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Exercise-induced muscle damage: The influence of genetic polymorphisms on inter-individual variability

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Palavras chave: Eccentric exercise, exertional muscle damage, creatine kinase (CK), skeletal muscle repair.

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Resumo

Existe uma variabilidade considerável relativamente à expressão fenotípica e apresentação de lesão muscular induzida pelo exercício, a qual não pode ser apenas predita com base na idade, raça, composição corporal e actividade ou inactividade física. Factores genéticos podem influenciar igualmente o desenvolvimento e progressão da lesão muscular induzida pelo exercício. Assim sendo, o objectivo da presente tese foi analisar a influência de polimorfismos de nucleótideo único (SNPs) em genes associados com mecanismos da lesão/reparação do músculo esquelético nos níveis sanguíneos de creatine kinase (CK) após exercício excêntrico. Quatro SNPs foram identificados como potenciais alvos: *ACE* (I/D), *IL-6* (-174 G/C), *CK-MM* (NcoI) e *TNF-α* (G-308A). Para a consecução destes objectivos, 3 estudos foram desenvolvidos envolvendo 70 estudantes saudáveis (42 homens e 28 mulheres; idade 22–32 anos), fisicamente activos e que não recebiam qualquer tratamento médico à altura. Os participantes eram caucasianos israelitas, com um rácio equivalente de cidadãos descendentes de Ashkenazi and não-Ashkenazi. Os resultados demonstraram que 3 SNPs, nomeadamente *ACE* (I/D), *IL-6* (-174 G/C) e *TNF-α* G-308A, estão associados com a resposta da CK ao exercício excêntrico. Com base nestes resultados, é possível concluir que a magnitude de lesão muscular induzida pelo exercício é condicionada igualmente por factores genéticos, em particular por SNPs presentes em genes associados com mecanismos de lesão e reparação do musculo esquelético. Estes resultados podem ajudar a identificar indivíduos que apresentam uma predisposição acrescida de lesões muscular-esqueléticas induzidas pelo exercício

Palavras-chave: Exercício excentrico, lesão muscular, creatina kinase (CK), reparação muscular-esquelética

Abstract

There is a wide interindividual variability in the phenotypic expression and presentation of exercise-induced skeletal muscle damage, which cannot be predicted based on age, race, body composition, physical activity, or inactivity, suggesting that genetic factors may also influence the development and progression of exercise-induced skeletal muscle damage. In this sense, the purpose of this thesis was to analyze the influence of candidate single nucleotide polymorphisms (SNPs) in genes closely associated with the mechanisms of skeletal muscle damage/repair on the blood levels of Creatine-kinase after eccentric exercise. Four SNPs were identified as potential targets: the *ACE* (*I/D*), *IL-6 (-174 G/C), CK-MM* (*Ncol*), and *TNF-α* (*G-308A*). To accomplish our goals three studies were conducted involving 70 healthy physical education students (42 males and 28 females; aged 22–32), physically active and not receiving any medical treatment. Participants were all Israeli Caucasians, with an equivalent ratio of Ashkenazi and non-Ashkenazi descent. The results showed that three SNPs, namely *ACE* (*I/D*), *IL-6 (-174 G/C),* and *TNF-α G-308A* are associated with CK (creatine kinase) response to eccentric exercise, whereas *CK-MM* (*Ncol*) SNP is not associated with CK response to eccentric exercise. Based on these results, it is possible to conclude that the magnitude of exercise-induced muscle damage is also conditioned by genetic factors, particularly by several SNPs in genes associated with the mechanisms involved in skeletal muscle damage and repair. These results might help to identify the individuals who are genetically predisposed to exercise-induced muscle damage.

Keywords: Eccentric exercise, exertional muscle damage, creatine kinase (CK), skeletal muscle repair.

List of abbreviations

ACE I/D- angiotensin-converting enzyme insertion/deletion polymorphism

ACE - angiotensin-converting enzyme

ANG - angiotensin

AT1R - angiotensin II type 1 receptor

ATP- adenosine triphosphate

Bp - base pairs

BMI - body mass index

CK - creatine kinase

FGF- fibroblast growth factor

HGF - hepatocyte growth factor

IL-1RA - Interleukin -1 receptor antagonist

IL-6 - Interleukin-6

IGF- insulin-like growth factor

LBM – Lean body mass

LIF- leukemia inhibitory factor

Mb - myoblobin

MVC - maximal voluntary isometric contraction

NIDDM - non-insulin-dependent diabetes mellitus

PCR- polymerase chain reaction

RAS - renin-angiotensin system

RFLP - restriction fragment length polymorphism

SNP- single nucleotide polymorphism

TNF-α - tumor necrosis factor α

TNF-α R1 - tumor necrosis factor α receptor 1

VO_{2max}- maximal oxygen consumption

This thesis is based on the following original articles:

1. Yamin, C., Amir, O., Sagiv, M., Attias, E., Meckel, Y., Eynon, N., Sagiv, M., Amir, R.E. (2007). ACE ID genotype affects blood creatine kinase response to eccentric exercise. J Appl Physiol 103: .2057-61

2. Yamin, C., Duarte, JA., Oliveira, JM., Amir, O., Sagiv, M., Eynon, N., Sagiv, M., Amir, RE. (2008). IL6 (-174) and TNF-α (-308) promoter polymorphisms are associated with systemic creatine kinase response to eccentric exercise. Eur J Appl Physiol 104: 579-86.

3. Yamin, C., Oliveira, J.M., Meckel, Y., Eynon, N., Sagiv, M., Ayalon, M., Duarte, JA (2010). CK-MM gene polymorphism does not influence the blood creatine kinase activity after exhaustive eccentric exercise. Int J Sports Med 31: 213-18.

General Introduction

Exercise-induced muscle damage is characterized by the breakdown of skeletal muscle cells accompanied by the leakage of muscle contents into the circulatory system [e.g. creatine kinase (CK) and myoblobin (Mb), among others] (Nosaka et al.,1992; Clarkson and Hubal, 2002; Lee and Clarkson, 2003). Clinical features are often nonspecific, and include loss of muscle strength, reduced range of motion, swelling, and delayed onset muscle soreness (Hojs et al., 1999). Disturbing complications, including acute renal failure, severe hyperkalemia, and cardiac arrhythmia and arrest (Milne, 1988; Beetham, 2000) may also occur.

Elevated levels of serum CK are commonly used to evaluate the severity of muscle damage (Houmard et al., 1990; Brancaccio et al., 2007; Khan, 2009), being the blood CK response considered a marker of muscle damage when its levels rise fivefold from peak normal values, which are normally between 15-167 U\L (Daher et al., 2005).

Markedly high blood levels of CK (between 300 – 10,174 U\L) are often seen following prolonged endurance activities (e.g., marathon running), and particularly following short exercises that involve intense resistive eccentric contractions (e.g., push-ups, squats, etc.) (Brancaccio et al. 2007; Devaney et al. 2007).

It is known that the magnitude of muscle damage is influenced by several conditioning factors such as type of exercise, gender, age, and level of training (Brancaccio et al., 2007). For instance, muscle damage induced by overexertion is frequently reported in healthy adolescent and adult athletes following habitual and/or strenuous exercise, such as in military training, weight lifting, and marathon running (Howatson and Someren, 2008; Proske and Allen, 2005; Senert et al., 1994). A gender difference in CK serum levels is an important factor that needs to be considered. In this regard, males often present higher values of CK at rest than females. In fact, following muscular exercise, sex-linked differences are still present (Amelink et al., 1988) and estrogen may be

an important factor in maintaining post-exercise membrane stability, thus limiting CK leakage from the muscle (Tiidus, 2009). An additional factor that might influence the magnitude of muscle damage is age. During aging, skeletal muscles become smaller and weaker, such that by the age of 70 years, the cross-sectional area of a range of muscles is reduced by 25–30% and muscle strength by 30–40% (Porter et al., 1995) as compared to young adults. Therefore old people are more susceptible to contraction-induced damage and take longer to recover from such damage than younger adults. (Close et al., 2005). The blood CK response to exercise is also influenced by body mass (Swaminathan et al., 1988) and physical activity status, with resting levels higher in athletes than in sedentary subjects, which might be explained by the regular training that athletes undergo (Brancaccio et al., 2007).

The inter-individual variability of muscle damage is huge and cannot be solely explained by the above-mentioned factors (Salvadori et al., 1992; Manfredi et al. 1993; Sherwood et al. 1996; Vincent and Vincent, 1997; Horská et al., 2000; Schwane et al., 2000). Indeed, several studies have demonstrated a large inter-subject variability in CK response to eccentric exercise (Newham et al., 1983; Nosaka et al., 1992; Nosaka and Clarkson, 1996). One study acknowledged a dramatically higher levels of CK (peak value ranged from 6,740 to 24,200 U\L) following eccentric exercise in six untrained female subjects (Nosaka et al., 1992), whereas another study (Nosaka and Clarkson, 1996) found a large variability among male subjects in peak CK response (236 - 25,244 U\L). Furthermore, following eccentric exercise, subjects who were high responders on the first exercise were also high responders when repeating the exercise one year later (Chen, 2006). The subjects who were high responders to eccentric exercise (greater than 10,000 $u \cdot L^{-1}$) also presented profound strength losses, swelling, and intense muscle pain (Sayers et al., 1999; Chen, 2006).

