processes have been captured by histological examination after strenuous exercise (Hikida et al. 1983; Jones et al. 1986; Round et al. 1987; Child et al. 1999), but satellite cell activity in highly damage muscle tissue assessed by immunohistochemistry has been poorly documented. Crameri et al. (2004) applied similar methodology as us, but found necrosis and myoblast fusion in only one out of eight subjects examined.

Possible retardation of regeneration processes in skeletal muscle tissue by NSAIDs, and especially COX-2 inhibitors (Bondesen et al. 2004; Warden 2005; Shen et al. 2006), has not been thoroughly investigated in humans. However, administration of indomethacin reduced satellite cell response after long distance running in endurance-trained subjects (Mackey et al. 2007). The discrepancy between our study, where the satellite cell response in the affected muscles was not detectably reduced by celecoxib, and that of Mackay et al. (2007), is not easily explained. However, the contradictory findings could be due to the large differences in the exercise protocols and subject populations, as well as the fact that different drugs were administered – a non-selective COX-inhibitor vs. our selective COX-2 inhibitor. Hence, any effect on satellite cell proliferation could be due to inhibition of both COX-1 and COX-2, which, inter alia, might markedly lower the tissue prostaglandin levels (in contras to our findings).

Our data suggest that the COX-2 inhibitor celecoxib (at high dosage: ~ 6 mg·$^{-}$kg^{1} for nine days) leaves regeneration and adaptation processes unaffected after exercise-induced muscle damage. However, the large inter-individual differences and the few subjects with considerable necrosis in the obtained biopsies, made any celecoxib effects of a more subtle nature elusive. Thus, further investigations are warranted, and it should be kept in mind that celecoxib may also have diverse COX-2 independent effects on a cell (e.g. (Glebov et al. 2006)).

NSAIDs, inflammation and the repeated-bout effect

The repeated-bout effect is well documented, and as expected, faster recovery of muscle function, as well as blunted muscle soreness and serum CK increases, were observed after the repeated exercise bout. The mechanisms behind the repeated-bout effect are, however, still ambiguous (McHugh 2003), but the inflammatory reaction might be causative. In an experiment with rats, Lapointe et al. (2002a) demonstrated that the repeated-bout effect was reduced when the accumulation of leukocytes had been blunted by the NSAID diclofenac in the recovery phase after the initial bout (Lapointe et al. 2002a). The study design of the present investigation resembled Lapointe et al.'s (2002a). However, we could not detect any clear anti-inflammatory effect of our COX-2 inhibitor (celecoxib). Moreover, the repeated-bout effect was seen both in subjects with large and low accumulation of leukocytes. Consequently, we question the importance of inflammation to the adaptation process after eccentric exercise in humans. In that sense, our findings are in agreement with other human studies where signs of myofibrillar remodelling have been observed, without leukocytes detectably present during the first days after eccentric exercise (Yu et al. 2002; Feasson et al. 2002).

Perspectives

Celecoxib intake (400 mg·day^{-1}) over nine days neither enhanced nor inhibited recovery, regeneration and adaptation processes after exercise-induced damage in skeletal muscles. However, the drug alleviated muscle soreness. Since the mechanisms behind DOMS are still

obscure, and it is unclear whether the drug works primarily on peripheral factors or in the central nervous system, further work is needed.

Of clinical relevance, short term use of celecoxib to reduce exercise-induced muscle damage can not be advocated. However, celecoxib might be a good choice when the intention is to reduce pain and promote rehabilitation and recovery after injuries such as ankle sprains (Ekman et al. 2002; Nadarajah et al. 2006), without detectable adverse effects on the musculature. The degree of damage from the exercise protocol applied in this study is not as extensive as after strain injuries (including myofibre rupture and intramuscular bleeding). Consequently, our conclusions should not without caution be extrapolated to other kinds of muscle injuries. It should also be noted that celecoxib, and other first generation COX-2 inhibitors, have been associated with increased risk of cardiovascular events, such as heart attacks (Dajani & Islam 2008). However, equivocal findings have been made, and the short term use (few weeks) of ≤ 400 mg per day for healthy people, not suffering from cardiovascular disease, seems to be reasonably safe (Kearney et al. 2006; Solomon et al. 2008).

CONCLUSION

This is the first study to investigate the effect of a COX-2 inhibitor on inflammation, recovery, regeneration and adaptation after exercised-induced muscle damage in humans. We found no effect of the COX-2 inhibitor on recovery of muscle function after damaging elbow flexor eccentric exercise. However, the drug reduced delayed onset muscle soreness. High doses of celecoxib during nine days after a bout of eccentric exercise had no detectable influence on the repeated-bout effect observed three weeks after the first bout. An extensive inflammatory response to unaccustomed eccentric exercise occurred in some subjects, several days after the exercise. The accumulation of leukocytes was apparently unaffected by celecoxib administration and seemed to be related to segments of necrotic and later regenerating myofibres in the exercised muscle. Thereby, our study provided insight into the basal regeneration processes in healthy human skeletal muscles.

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TABLE AND FIGURE LEGENDS

Table 1

Descriptive characteristics of the subjects. Values are means±SD.