In recent years, it has been suggested that genetic factors might play an important role in the development of exercise-induced muscle damage (Clarkson et al., 2005; Devaney et al., 2007; Heled et al., 2007). Indeed, it is

recognized that several inherited genetic muscular disorders, such as McArdle's disease and Duchenne's muscular dystrophy, predispose individuals to develop exercise-induced muscle damage (Zager et al., 1996). Other more rare causes of muscle damage include inherited diseases of glucose, lipids, proteins, or nucleoside metabolism that lead to a reduced energy source for the working muscles (Khan, 2009). In most cases, the primary common pathway for muscle fiber disintegration is the imbalance between energy demand and energy production/supply, leading to deficient delivery of adenosine triphosphate (ATP), and compromising cell physiology and integrity (Howatson and Someren., 2008). These disorders are usually expressed during childhood and should be suspected if muscular weakness or myoglobinuria frequently recur, or appear in association with events that are unlikely to precipitate muscle damage in healthy subjects (Tonin et al., 1990).

Regarding apparently healthy people, comprehensive study in twins demonstrated a positive relationship between the severity of exercise-induced muscle damage and genetic polymorphisms, suggesting that genetic influence should also be considered (Meltzer et al., 1978). In recent years, the previously mentioned inter individual susceptibility to exertional muscle damage has usually been detected by studies of genetic association. This kind of study is commonly used to test the influence of variation at a given candidate locus on specific performance traits. These genetic variations, also known as single nucleotide polymorphisms (SNPs), might lead to different CK level responses and to other muscle damage phenotypes with any type of exercise. Apart from the relatively well identified genetic diseases, several gene polymorphisms described in healthy people might be considered as potential candidates to explain inter-subject variability, such as those encoding energy metabolic enzymes or those involved in the control of muscle proteolysis, inflammatory reaction or repair mechanisms (Stewart and Rittweger, 2006).

The muscle-specific isoenzyme of CK (CK-MM) is localized at the M line and at the sarcoplasmic reticulum of myofibrils (Brancaccio et al., 2007; Echegaray and Rivera, 2001). Heled et al. (2007) found that one genotype of

the *CK-MM NcoI* polymorphism was associated with a risk of exaggerated CK response to exercise. Individuals with the *AA* genotype (normal homozygous) had a six-fold higher risk compared to those who include the rare allele (*G*) polymorphism (*GG* and *AG*) for exhibiting an exaggerated CK response to exercise. Up to this point, this is the only study to suggest that this particular polymorphism might be involved in the process of developing exercise-induced muscle damage. However, because of several flaws identified in that study, their conclusions might be questioned, raising doubts about the real influence of *CK-MM NcoI* polymorphism on the magnitude of muscle damage induced by exercise.

Besides its immune function, interleukin-6 (IL-6) seems to be involved in muscle repair following exercise-induced damage (Cantini et al., 1995; Helge et al., 2003; Weigert et al., 2007; McKay et al., 2008; Serrano et al., 2008). The IL-6 exerts anti-inflammatory effects by inhibiting the production of the pro-inflammatory cytokine TNF-α (tumor necrosis factor α), and stimulating the synthesis of other anti-inflammatory cytokines such as IL-1 receptor antagonist (IL-1RA), IL-10 and soluble TNF-α receptor1 (TNF-α R1). Thus, during exercise IL-6 indirectly restricts the inflammatory process. A functional-*174G/C* polymorphism of *IL-6* has been identified (Fishman et al., 1998), in which the *G* allele rather than the *C* allele, has been associated with an increased transcriptional response *in vitro* (Fishman et al., 1998; Terry et al., 2000) and *in vivo* (Bennermo, 2004). The pro-inflammatory TNF-α was previously described as a critical component for initiating the breakdown and subsequent removal of damaged muscle fragments following eccentric exercise (Cannon and St Pierre, 1998). A single nucleotide exchange of a guanine by an adenine (*G/A*) at promote region *-308 of TNF-α* has been associated with increased TNF-α production and higher C-reactive protein circulating levels (Bayley et al., 2004; Wilson et al., 1997). Due to the crucial role of IL-6 and TNF-α in muscle repair and muscle damage, respectively, it could be hypothesized that SNPs in these particular genes might be involved in the predisposition to exercise-induced muscle damage.

The skeletal muscle expressed ACE (angiotensin-converting enzyme) has a metabolic role through exercise (Jones and Woodes, 2003). ACE is a key component of the Renin-Angiotensin system (RAS), generating the vasoconstrictor angiotensin (ANG) II and degrading kinins which have a vasodilator effect (Coates, 2003). Within the muscle ANG II affects metabolism (Brink et al., 1996) and seems to act as a growth factor during the skeletal muscle hypertrophic response to mechanical load (Gordon et al., 2001). A functional polymorphism of the human *ACE* gene was recognized in which the presence of *I* allele (insertion) but not *D* allele (deletion) of a 287-bp Alu 2 repeat element in intron 16 was connected with lesser enzyme activity in circulation and in tissues (Danser et al., 1995; Rigat et al., 1990). Because there is considerable RAS activity in skeletal muscle involved in the regulation of muscle metabolism, vascular tone and injury responses, it can be presumed that the *ACE ID* genotype may also contribute to the development of exertional muscle damage and, therefore, may affect CK blood response to strenuous exercise.

Therefore, the aims of this thesis are the following:

1. To analyze the association between *ACE* (*I/D*) genotype and the CK response following eccentric contractions. (**paper I**).

2. To analyze the association of *IL6 G-174C* and *TNF-α G-308A* SNPs with systemic CK response to strenuous exercise, highlighting the role of inflammatory response on the physiopathology of exercise-induced muscle damage. (**paper II**).

3. To analyze the association of *CK-MM NcoI* polymorphisms with the magnitude of CK response, focusing on the susceptibility of muscle cell to exertional muscle damage (**paper III**).

Understanding the relationship between genetic variants and exercise-induced muscle damage may eventually make it possible to identify subjects who are more susceptible to develop this condition, and possibly prevent the

co-morbidities and life-threatening complications (e.g., acute renal failure) associated with this syndrome.

Based on the accumulated evidence, we hypothesize that four SNPs [*(ACE (I/D), IL-6 (-174 G/C), CK-MM (NcoI), and TNF-α (G-308A)*] might influence the development and the magnitude of exercise-induced muscle damage.

Theoretical background

Eccentric contraction seems to be the main trigger for the development of muscle damage (Lovering and De Deyne, 2004). Apart from the mechanical overload imposed to the sarcolemma and to membranes of sarcoplasmic reticulum, it is assumed that metabolic overload with its enhanced temperature and oxidative stress might also contribute to explaining the injury imposed by exercise on skeletal muscle, through the enhancement of structural and functional degradation of lipids and proteins that might compromise the selective permeability of cell membranes, as well as other cellular functions, (Pattwell and Jackson, 2004). This situation leads to a focal Ca^{+2} overload within muscle fiber due to cell inability to maintain its homeostasis. As a consequence, a number of Ca^{+2} dependent proteolytic and phospholipolytic pathways native to the muscle fiber will be activated, which respectively degrade structural and contractile protein and membrane phospholipids (Lovering and De Deyne, 2004). In addition, elevated intracellular Ca^{+2} can also disrupt normal mitochondrial respiration and cause sarcomere contraction (Tee and Bosch, 2007). These autogenic mechanisms will be followed by an inflammatory response in order to remove injured tissue debris and to stimulate regeneration of the damaged fibers (Miles et al., 2008). The inflammatory response, controlled by pro and anti-inflammatory cytokines, results in the muscle infiltration of activated leukocytes and in the release of proteases and free radicals, which contributes to aggravating the degenerative condition of skeletal muscle, in a process widely known as extrinsic degeneration (Malm, 2002; Suzuki et al., 2002). Pro-inflammatory cytokines include tumor necrosis factor-alpha (TNF-α) and interleukin (IL)-1β, which are known to be associated with inflammatory response after exercise-induced muscle damage (Ostrowski et al., 2001; Pedersen et al., 2001). In contrast, anti-inflammatory cytokines such as IL-4, IL-10, and IL-1 receptor antagonist (IL-1ra) inhibit inflammatory responses by reducing the activity of pro-inflammatory cytokines (Malm, 2002; Suzuki et al., 2002). Although not yet fully clarified, it is accepted that IL-6 can act both as a pro- and/or anti-inflammatory cytokine, depending on circumstances (Boneberg and Hartung, 2002; Malm, 2002; Pajkrt et al., 1997;

Pedersen et al., 2001).

Both pro- and anti-inflammatory cytokines increase dramatically following different types of exercise. It is indicated that intense exercise is associated with 1) systemic release of leukocytes and cytokines, 2) alterations in muscular leukocyte receptor expression, 3) muscle leukocyte infiltration and the production of pro-inflammatory cytokines within the damaged muscle tissue, and d) reduction in functional muscular activity (Peake et al., 2005). Interestingly, IL-6 is also produced by the skeletal muscle following exercise, and may act indirectly to restrict muscle inflammation by stimulating anti-inflammatory cytokines production (IL-1ra and IL-10) (Petersen and Pedersen, 2005), and by inhibiting the production of pro-inflammatory mediators like TNF-α and IL-1β (Aderka et al., 1989; Di Poi and Ferraccioli, 1999; Gabay et al., 1997; Xing et al., 1998). Therefore, exercise is associated with a simultaneous increase of both pro- and anti-inflammatory cytokines. This concomitant increase of pro- and anti-inflammatory mediators is probably needed to limit the level and extension of inflammatory process, in order to enhance muscle recovery, maintain muscular capacity, and improve physical performance. The process of inflammatory responses following muscle damage is illustrated in Figure 1. Neutrophils, natural killer cells and lymphocytes are mobilized into circulation after exercise, and soon invade the damaged muscle tissue (Peake et al., 2005). Cytokines are also released into systemic circulation during and immediately after eccentric exercise (Suzuki et al., 2002). Neutrophils are progressively replaced in damaged muscle tissue by macrophages, and pro-inflammatory cytokines are produced in the muscle within one day after eccentric exercise. These inflammatory responses remove cellular debris, favoring the regeneration of muscle fibers after eccentric exercise (Peake et al., 2005).