Table 2

Quantitative immunohistochemistry: Number of neutrophilic granulocytes (CD66b), monocytes/macrophages (CD68) and satellite cells/myoblasts (CD56) in muscle cross sections from the exercised muscle. Values are medians and full ranges. N = 8-14 in each group per time point. The p-values stem from group comparisons.

Figure 1

Overview of the study. The dots, x's and # show the time points for tests and measurements. Note the drug administration after bout 1 only.

Figure 2

Changes in force-generating capacity of the elbow flexors exposed to two bouts of maximal eccentric exercise (same arm) and no exercise (control). Figure 2 A) Changes in maximal voluntary isometric torque (90°) and Figure 2 B) changes in maximal voluntary isokinetic concentric torque ($60°\cdot s^{-1}$). N-celecoxib = 12; n-placebo = 15. Error bars are SEM. # denotes difference from baseline values (before bout 1). The area under the curve (AUC) was larger for bout 1 than 2 for both groups ($p < 0.01$).

Figure 3

Muscle soreness after two bouts of eccentric exercise, evaluated by VAS (visual analogue scale; 0-100 mm). N-celecoxib = 12; n-placebo = 15. Error bars are SEM. # denotes difference from baseline. The area under the curve (AUC) was larger for bout 1 than 2 for both groups ($p < 0.01$). The AUC was smaller for the celecoxib group than for the placebo group for both bouts (bout 1: $p = 0.04$; bout 2: $p = 0.06$).

Figure 4

Scintigram illustrated. The image shows accumulation of radiolabelled autologous leukocytes in the exercised arm muscles, most prominent in the lower/middle part of the upper arm elbow flexors (dark areas reflect high radioactivity). The figure shows the relative differences in radioactivity between the exercised arm and the non-exercised, control arm six and 20 hours after exercise. N-celecoxib = 9; n-placebo = 13. Error bars are SEM. * denotes difference between the six and 20 hours values.

Figure 5

Immunohistochemical experiments illustrated. The images are from a subject in the placebo group (but similar pictures were obtained from those in the celecoxib group). Left image (A): CD68 staining of a sample obtained 168 hours (one week) after bout 1 (CD68 = red, laminin = green, nuclei = blue [DAPI]). The arrow points to a myofibre that is heavily infiltrated by $CD68^+$ cells (primarily monocytes/macrophages). Note that this myofibre was negative for dystrophin (not shown) and probably in a stage of necrosis. The presence of rounded and apparently swollen myofibres suggests that the myofibres were highly stressed in this sample. Right image (B): CD68 staining on a sample obtained 1 hour after bout 2, i.e., three weeks after bout 1. Note the small myofibres with centrally located nuclei (arrow), indicating regenerated myofibres, surrounded by $CD68^+$ macrophages. These myofibres were also positive for CD56 (see Figure 6). Note that the $CD68^+$ cells were most likely remnants from the damage reaction to bout 1 (not bout 2). Thus, the present $CD68^+$ cells are probably macrophages/histiocytes that served regenerating myofibres. There was little or no specific staining in the control samples (lower right corner). Scale bars = 50 µm.

Figure 6

Immunohistochemical experiments illustrated (CD56 = green, Ki67 = red, nuclei = blue). Left column of images (A, C): Tissue samples from a subject in the celecoxib group. Right column of

images (B, D): Tissue samples from a subject in the placebo group. The top row of images (A, B) shows high satellite cell/myoblast activity and $Ki67^+$ cells one week after bout 1. The inserted image on image A shows accumulation and apparent fusion of myoblasts ($CD56^+$), as well as cells that seem to go through cell division (pink stain = Ki67 [red] + DAPI [blue]). The inserted images on image B show (i) a normal looking satellite cell, i.e., with CD56 staining around the nucleus, which could be found in samples from both control and exercised muscles, and (ii) a satellite cell positive for Ki67 (sample from a exercised muscle). The second row of images (C, D) shows tissue samples obtained 48 hours after bout 2, i.e., three weeks after bout 1. Note the CD56 positive myofibres with relatively small diameter and centrally located nuclei (arrows), which indicate that these are regenerated myofibres. On image C there is a $Ki67^+$ nucleus apparently inside the myofibre (arrowhead). Hence, this seems to be part of the regenerating process after bout 1 (not bout 2). Scale bars = 50 μm.

Figure 7

Creatine kinase in serum. N-celecoxib = 12; n-placebo = 15. Error bars are SEM. # denotes difference from baseline values. The area under the curve (AUC) was larger for bout 1 than bout 2 for both groups ($p < 0.01$).

Table 1.

	Group	N	Age (yr)	Height (m)	Weight (kg)	Celecoxib mg·kg^{-1}
Males	Celecoxib	8	28 ± 5	1.81 ± 0.03	78 ± 7	5.1 ± 0.5
	Placebo	14	26 ± 4	1.82 ± 0.06	77 ± 6	
Females	Celecoxib	7	28 ± 5	1.65 ± 0.03	59 ± 6	6.9 ± 0.7
	Placebo	4	23 ± 5	1.67 ± 0.03	62 ± 3	
♀+♂	Celecoxib	15	28 ± 5	1.73 ± 0.08	69 ± 12	6.0 ± 1.1
	Placebo	18	25 ± 4	1.79 ± 0.08	74 ± 8	

Table 2.

Positive cells pr 100 myofibre	Bout #	Time point	Celecoxib	Placebo	P-value celecoxib *vs.* placebo
CD66b	Bout 1	Early	0 (0-3)	0 (0-29)	P = 0.8
		Late	0 (0-2)	0 (0-3)	P = 0.9
	Bout 2	Early	0 (0-2)	0 (0-3)	P = 0.6
CD68	Bout 1	Early	21 (6-81)	25 (10-133)	P = 0.9
		Late	35 (14-150)	45 (7-968)	P = 0.7
	Bout 2	Early	27 (2-94)	47 (15-104)	P = 0.2
CD56	Bout 1	Early	11 (7-18)	12 (8-23)	P = 0.6
		Late	16 (10-50)	20 (7-103)	P = 0.7
	Bout 2	Early	13 (11-25)	14 (10-49)	P = 0.5

Figure 1.

BOUT 1		**Pre exercise**								**Post exercise**				*1 week*		*3 weeks*
				Day 1						Day 2	Day 3	Day 4	Day 5	Day 8	Day 10	Day 22 (**Bout 2**)
Hours		*-1*	*0*	*1*	*2*	*5*	*6*	*8*	*20*	*24*	*48*	*72*	*96*	*168*	*216*	*503*
Drug administration																
Tests:	Fam. tests		**Exercise Bout 1**													
Muscle function	•	•	•			•				•	•	•	•	•	•	•
Muscle soreness	x	x	x			x				x	x	x	x	x	x	x
Muscle swelling	•	•	•			•				•	•	•	•	•	•	•
Resting arm angle	x	x	x			x				x	x	x	x	x	x	x
Blood sample		•		•				•	•	•	•	•	•	•	•	•
Scintigraphy							x		x							
Biopsy				#1							#2		#3	#4		
Microdialysis					x					x	x					

BOUT 2	**Pre exercise**							**Post exercise**				*1 week*	
			Day 1					Day 2	Day 3	Day 4	Day 5	Day 8	Day 10
Hours	*-1*	*0*	*1*	*5*	*6*	*8*	*20*	*24*	*48*	*72*	*96*	*168*	*216*
Tests:		**Exercise Bout 2**											
Muscle function	•	•		•				•	•	•	•	•	•
Muscle soreness	x	x		x				x	x	x	x	x	x
Muscle swelling	•	•		•				•	•	•	•	•	•
Resting arm angle	x	x		x				x	x	x	x	x	x
Blood sample	•		•			•	•	•	•	•	•	•	•
Scintigraphy					x		x						
Biopsy			#5						#6				

Figure 2 A)