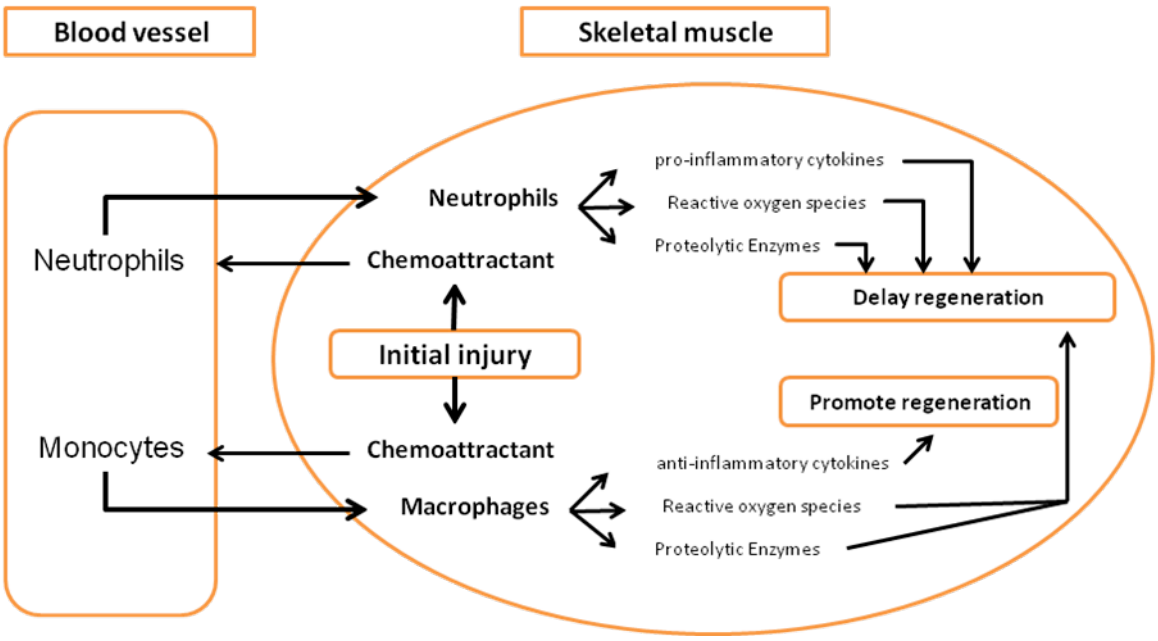

Figure 1. Steps of inflammatory response following eccentric exercise-induced muscle damage.

In response to muscle damage, myogenic satellite cells exit their quiescent state, become "activated" and proliferate, with further migration to the site of injury in order to replace damaged areas by fusion with adjacent muscle fibers, or fusing together to produce new muscle fibers (Figure 2) (Karalaki et al., 2009). Regulation of the myogenic satellite cell population is primarily mediated by the production of inflammatory cytokines in the damaged muscle and by growth factors that are produced and released locally in response to the muscle injury (Zammit et al., 2002). Growth factors also play an important role in cellular proliferation and differentiation in response to skeletal muscle damage. For instance, Insulin-like growth factors (IGFs) are members of a family of proteins involved in satellite cell differentiation and proliferation (Florini et al., 1996; Haugk et al., 1995). In animal models, IGFs have been found to increase in response to degeneration and regeneration, thereby influencing satellite cell activation (Adams, 2002; Bamman et al., 2001; Florini et al., 1996; Levinovitz et al., 1992). Indeed, Keller et al. (1999) demonstrated that after contraction-induced damage of the soleus muscle, there was a significant increase in IGF-I and IGF-II during the days following the injury. The expression

of developmental myosin heavy chain also increased and was co-expressed with both IGFs. In addition to the IGFs, circulating cytokines such as IL-6 also appear to increase myogenic satellite cell proliferation and play an integral role in the repair process following muscle damage (Hibi et al., 1996). Another mechanism involved in muscle tissue repair is related to the renin-angiotensin system. RAS plays an important role in human body fluid homeostasis and in cardiac left ventricular remodeling. Angiotensin-converting enzyme, a key enzyme of this system, is widely expressed in human tissues, including skeletal muscle, and may play a metabolic role during exercise (Jones and Woods, 2003). Angiotensin II, one of the RAS products, has known metabolic effects (Brink et al., 1996), and is recognized as a necessary growth factor for skeletal muscle hypertrophy in response to mechanical loading (Gordon et al., 2001). Most of Angiotensin II (Ang II) physiological, metabolic, and pathophysiological activities are mediated through the Angiotensin II type 1 receptor (AT1R), which is also the only Ang II receptor present in human skeletal muscle (Jones and Woods, 2003).

Other growth factors seem to play important roles in muscle tissue repair, such as Hepatocyte growth factor (HGF), Fibroblast growth factor (FGF), and Leukemia inhibitory factor (LIF). HGF was found to be an integral regulator of multiple myogenic satellite cell function during skeletal muscle regeneration and hypertrophy (Hawk et al. 2001) and FGF has a significant regulatory effect on myogenic satellite cell activity. Currently, at least 10 isoforms of FGF have been identified. Among these isoforms, FGF-6 exhibits high expression levels, which appear to be limited to muscle lineage (Delapeyriere, 1993). LIF belongs to a family of cytokines that play a critical role in the muscle tissue repair process. It has been shown that release of LIF is significantly increased in response to muscle damage, and that LIF increases myogenic satellite cell proliferation both in vitro and in vivo (Kurek et al., 1997).

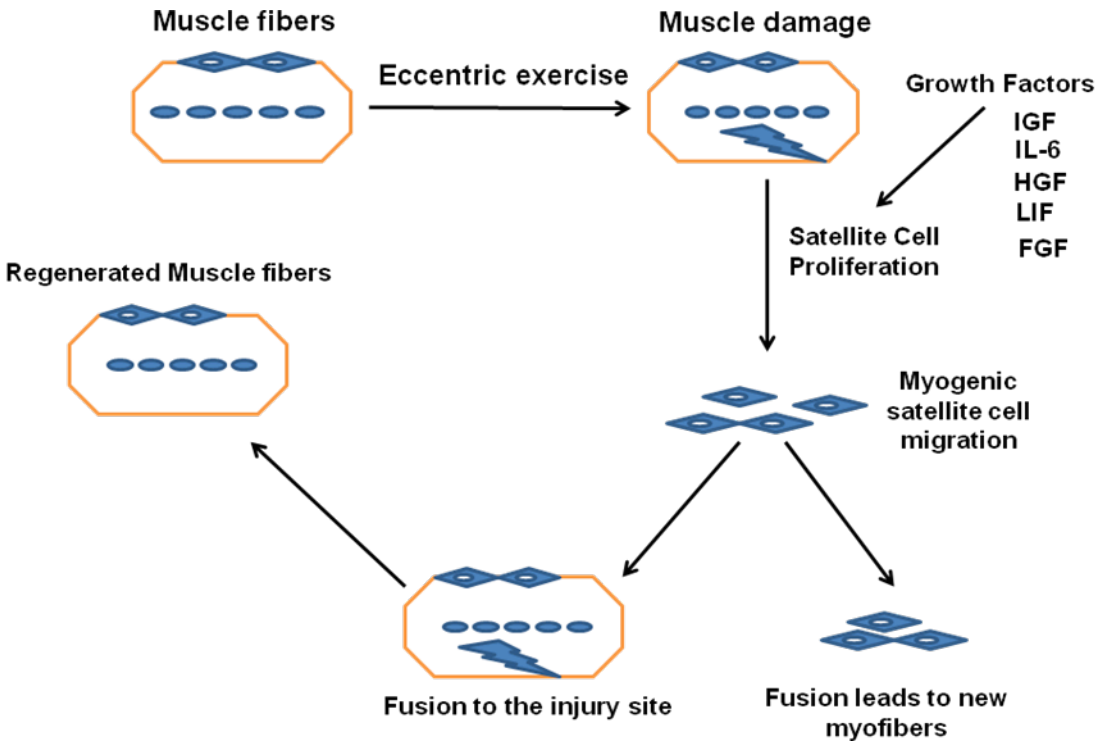

Figure 2. Satellite cell response to exercise-induced muscle damage (IGF, insulin-like growth factor; IL-6, interleukin-6; HGF, hepatocyte growth factor; FGF, fibroblast growth factor; LIF, leukemia inhibitory factor).

Based on the above-mentioned mechanisms underlying exercise-induced muscle damage, it is possible to point out several genes which are directly or indirectly related to the daily maintenance of muscle tissue homeostasis. Among all the potential candidates, several gene SNPs have already been associated with the magnitude of markers of exertional muscle damage, such as those evolving the Myosin light chain kinase [*MLCK*, (*C37885A and C49T*)], *CK-MM NcoI*, *IGF-II* (*C13790G*), *IGF II* (*ApaI, G17200A*), *IGF2AS* (*G11711T*)], *ACE (I/D)*], *IL-6 (-174 G/C*)] and TNF-α (*G-308A*)]. Theoretically, many other genes and polymorphisms could be also linked with the enhanced susceptibility to exertional muscle damage, however, further studies are needed to demonstrate the association.

MLCK is a protein linked with muscle fiber-type that participates in the phosphorilation of myosin's regulatory light chain (RLC) (Sweeney et al., 1993).

Although the binding of Ca^{+2} to tropomyosin-troponin is the primary regulator of skeletal muscle contraction, RLC plays an important regulatory role in muscle force development (Sweeney and Stull, 1990; Szczesna et al., 2002). Clarkson et al. (2005) found that individuals who have the *MLCK C49T* rare mutant homozygous (*TT*) had a significantly greater increase in blood CK and myoglobin levels compared to heterozygous individuals (*CT*) and normal homozygous (*CC*), following eccentric exercise. In addition, those heterozygous for *MLCK C37885A (CA)* had greater strength loss, and a significantly greater increase in CK compared with the homozygous (*CC*) wild type. The given explanation for the increased muscle damage is that *MLCK C49T* and *C37885A* polymorphisms increase the ability of RLC phosphorilation in type 2 fibers, thereby increasing the ability to generate more tension during the stretch of eccentric muscle contraction. Since muscle damage is related to myofibrilar strain (Clarkson et al., 2005), this would increase the magnitude of muscle damage (Figure 3).

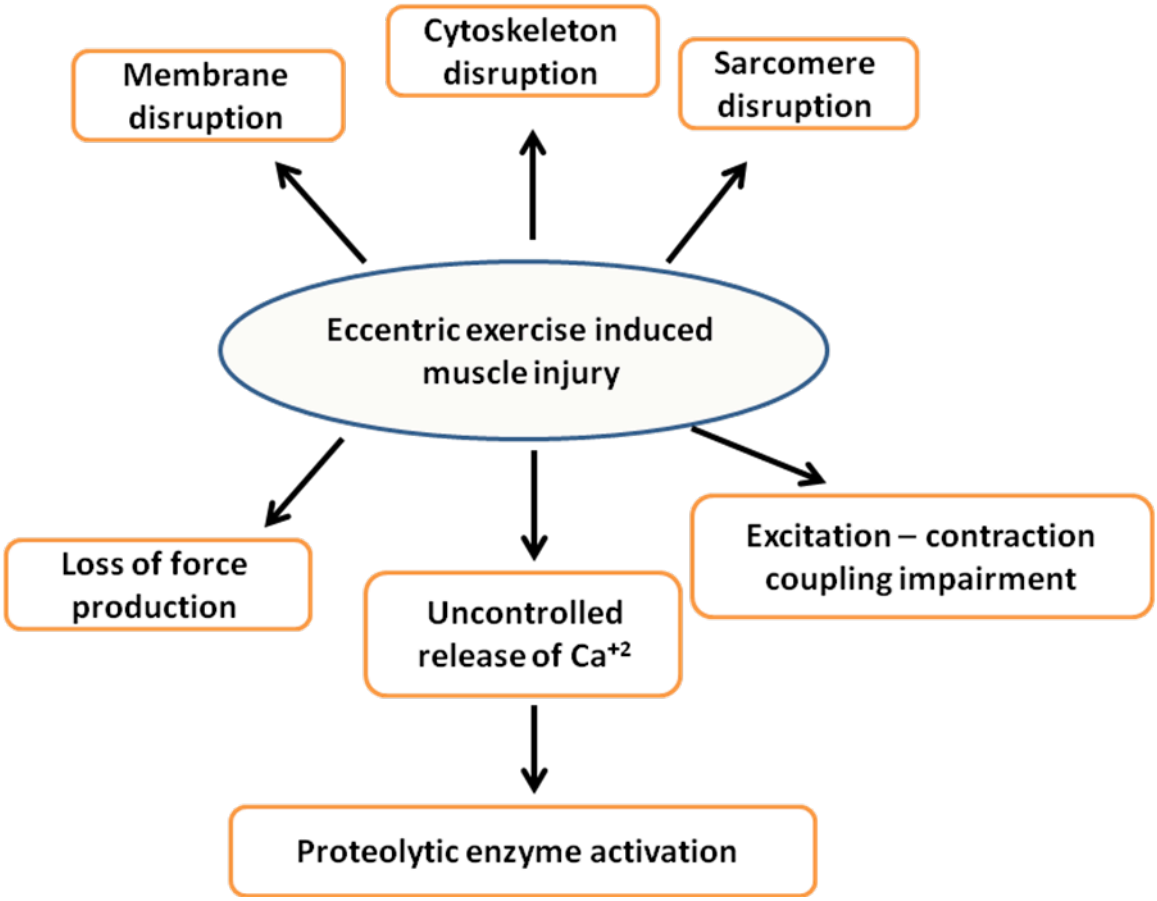

Figure 3. Summary of early changes in eccentric contraction induced muscle injury.

There are three different CK isoenzymes (CK-MM, CK-BB CK-MB) differing in predominance according to tissues (Brancaccio et al., 2007). For instance, CK-BB is located mainly in the brain, and has very little circulatory significance. In contrast, skeletal muscle highly expresses CK-MM (98%) and CK-MB at low levels (1-2%), and the myocardium (heart muscle) expresses CK-MM at 70% and CK-MB at 30% (Echegaray and Rivera, 2001). Following exercise, not only can an increase of CK-MM be seen, but also a significant increase of CK-MB can be detected in the blood. Several possible mechanisms can clarify why the *CK-MM* polymorphism may affect the CK response to exercise. First, there is a linkage between the *CK-MM NcoI* polymorphism and the gene encoding the Ryanodine receptor 1 (RYR1). The *RYR1* gene is

located in the same chromosome region as the *CK-MM* gene (19q13.3), and has been associated with susceptibility to exertional muscle damage (Wappler et al., 2001). Second, inconsistent expression of CK-MM, and possibly altered muscle cell function may originate in the 3' untranslated region, which influences intracellular localization of its mRNA (Zhou et al., 2006).

Insulin-like growth factors are a family of proteins involved in satellite cell differentiation and proliferation (Florini et al., 1996; Haugk et al., 1995). Devaney et al. (2007) found that in human males *IGF-II (C13790G), IGF-II ApaI (G17200A)*, and *IGF2AS (G11711T)* gene polymorphisms were significantly associated with indicators of muscle damage. They found that: 1) Homozygous (*GG*) for the rare *IGF-II C13790G* polymorphisms and the rare genotype (*AA*) for the *ApaI (G17200A)* SNP demonstrated the greatest strength loss immediately after exercise, the greatest post-exercise muscle soreness, and the highest post-exercise serum CK activity; and 2) Homozygous (*TT*) wild type for *IGF2AS (G11711T)* had the greatest strength loss and muscle soreness and a greater CK and myoglobin response to exercise. The main associations of *IGF-II* SNPs with indicators of muscle damage could be due to the role of IGFs in muscle repair. Sayer et al. (2002) and O'Dell et al. (1997) reported that mean serum IGF-II concentration in homozygous individuals for the *IGF-II ApaI* rare genotype (*AA*) was higher than concentrations found in homozygous wild type individuals (*GG*). Gregorevic et al. (2002) suggested that IGF-I administration alters membrane-bound receptors/channels that regulate voltage- and ion-dependent calcium release in muscle cells. Thus, skeletal muscles of the *IGF-II* genotype groups may vary in their exposure to circulating and local muscle IGF-II, which could alter their contractile abilities, enable them to generate more force during contraction and place greater strain on the muscle, explaining the significant associations between genotype and muscle damage.

ACE is expressed in human tissues, including skeletal muscle, and plays a metabolic role during exercise (Jones and Woodes, 2003). Angiotensin II affects metabolism (Brink et al. 1996) and serves as a growth factor during skeletal muscle hypertrophic response to mechanical load (Gordon et al., 2001).

A functional polymorphism of the human *ACE* gene was identified in which the presence (*insertion - I allele*), and not of the absence (*deletion - D allele*) of a *287-bp Alu 2* repeat element in *intron 16* was associated with lower circulation and tissue enzyme activity (Danser et al., 1995; Rigat et al., 1990). This leads to elevated levels of Ang II and aldosterone along with a decreased half-life of bradykinin (Baudin, 2002; Williams et al., 2004). Several potential mechanisms may explain how the *ACE* genotype influences individuals' CK response to strenuous exercise. The increased ACE activity associated with the *DD* genotype may lead to enhanced production of Ang II, which is the predominant biological product of RAS mediating many of the local effects of ACE on skeletal muscle. Ang II is a necessary factor for vascular smooth muscle growth and capillary density in skeletal muscle (Jones and Woodes, 2003). Ang II has a direct hypertrophic effect on skeletal muscle, and AT1R-mediated Ang II is crucial for optimal overload-induced skeletal muscle hypertrophy (Figure 4) (Gordon et al., 2001). Furthermore, Ang II has been shown to regulate oxygen consumption and affect muscle energy expenditure (Cassis et al., 2002). Higher VO_2max levels have been associated with *ACE D* allele (Zhao et al., 2003), indicating an improved oxidative capacity. Although most previous studies demonstrated that the *ACE I* allele (lower ACE activity, high kinin ligand generation, and increased half-life of bradykinin) is associated with increased skeletal muscle metabolic efficiency and perhaps improved contractile function (Boushel et al., 2002; Henriksen et al., 1999; Shen et al., 1995; Shiuchi et al., 2001), local RAS activity in skeletal muscle is much more complex. ACE is not only involved in Ang II production and bradykinin degradation, but also regulates the levels of Ang (1-7) peptide, which is known to cause vasodilatation (Jones and Woodes, 2003). Thus, it is possible that the protective effects of the ACE D allele against exercise-induced skeletal muscle damage are mediated through the fine-tuning that regulates the levels of both Ang II and Ang (1-7). An additional explanation for our results may rely on the mechanisms underlying the process of muscle damage. In exercise-induced muscle damage, the initial inciting event (whether mechanical stretch or excitation-contraction uncoupling) is accompanied by the uncontrolled movement of Ca^{+2} into the

sarcoplasm, triggering the intrinsic degeneration process (Proske and Allen, 2005). Bradykinin receptor B2 (BDKRB2) activation can lead to transient rises in inositol 1,4,5-triposphate (Rabito et al., 1996), which is involved in excitation-contraction coupling through increases in cytoplasmic Ca^{+2} (Lopez and Parra, 1991). Bradykinin stimulates glucose uptake in the presence of insulin, a process apparently related to alteration in intracellular Ca^{+2} concentrations (Kudoh and Matsuki, 2000). Moreover, data suggest that this process is enhanced by the inhibition of ACE (Kudoh and Matsuki, 2000). Interestingly, there is evidence that Ang II can affect both sympathetic and neuromuscular transmission (Jones and Woodes, 2003). Thus, it is conceivable that the ACE genotype affects CK response induced by exercise through the regulation of the excitation-contraction coupling process.