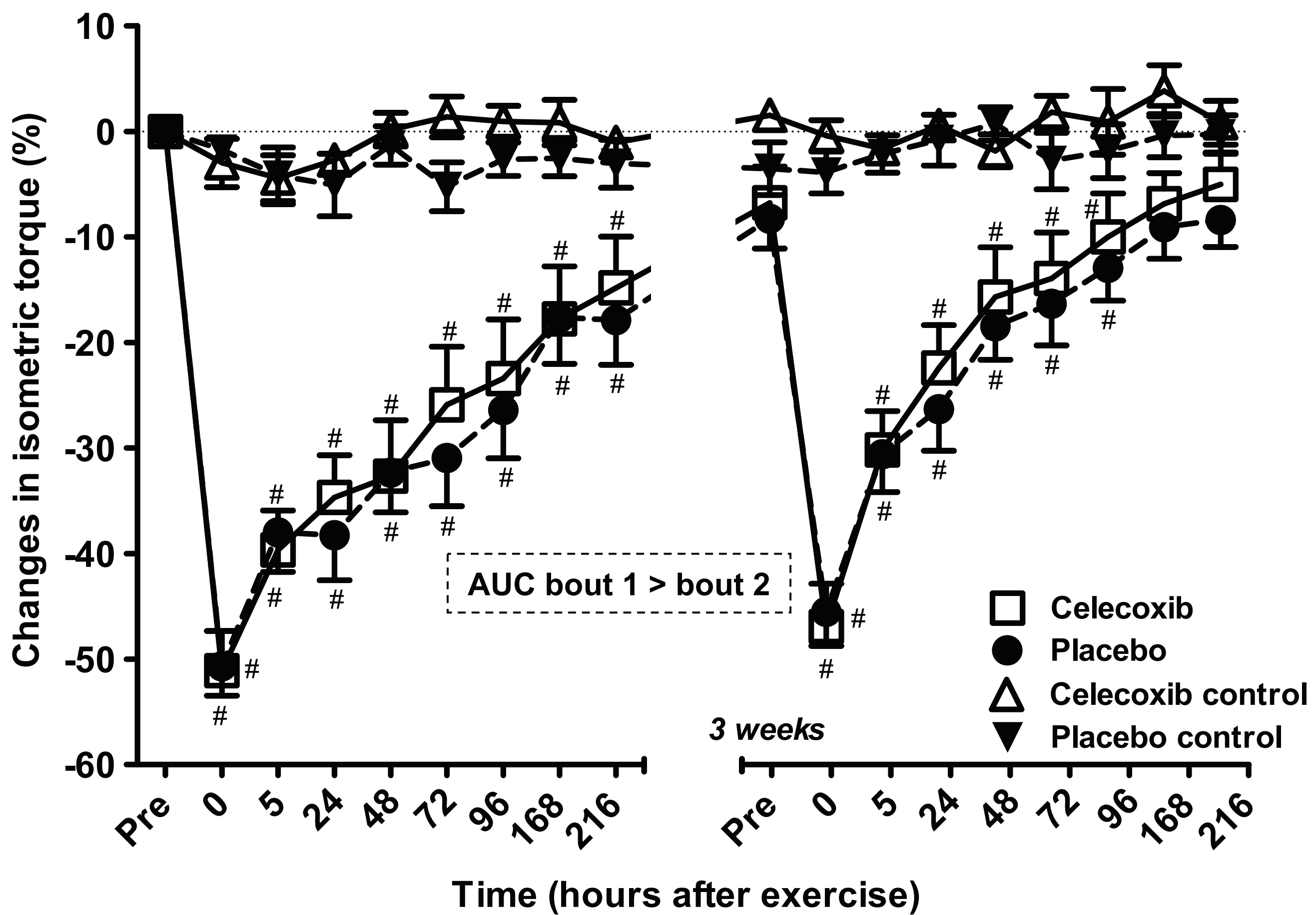


Figure 2 B)