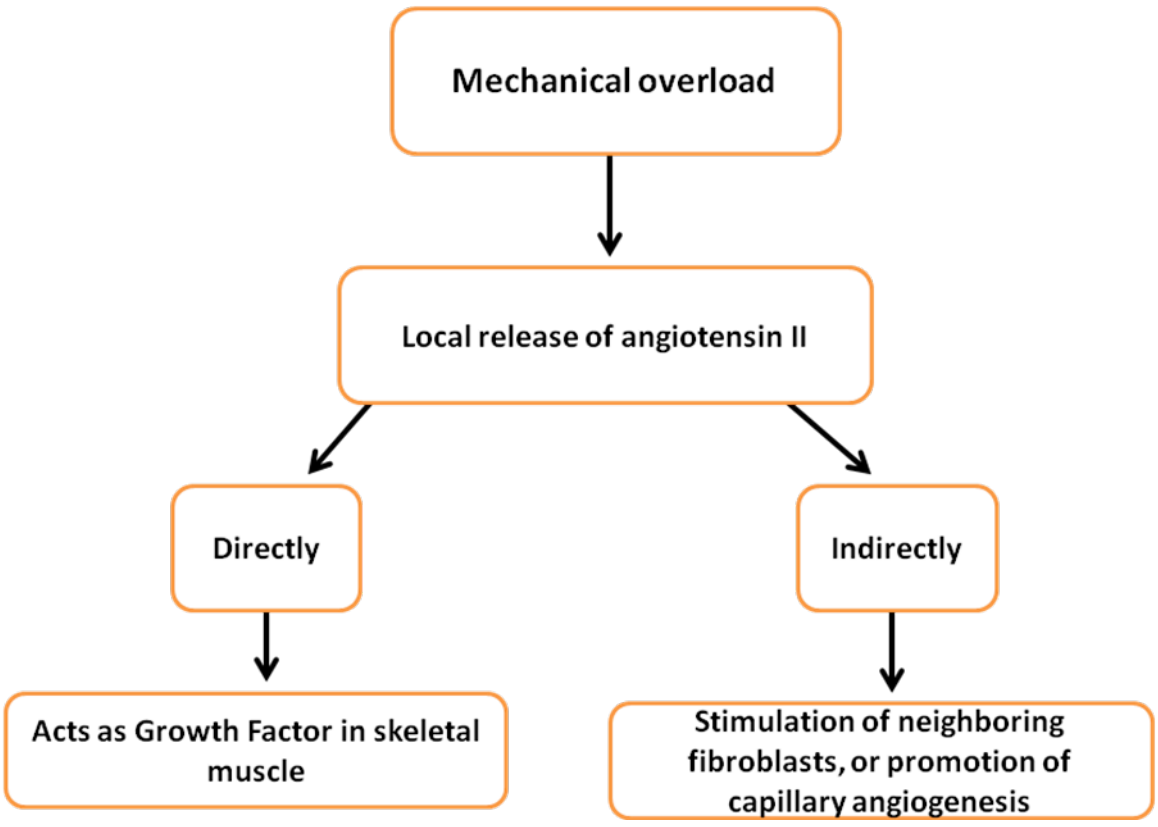

Figure 4. The possible mechanisms by which ANG II contributes to overload-induced skeletal muscle hypertrophy.

The IL-6 family of cytokines may act both as pro- and anti- inflammatory cytokines (Boneberg and Hartung, 2002; Malm, 2002; Pajkrt et al., 1997; Pedersen et al., 2001). IL-6 also acts as a myokine produced within skeletal muscles following resistive eccentric exercise. Furthermore, IL-6 increases myogenic satellite cell proliferation and plays an integral role in the exercise-induced muscle damage repairing process (Hibi et al., 1996). The *TNF-α* and *IL-6* genotype can either directly affect CK synthesis, or influence the magnitude of the reactive inflammatory process. The IL-6 response to exercise depends on the type of polymorphism *(-174 G/C)* within the *IL-6* promoter (*G*-allele or *C*-allele). In healthy subjects, the *IL-6 G-174C* polymorphism has been shown to influence IL-6 transcription and circulating IL-6 levels (Schotte et al., 2001). A functional *G/C* polymorphism at position -174 of the *IL-6* promotor in the 5' flanking region was associated with a significantly higher expression of the luciferase reporter vector assay (*-174 G construct*), compared to the *-174C* construct. In addition, the *G* allele was associated with a higher level of plasma IL-6 in healthy adults (Fishman et al., 1998). The indirect increase of IL-6 activity associated with the *GG* genotype reduces inflammation by stimulating the production of anti-inflammatory cytokines including IL-1ra, IL-10, cortisol and soluble TNF-α receptors (Petersen et al., 2005), and by inhibiting the production of pro-inflammatory cytokines like TNF-α and IL-1 (Aderka et al., 1989; Di Poi and Ferraccioli, 1999; Gabay et al., 1997; Xing et al., 1998). Suppression of pro-inflammatory cytokines by anti-inflammatory cytokines decreases circulating CK concentrations (Hirose et al., 2004). Increased production of IL-6 by skeletal muscle following acute exercise (Petersen et al., 2005) can also improve glucose utilization and sustain energy demand of the muscle by enhancing the activity of adenosine monophosphate-activated protein kinase (Carey et al., 2006). This effect may be related to IL-6 inhibition of the exercise-induced TNF-α response, since TNF- α has been linked to insulin resistance and inhibition of glucose uptake by the skeletal muscle (Penkowa et al., 2003). Thus it is possible that the protective effects of the IL-6 -*174 G* genotype against exercise-induced skeletal muscle damage are due mainly to increased glucose uptake by the skeletal muscle, restricted

inflammation by stimulation of anti-inflammatory cytokines production, and inhibition of inflammatory cytokines production all of which are known to play an integral role in post-exercise muscle damage repair.

TNF-α is a pro-inflammatory cytokine, which is considered to promote inflammatory responses associated with muscle damage (Ostrowski et al., 2001; Pedersen et al., 2001). It has been found that the rare *TNF-α-308A* allele is a stronger inducer of gene expression than *TNF-α-308G* allele (Wilson et al., 1995). Figure 5 illustrates the hypothetical role of TNF-α and Il-6 in muscle damage and repair.

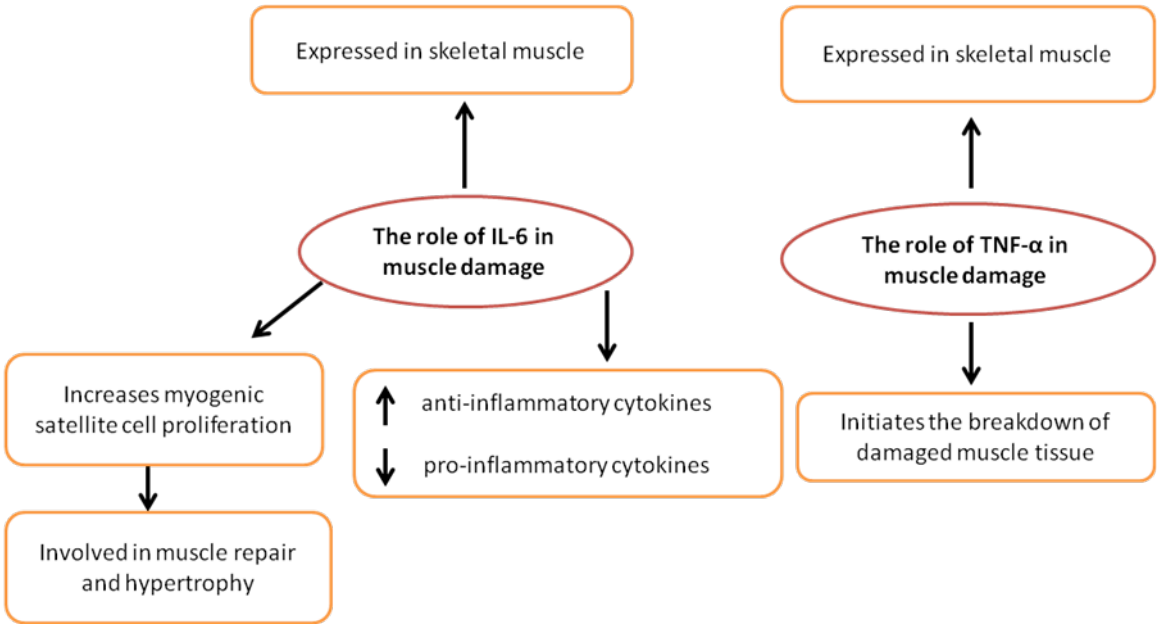

Figure 5. Possible influence of TNF-α and Il-6 in exercise-induced muscle damage.

In summary, severe exertional muscle damage may occur in susceptible individuals following strenuous exercise such as military basic training, intense weight lifting, and marathon running. Identification of individual predisposing factors for the development of severe exercise-induced muscle damage may prevent the development of potentially life-threatening renal complications. The

wide variability of the phenotypic expression and presentation of exertional muscle damage is not solely explained by age, race, body composition, and the individual's fitness level, with accumulated literature evidence suggesting that genetic factors may also influence its development.

Experimental work

Paper I

ACE ID genotype affects blood creatine kinase response to eccentric exercise.

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ACE ID genotype affects blood creatine kinase response to eccentric exercise

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Yamin C, Amir O, Sagiv M, Attias E, Meckel Y, Eynon N, Sagiv M, Amir RE. *ACE ID* genotype affects blood creatine kinase response to eccentric exercise. *J Appl Physiol* 103: 2057–2061, 2007. First published September 20, 2007; doi:10.1152/japplphysiol.00867.2007.—Unaccustomed exercise may cause muscle breakdown with marked increase in serum creatine kinase (CK) activity. The skeletal muscle renin-angiotensin system (RAS) plays an important role in exercise metabolism and tissue injury. A functional insertion (*I*)/deletion (*D*) polymorphism in the angiotensin I-converting enzyme (*ACE*) gene (rs4646994) has been associated with ACE activity. We hypothesized that *ACE ID* genotype may contribute to the wide variability in individuals' CK response to a given exercise. Young individuals performed maximal eccentric contractions of the elbow flexor muscles. Pre- and postexercise CK activity was determined. *ACE* genotype was significantly associated with postexercise CK increase and peak CK activity. Individuals harboring one or more of the *I* allele had a greater increase and higher peak CK values than individuals with the *DD* genotype. This response was dose-dependent (mean ± SE U/L: *II*, 8,882 ± 2,362; *ID*, 4,454 ± 1,105; *DD*, 2,937 ± 753, ANOVA, $P = 0.02$; $P = 0.009$ for linear trend). Multivariate stepwise regression analysis, which included age, sex, body mass index, and genotype subtypes, revealed that *ACE* genotype was the most powerful independent determinant of peak CK activity (adjusted odds ratio 1.3, 95% confidence interval 1.03–1.64, $P = 0.02$). In conclusion, we indicate a positive association of the *ACE ID* genotype with CK response to strenuous exercise. We suggest that the *II* genotype imposes increased risk for developing muscle damage, whereas the *DD* genotype may have protective effects. These findings support the role of local RAS in the regulation of exertional muscle injury.

genetics; *ACE*; insertion/deletion; renin-angiotensin system; eccentric exercise

IT IS WELL ESTABLISHED THAT strenuous exercise may lead to skeletal muscle damage, a condition known as exertional rhabdomyolysis (29, 45). Clinical features include muscle cramps, pain, and a feeling of fatigue accompanied by elevation in levels of serum creatine kinase (CK) following strenuous exercise. In most cases, this muscle damage is repairable without any serious complications and results in muscle adaptation. However, in rare situations, muscle fiber breakdown and the subsequent leakage of muscle contents into blood circulation may lead to renal failure and become a clinically potentially life-threatening condition (22, 29, 51).

Eccentric (muscle lengthening) exercise-induced rhabdomyolysis has been well described (9, 14). However, there is wide variability in both the range of increase in plasma CK and the degree of muscle damage (25, 31) in response to a given exercise. There is growing evidence for a genetic contribution to the phenotypic responses of exertional muscle damage (8, 13).

The renin-angiotensin system (RAS) plays an important role in human body fluid homeostasis and left ventricular remodeling. The angiotensin-converting enzyme (ACE) is a key component in the RAS, generating the vasoconstrictor angiotensin (ANG) II and degrading vasodilator kinins (11). ACE is widely expressed in human tissues, including skeletal muscle, and may play a metabolic role during exercise (20). ANG II has known effects on metabolism (6) and is a recognized growth factor necessary for the hypertrophy of skeletal muscle in response to mechanical load (16). Most of its known physiological and pathophysiological activities are mediated through the ANG II type 1 receptor (AT1R), which is also the only ANG II receptor present in human skeletal muscle (20).

A functional polymorphism of the human *ACE* gene has been identified in which the presence (insertion: *I* allele), rather than the absence (deletion: *D* allele), of a 287-bp *Alu* repeat element in intron 16 (rs4646994) is associated with lower enzyme activity in both serum and tissue (12, 39), resulting in greater production of ANG II and aldosterone and a decreased half-life of bradykinin (4, 52).

ACE ID polymorphism has been extensively studied in relation to human physical performance. Several reports from European and US Caucasian populations suggested the association of the *I* and the *D* alleles with endurance (1, 15, 30–32, 42) and sprint performance (31, 32, 53), respectively. However, some studies in which elite athletes were drawn from diverse sporting disciplines, requiring mixed skills, have failed to demonstrate any association with the *ACE* genotype (37, 46). Of note, in a different ethnic population of Israeli Caucasians, the *ACE DD* genotype has been associated with elite endurance athletes (2).

Because there is considerable RAS activity in skeletal muscle that is involved in the regulation of muscle metabolism, vascular tone, and injury responses, we hypothesized that the *ACE ID* genotype may contribute to the development of exertional rhabdomyolysis and therefore may affect CK response to strenuous exercise. To test our hypotheses, in the present study, we used repetitive eccentric contractions as an exercise model for inducing exertional rhabdomyolysis in healthy young individuals. We investigated the association of *ACE ID* alleles with CK response to exercise.

MATERIALS AND METHODS

Subjects. Seventy healthy physical education students (42 men and 28 women; aged 22–32 yr) volunteered for the study. Participants

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were physically active, but none were well trained or competitive athletes. All were healthy nonsmokers and were not receiving any medical treatment. Exclusion criteria included an occupation that required heavy weight lifting, participating in a resistance training program in the previous 6 mo, baseline blood values outside of the normal range (men: 24–195 U/l; women: 24–170 U/l), known muscle disorders, and any existing myopathy. Participants were all Israeli Caucasians, with an equivalent ratio of Ashkenazi and Non-Ashkenazi descent.

The study was approved by the Institution Review Board (Helsinki Committee) of the "Hillel-Yafe" Medical Center, and all participants gave written, informed consent before inclusion in the study and the start of any study-related procedures.

Eccentric exercise protocol. Subjects performed one set of 50 maximal eccentric contractions of the elbow flexor muscles of their nondominant arm using the BIODEX dynamometer (BIODEX System 3). Subjects were seated with the arm supported and were stabilized at the waist and the chest. Starting with the elbow flexed to 50° and ending at an angle of 170°, each subject performed 50 maximal eccentric movements of the elbow flexors at 120°/s (each contraction lasted 3 s). During each movement, subjects were verbally encouraged to produce a maximal effort to resist the ability of the dynamometer to extend the elbow. Subjects were given a 10-s rest between each contraction, during which time the dynamometer arm returned passively to the starting position.

Subjects were instructed to drink water before the exercise session and encouraged to maintain hydration and to monitor their urine color throughout the study. They were instructed to call the 24-h study phone to report whether their urine changed from clear or yellow to a brownish color (no subject experienced darkened urine). Subjects were followed-up and instructed not to participate in any strenuous physical activity until their CK values had returned to near normal.

CK analysis. Blood samples were drawn from an antecubital vein by venipuncture for genotypic analysis and to determine whole blood CK activity pre- and 3, 24, 48, 72, 96, 120, and 168 h postexercise. CK activity was determined by use of a commercially available Reflotron CK assay using the Reflotron system (18).

Genotyping for ACE ID polymorphism. Genomic DNA was extracted from peripheral blood leukocytes using a standard protocol (40). We used a PCR-based method for genotyping the *ACE ID* polymorphism, as previously described (26). Briefly, this method yields a PCR fragment of 319 bp and 597 bp in the presence of the *D* and the *I* alleles, respectively. PCR fragments were amplified from ≈20 ng of each DNA sample used as a template in 20-μl polymerase chain reactions (PCR) containing 0.2 U *Taq* polymerase, 1× reaction buffer, 0.2 mM concentration of each deoxynucleotide triphosphate, and 10 pmol of each of the following primers: GCCCTGCAAGGTGTCTGCAGCATGT (*ACE* sense) and GGATGGCTCTCCCCGCCTTGTCTC (*ACE* antisense). The initial denaturation at 95°C for 5 min was followed by 35 cycles of 94°C for 30 s, 58°C annealing for 30 s, and 65°C elongation for 45 s. To avoid misclassification of *ID* genotypes into *DD* genotypes, a second PCR was performed using *I*-specific primers: TGGGACCACAGCGCCCGCCACTAC (*I*-specific sense) and TCGCCAGCCCTCCCATGCCCATAA (*I*-specific antisense). This PCR yields a 335-bp fragment only in the presence of the *I* allele and no product in sample homozygotes for the *D* allele. Genotyping was performed by experienced staff. PCR scores by two independent investigators who were blind to subject data correlated well ($r^2 = 0.991$).