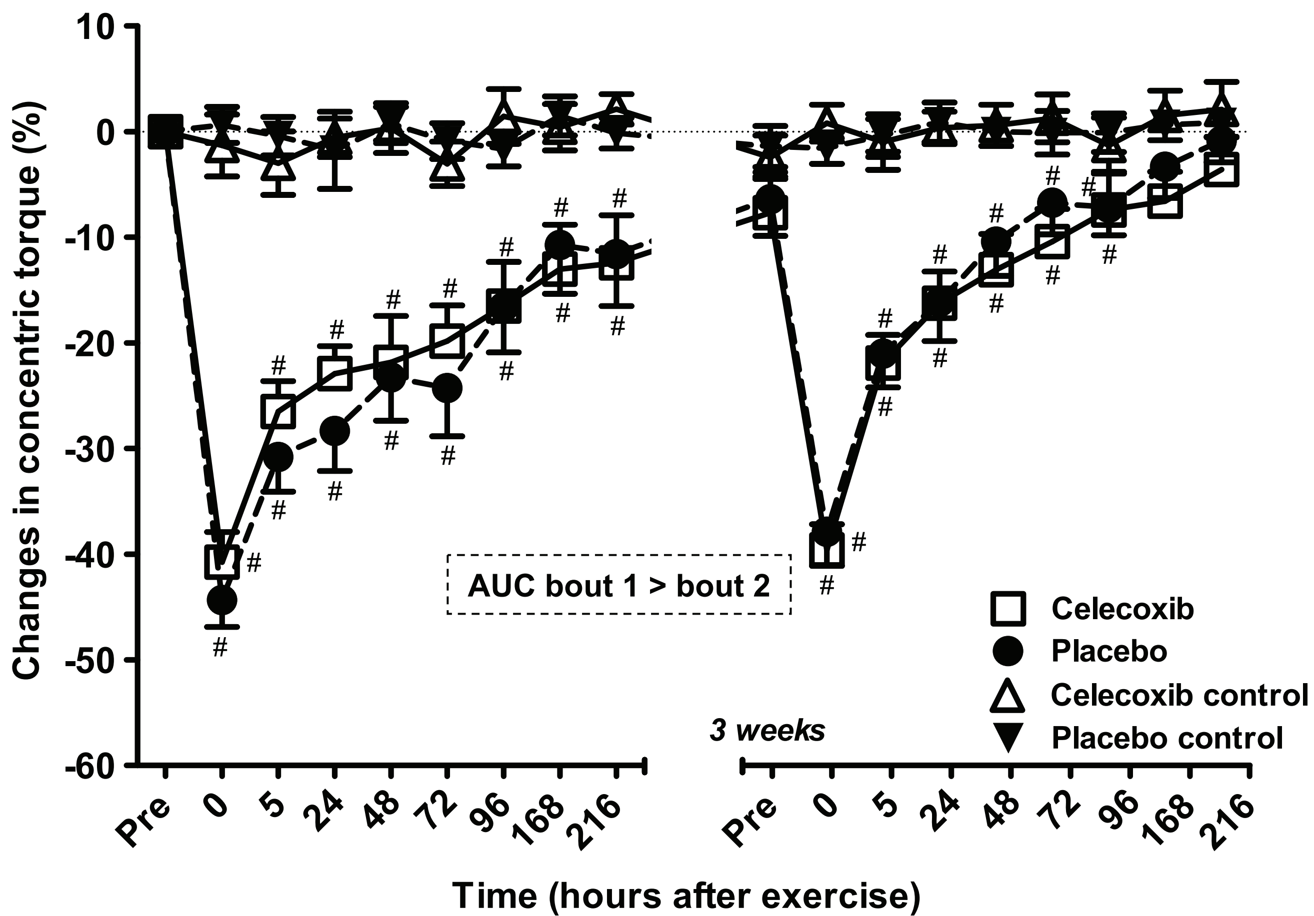


Figure 3