Data analysis. The SPSS statistical package version 13.0 was used for statistical evaluation (SPSS, Chicago, IL). Pre- and postexercise CK values were analyzed for their association with *ACE* genotypes. A χ^2 test was used to confirm that observed genotype frequencies were in Hardy-Weinberg equilibrium. Normality of each quantitative variable was tested using the Shipiro-Wilk normality test. Genotype subtype comparisons were made by ANOVA and the Kruskal-Wallis test (asymmetrical data distribution). Continuous variables were compared according to genotype group by linear ANOVA. Genotype distribution across levels of CK response was compared by χ^2 for linear trend. Physiological parameters and genotype data were used in multivariate analysis by the use of forward stepwise regression to determine the model that best predicted CK response to eccentric exercise and to evaluate whether the number of *ACE* alleles carried by each subject had statistical influence on laboratory parameters. To account for any possible effects of age, sex, or body mass index (BMI), analyses were covaried for these parameters. Asymmetrically distributed variables were log transformed before regression analysis. Continuous data are presented as means ± SD or as means ± SE. Square multiple correlation coefficients (R^2) were calculated. Two-tailed values of $P < 0.05$ were considered statistically significant.

RESULTS

Subjects were 25 ± 3 yr in age, 171 ± 8 cm in height, and 67 ± 10 kg in mass. The data on allele and genotype frequencies in the study population are shown in Table 1. There was no deviation from the Hardy-Weinberg equilibrium (allele frequency *ACE I/D* = 0.37/0.63, expected genotype frequencies % *II/ID/DD* = 14%/47%/39%; $\chi^2 = 0.03$; $P = 0.98$). Ashkenazi and Non-Ashkenazi descendants did not differ by *ACE* genotype. There was no relationship of age or body mass to *ACE ID* genotypes (Table 1). We, like others (8, 17), analyzed both sexes. Of the study population, 42 (60%) were men and 28 (40%) were women, and there was no difference in sex distribution among any of the genotype subtypes (Table 1). CK activity in response to the eccentric exercise regimen for the study population is shown in Fig. 1. Briefly, the baseline CK values were 157 ± 11 U/l (mean ± SE), and the average peak CK activity, which was noted 96 h postexercise (in all sub-

Table 1. *ACE ID allele and genotype frequencies in the study group with subjects' physical characteristics*

	ACE II	*ACE ID*	*ACE DD*	Allele *I*	Allele *D*	*P* Value
All, $n = 70$	10 (0.14)	32 (0.46)	28 (0.40)	52 (0.37)	88 (0.63)	
Men, $n = 42$	5 (0.12)*	21 (0.50)*	16 (0.38)*	31 (0.37)†	53 (0.63)†	
Women, $n = 28$	5 (0.18)*	11 (0.39)*	12 (0.43)*	21 (0.37)†	35 (0.63)†	
Age, yr	24±3	25±3	25±3			0.18
Height, cm	171±6	171±9	171±7			0.91
Weight, kg	67±9	67±10	67±10			0.98
LBM, kg	55±11	55±11	54±11			0.93
BMI, kg/m^2	22.6±1.6	22.7±1.6	22.8±9			0.96

Values are absolute and relative (in parentheses) frequencies, and means ± SD for continuous variables. ACE, angiotensin-converting enzyme; *I*, insertion allele; *D*, deletion allele; *DD*, homozygotes for the deletion allele; *II*, homozygotes for the insertion allele; *ID*, heterozygotes. *$\chi^2 = 0.93$; degrees of freedom = 2; $P = 0.62$ for genotype frequencies in men vs. women. †$\chi^2 = 0.01$; degrees of freedom = 1; $P = 0.91$ for allele frequencies in men vs. women.

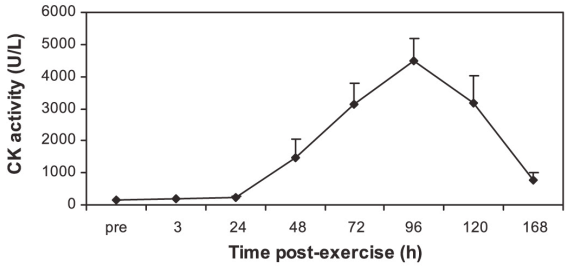

Fig. 1. Creatine kinase (CK) activity in response to the eccentric exercise regimen for the study population. Subjects' average serum CK activity at baseline and at different points in time postexercise.

jects), was 4,480 ± 705 U/l (mean ± SE). Sex had no influence on CK response.

To determine genotype-phenotype correlations, we compared subjects' phenotypes among *ACE* genotypes. There was no difference in baseline CK activity in relation to genotype subtypes (Table 2). However, the response to eccentric exercise was genotype dependent. The *ACE I* allele was significantly associated with peak CK activity as well as with the increase in CK activity (delta CK activity = peak CK activity-baseline CK activity). Individuals harboring one insertion allele (*ID*) had higher peak values of CK activity and a higher increase in CK activity than individuals having only the deletion allele (*DD*), and those individuals who had two alleles with the insertion (*II*) manifested the highest values of peak CK activity and the highest increase in CK activity (Table 2; $P = 0.02$ for ANOVA, $P = 0.009$ for linear trend). In a stepwise multivariate linear regression model that included age, sex, BMI, and genotype subtypes, *ACE ID* polymorphism was the most powerful independent determinant of peak CK activity (adjusted odds ratio = 1.3; 95% confidence interval = 1.03–1.64; $P = 0.02$).

DISCUSSION

We tested whether *ACE ID* genotype was associated with skeletal muscle response to strenuous eccentric exercise in healthy young men and women. The major finding of the present study was that individuals with one or more *I* allele of the *ACE* gene (*ACE II/ID*) had a greater increase in CK activity and higher peak CK levels compared with individuals homozygous for the *ACE D* allele. This response was dose-dependent, since peak CK values were highest in *II*, intermediate in *ID*, and lowest in *DD* genotypes. We suggest that the *ACE* genotype may influence CK response to eccentric contractions with the *ACE II* genotype having increased risk for developing exertional rhabdomyolysis. Conversely, it seems that the *ACE DD* genotype may favorably confer protective effects against exercise-induced muscle injury.

Several potential mechanisms may explain how the *ACE* genotype influences individuals' CK response to strenuous exercise. The increased ACE activity associated with the *DD* genotype may lead to enhanced production of ANG II, which is the predominant biological product of RAS, mediating many of the local effects of ACE on skeletal muscle. ANG II is a necessary factor in mediating vascular smooth muscle growth and capillary density in skeletal muscle (20). ANG II has a direct hypertrophic effect on skeletal muscle, and AT1R-mediated ANG II is crucial for optimal overload-induced skeletal muscle hypertrophy (16). Moreover, ANG II has been shown to regulate oxygen consumption and affect muscle energy expenditure (7), and higher maximal oxygen consumption levels have been associated with the *ACE D* allele (36, 54), indicating an improved oxidative capacity. Although most previous studies associated the *ACE I* allele (lower ACE activity, high kinin ligand generation, and increased half-life of bradykinin) with increased skeletal muscle metabolic efficiency and perhaps improved contractile function (5, 18, 43, 44), local RAS activity in skeletal muscle is much more complicated. ACE is not only involved in ANG II production and bradykinin degradation, but it also regulates the levels of ANG (1–7) peptide, which is known to cause vasodilating effects (20). Thus it is possible that the protective effects of the *ACE D* allele against exercise-induced skeletal muscle damage are mainly mediated through the fine tuning of regulating the levels of ANG II and ANG (1–7).

Another explanation for our findings may rely on the mechanisms underlying the process of muscle damage. In exertional rhabdomyolysis, the initial inciting event (whether mechanical stretch or excitation-contraction uncoupling) is accompanied by the uncontrolled movement of Ca^{2+} into the sarcoplasm, triggering the next stage in the damage process (34, 50). Bradykinin receptor B_2 activation can lead to transient rises in inositol 1,4,5-trisphosphate (35), which is involved in excitation-contraction coupling via increases in cytoplasmic Ca^{2+} (27). Bradykinin stimulates glucose uptake in the presence of insulin, a process related to alteration in intracellular Ca^{2+} concentration (24). Moreover, data suggest that this process is enhanced by the inhibition of ACE (23). Interestingly, there is evidence that ANG II can affect both sympathetic and neuromuscular transmission (20). Thus it is conceivable that *ACE* genotype affects CK response via involvement in the regulation of the excitation coupling process.

Heled et al. (17) have recently published a similar study in which no association of *ACE* genotype with exertional rhabdomyolysis was found. The disagreement between these results and our findings is not uncommon when using population-

Table 2. *Subjects' phenotypes in relation to ACE genotype*

	ACE II ($n = 10$)	*ACE ID* ($n = 32$)	*ACE ID + II* ($n = 42$)	*ACE DD* ($n = 28$)	ANOVA *P* Value
Baseline CK activity, U/l	147±27	158±15	155±13	161±19	0.92/0.82* 0.71†
Peak CK activity, U/l	8,882±2,361	4,454±1,105	5,508±1,040	2,937±753	0.02/0.07* 0.009†
Delta CK activity, U/l	8,735±2,352	4,296±1,108	5,353±1,041	2,778±757	0.02/0.07* 0.009†

Values are reported as means ± SE. **P* value for CK activity in *ACE II + ID* vs. *ACE DD*. †*P* value for linear trend.

association studies and may be attributable to different experimental designs and study cohorts. Data suggest that the effect of *ACE* genotype on physical performance may depend on the type of exercise (28). Moreover, it is well known that the frequencies of the *ACE ID* alleles vary considerably among different control populations (3), and the influence of varying genetic background may obscure a true association. To support our findings, we have recently demonstrated in Israeli elite athletes an association of the *ACE D* allele and *DD* genotype with endurance performance (2). Of note, our individuals were physically active, but none were well trained or competitive athletes. More importantly, they were not engaged in any resistance training programs and were not highly active. Thus it seems that in the Heled et al. study (17) participants were very active and their fitness level was higher than ours. This is also supported by the relatively lower average increase in CK level of their high responders group compared with our average delta CK levels (1,048 vs. 4,480 U/l). Given that fitness level influences the degree of exercise-induced muscle damage (10) and since the effect of ACE polymorphism depends on individuals' fitness levels as well (28), the association between ACE genotype and CK response to exercise may become prominent only in sedentary individuals performing a highly intense effort, as in our study.