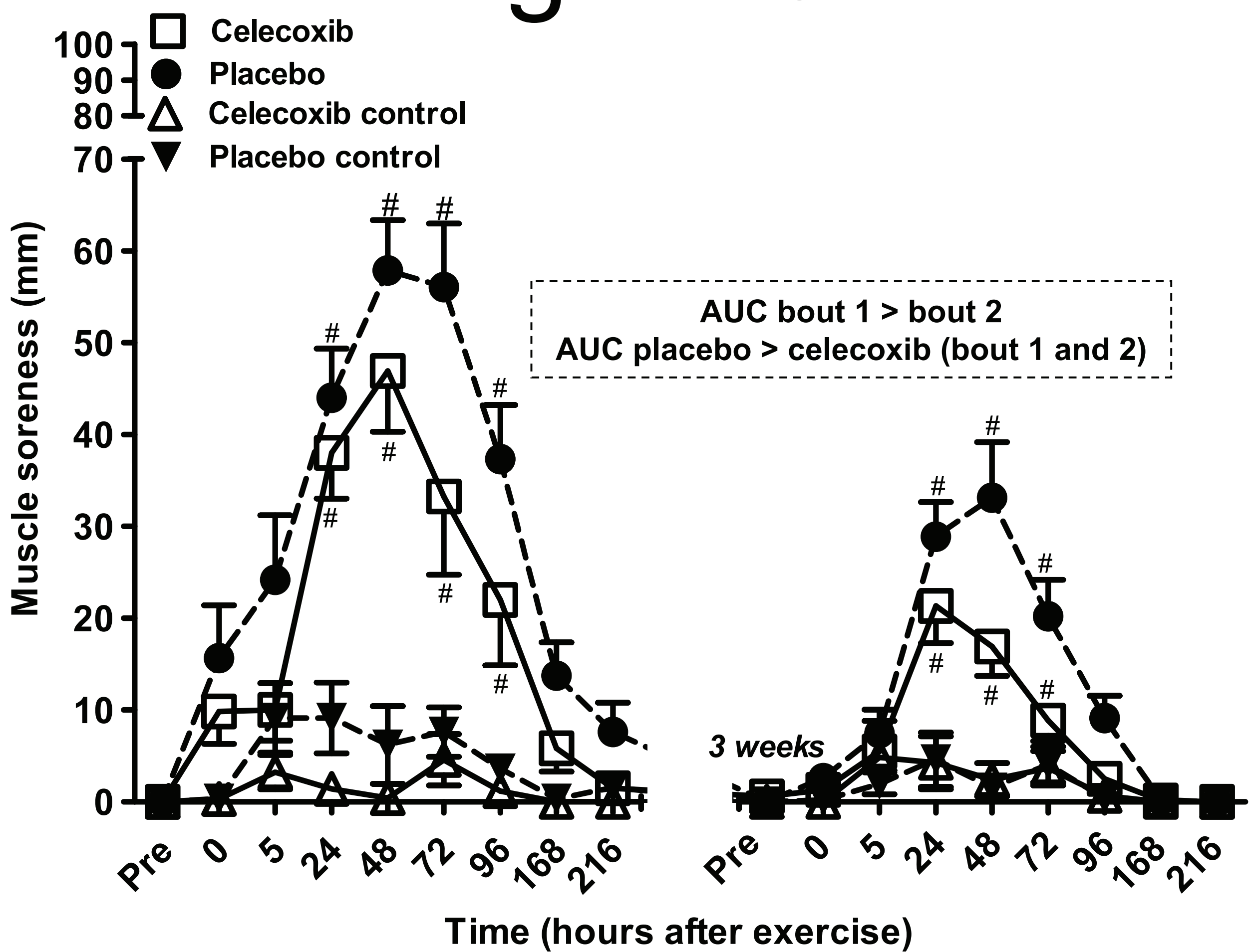
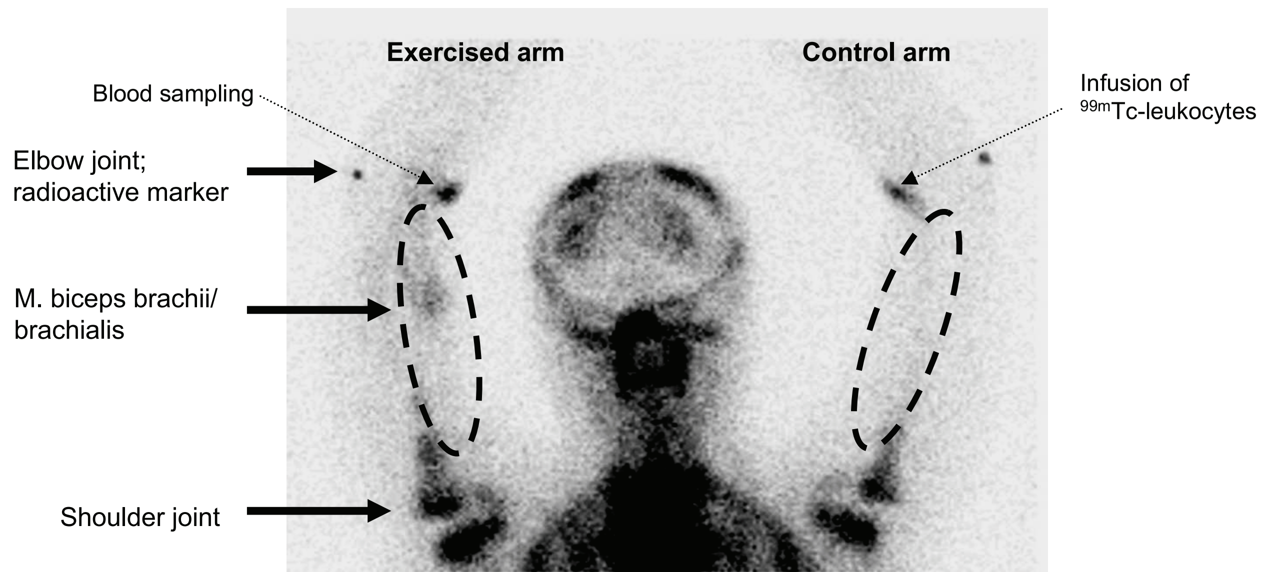


Figure 4



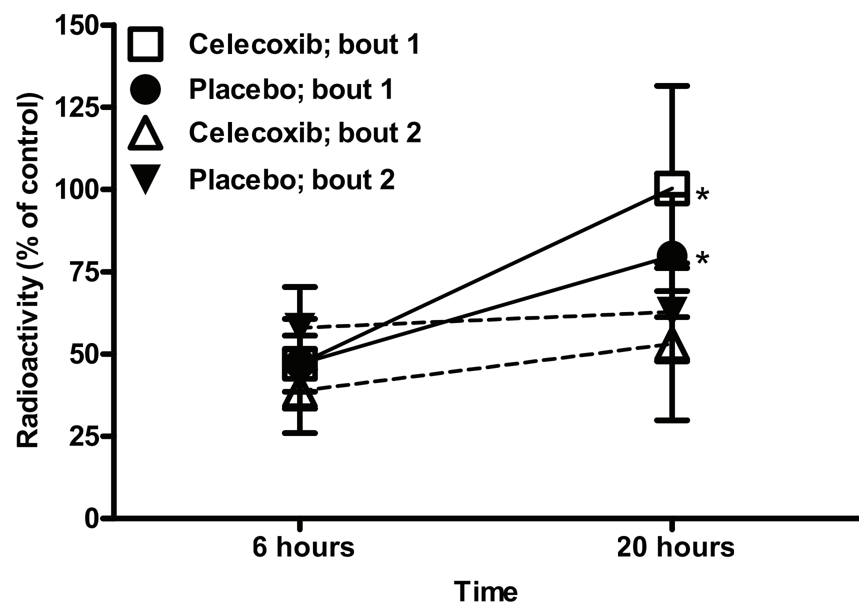


Figure 5

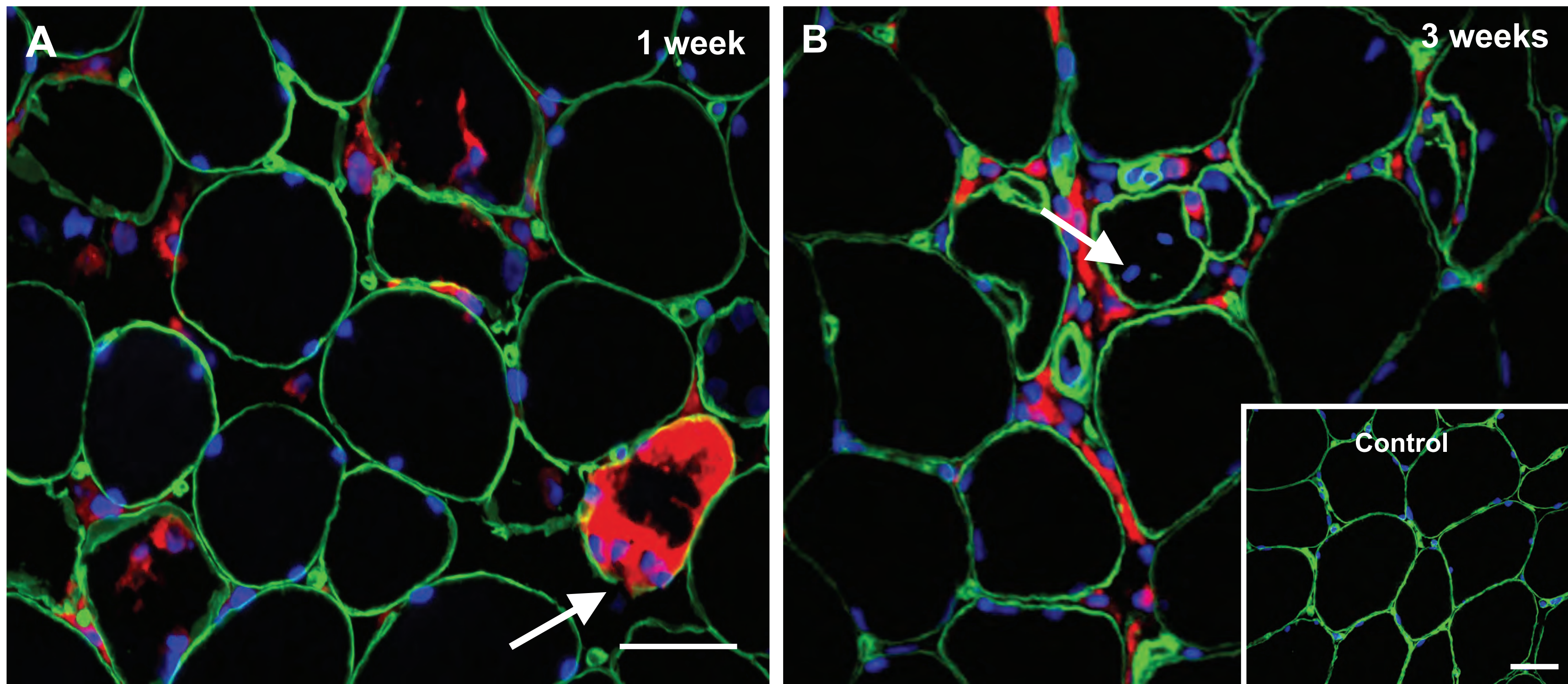


Figure 6

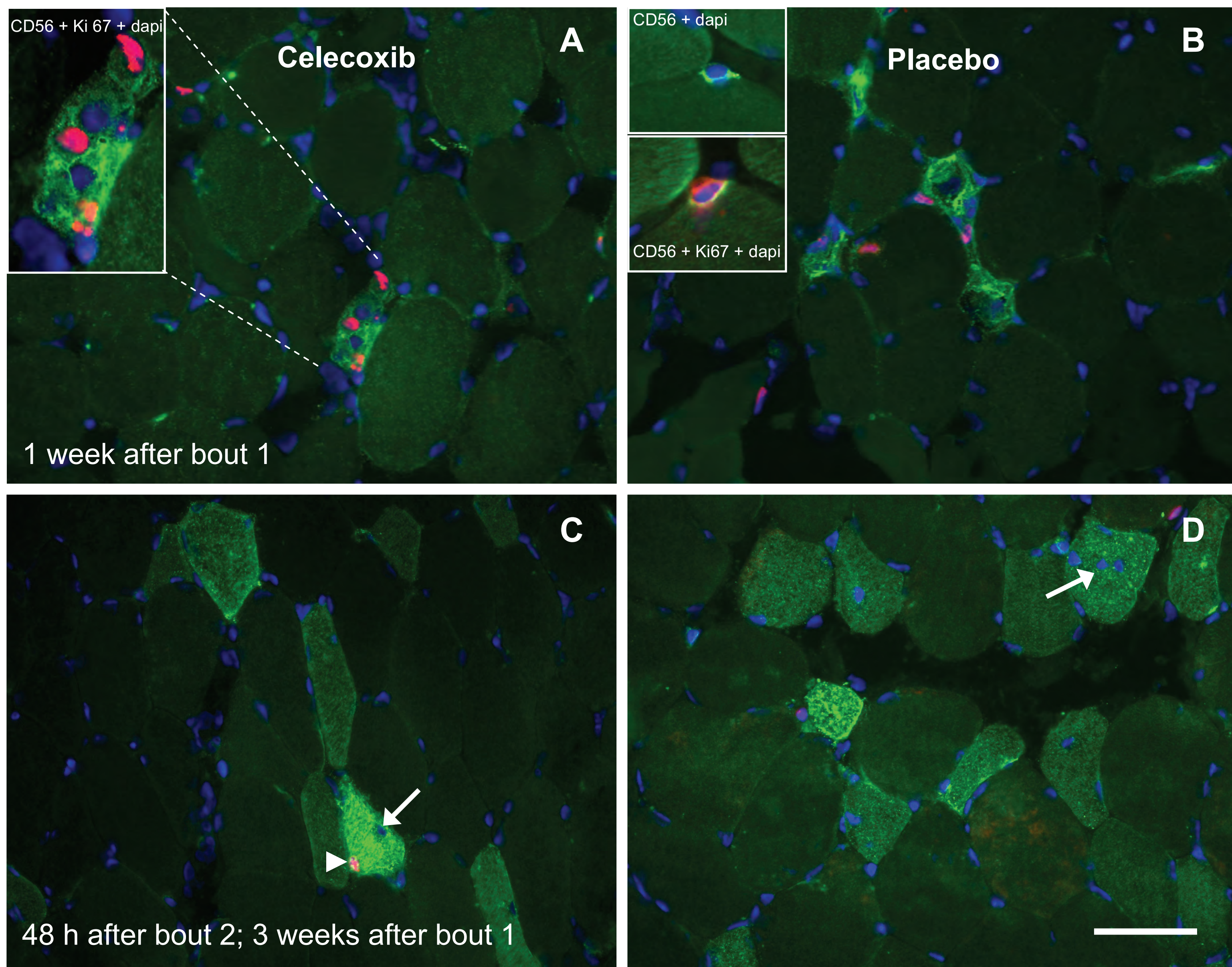
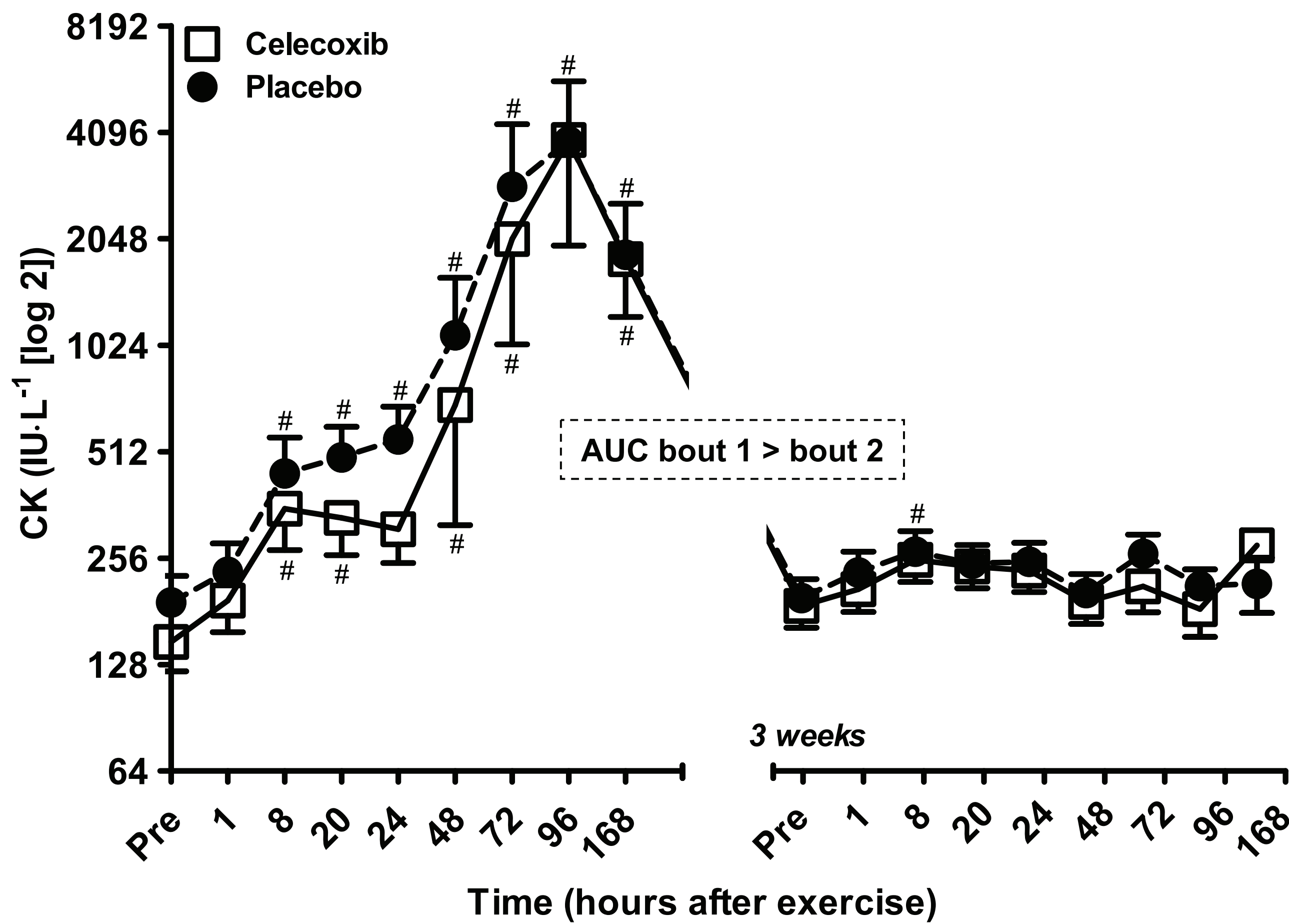


Figure 7



PAPER III

Paulsen G, Vissing K, Kalhovde JM, Ugelstad I, Bayer ML, Kadi F, Schjerling P, Hallen, J, Raastad T. Maximal eccentric exercise induces a rapid accumulation of small heat shock proteins on myofibrils and a delayed HSP70 response in humans. Am.J Physiol Regul.Integr.Comp Physiol 2007 Aug;293(2):R844-R853.

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PAPER IV

Paulsen G, Lauritzen F, Bayer ML, Kalhovde JM, Ugelstad I, Owe GS, Bergersen LH, Hallén J, Raastad T. Involvement of HSP27, B-crystallin and HSP70 in the repeatedbout effect after eccentric exercise in humans. J Appl. Physiol; In revision

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