Finally, it is still possible that *ACE ID* polymorphism is one of many genetic variants contributing to the observed variance in muscle CK response or that it is in strong allelic association with functional variants in adjacent genes and that these are responsible for the observed associations with *ACE* genotype (36). Likewise, it has been proposed that the higher ACE activity associated with the *ACE DD* genotype (12, 39) is perhaps caused by an *Alu*-associated transcription silencer (47, 48) or by an identified variant of the of the *ACE* gene that is in linkage disequilibrium with the *Alu* insertion/deletion polymorphism (21, 38, 49). However, the medical literature is still debating whether this candidate polymorphism is located in the promoter region (38, 49) or in downstream sequences of the *ACE* gene (21). The inconsistency regarding *ACE* haplotypes represents some of the difficulties in studying different populations, and the sequencing of coding and noncoding regions of the *ACE* gene in other populations with different evolutionary histories may reveal alternative polymorphisms. Rieder et al. (38) found some differences in sequence variation between African-Americans and European-Americans. In his analysis, a major genetic subdivision in the deletion haplotypes was evident only in European-Americans, further supporting the notion that some haplotypes that are present in one population may not apply to other populations. Clearly, more work on large sample sizes is needed to confirm our observations and to better clarify the pathways through which *ACE ID* genotype associates with exertional muscle damage.

In conclusion, our data suggest a positive association between the *ACE* genotype and CK response to repetitive eccentric contractions and further support the role of local RAS in the regulation of exertional muscle injury.

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Paper II

IL6 (-174) and TNF-α (-308) promoter polymorphisms are associated with systemic creatine kinase response to eccentric exercise.

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ORIGINAL ARTICLE

IL6 (-174) and *TNFA* (-308) promoter polymorphisms are associated with systemic creatine kinase response to eccentric exercise

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Abstract Exertional rhabdomyolysis is a complex and poorly understood entity. The inflammatory system has an important role in muscle injury and repair. Serum creatine kinase (CK) is often used as systemic biomarker representing muscle damage. Considerable variation exists in CK response between different subjects. Genetic elements may act as predisposition factors for exertional srhabdomyolysis. Based on their biological activity, we hypothesized that in healthy subjects *IL6* G-174C and *TNFA* G-308A promoter polymorphisms would be associated with CK response to exercise. We determined serum CK activity pre- and post-maximal eccentric contractions of the elbow flexor muscles. *IL6* G-174C and *TNFA* G-308A genotypes were analyzed for possible relationship with changes in serum CK activity. *IL6* G-174C genotype was associated with CK activity in a Dose-Dependent fashion. Subjects with one or more of the -174C allele had a greater increase and higher peak CK values than subjects homozygous for the G allele (mean ± SE U/L: GG, 2,604 ± 821; GC, 7,592 ± 1,111; CC, 8,403 ± 3,849, ANOVA $P = 0.0003$ for GG + GC genotypes versus CC genotype, $P = 0.0005$ for linear trend). *IL6*-174CC genotype was associated with a greater than threefold increased risk of massive CK response (adjusted odds ratio 3.29, 95% confidence interval 1.27–7.85, $P = 0.009$). A milder association ($P = 0.06$) was noted between *TNFA* G-308A genotype and CK activity. In conclusion, we found a strong association of the *IL6* G-174C genotype with systemic CK response to strenuous exercise. Data suggest that homozygosity for the *IL6*-174C allele is a clinically important risk factor for exercise-induced muscle injury, further supporting the central role of cytokines in the reactive inflammatory process of muscle damage and repair.

Keywords Genetics · Inflammation · Polymorphisms · Cytokines · Eccentric exercise

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Introduction

Unaccustomed exercise often causes damage in skeletal muscle that is marked by loss of muscular strength, reduced range of motion, muscle soreness and swelling, and elevated blood concentrations of muscle proteins [creatine kinase (CK), myoglobin] (Milne 1988; Sinert et al. 1994), a condition known as exertional rhabdomyolysis. There is an adaptation process to exercise-induced muscle injury such that less damage occurs when repeated bouts of exercise are performed by the same group of muscles within 6 months (McHugh 2003; Nosaka et al. 2001).

The immune system is involved in the degeneration and regeneration process of muscle after exercise-induced muscle damage (MacIntyre 2001; Tidball 2005). Within the injured muscle tissue there is leukocyte infiltration and local production of the pro-inflammatory cytokines interleukin (IL)-1β, tumor necrosis factor-α (TNF-α) and IL-6. IL-1β and TNF-α are crucial for initiating the breakdown and the subsequent removal of damaged muscle fragments

(Cannon and St Pierre 1998; Tidball 2005), while IL-6 is an important mediator of the release of other cytokines (TNF-α and IL-1β), which act subsequently to amplify the inflammatory reaction (Barton 1996; Hamada et al. 2005; Hirose et al. 2004). Systemic release of leukocytes and cytokines commonly accompanies the local inflammatory response. Growing evidence suggests that IL-6 exerts anti-inflammatory effects as well by inhibiting the production of the pro-inflammatory cytokine TNF-α, and stimulating the synthesis of other anti-inflammatory cytokines such as IL-1 receptor antagonist (IL-1RA), IL-10 and soluble TNF-α receptor 1 (TNF-αR1). Thus, during exercise IL-6 indirectly acts to restrict the inflammatory process.

Eccentric exercise (lengthening contractions) -induced muscle injury and the associated inflammatory reaction have been extensively studied (Bruunsgaard 1997; Hirose et al. 2004; Malm et al. 2000; Peake et al. 2005; Steinberg et al. 2007). Inflammatory responses to muscle damage are dependent on age, eccentric-exercise type, previous eccentric loading (repeated bout effect), anti-oxidant supplements, intracellular calcium homeostasis and anti-inflammatory drugs (reviewed in Peake et al. 2006). Systemic cytokine concentrations seem to be higher and return to baseline more rapidly following eccentric exercise that involves a large muscle mass as compared to local eccentric exercise using isolated muscle groups (Peake et al. 2006). Following repeated bouts of exercise there are no major differences in cytokine responses, while aging seems to be associated with impaired cytokine responses (Toft et al. 2002). The medical literature is still debating the correlation between contraction-induced muscle injury and the severity of the inflammatory response. Although the inflammatory reaction usually has a beneficial impact on tissues by removing cellular debris and favoring tissue regeneration, it must be emphasized that it may also have a deleterious effect, especially when it is exaggerated and/or out of control. Overall, it seems that inflammation is proportional to the extent of muscle damage and plays a major role in regulating muscle adaptability to exercise (Bruunsgaard 1997; Greiwe et al. 2001; Tidball 2005).

Current evidence supports the contribution of genetic elements to the process of exercise-induced muscle damage and repair (Clarkson et al. 2005; Dennis et al. 2004; Devaney et al. 2007). Moreover, inflammatory responses show high inter-individual variability and have been associated with cytokine gene variations (Dennis et al. 2004; Malm et al. 2000). The 233-amino acid TNF-α protein is coded by the 4-exon *TNFA* gene (1,585 bp) located at chromosome 6p21. A single nucleotide substitution of a guanine by an adenine at promote region -308 of *TNFA* (rs361525) has been related with increased TNF-α production and higher C-reactive protein levels (Bayley et al. 2004; Wilson et al. 1997). The *TNFA* G-308A polymorphism has been associated with cardiovascular phenotypes (Padovani et al. 2000; Sookoian et al. 2005), pulmonary sarcoidosis (Swider et al. 1999; Wilson et al. 1997), ischemic stroke in children with sickle cell anemia (Hoppe et al. 2007), malaria and mucocutaneous leishmaniasis (Cabrera et al. 1995; McGuire et al. 1994). The human IL-6 gene (*IL6*) is mapped to chromosome 7p21–24 with an upstream promoter containing 303 bp. A functional-174G/C polymorphism of *IL6* has been identified (rs13447445) (Fishman et al. 1998), in which the G rather than the C allele has been associated with an increased transcriptional response in vitro (Fishman et al. 1998; Terry et al. 2000) and in vivo (Bennermo 2004). *IL6* G-174C gene variation has been associated with the prevalence, incidence, and/or prognosis of a variety of disease states, such as Alzheimer's disease, atherosclerosis, cardiovascular disease, cancer, non-insulin-dependent diabetes mellitus (NIDDM), osteoporosis, sepsis, and systemic-onset juvenile chronic arthritis (Bennermo 2004).

The prominent role of the inflammatory response in exercise-induced muscle injury/repair and the contribution of gene variation to the inflammatory response in other systems led us to hypothesize that in healthy young individuals, the magnitude of CK response (which reflects the degree of the inflammatory reaction) to repetitive eccentric contractions would be associated with specific cytokine gene single nucleotide polymorphisms (SNPs). For the current study we selected the analysis of *IL6* G-174C and *TNFA* G-308A SNPs, because these are both variations of gene promoters, implying transcriptional regulation and suggesting possible association with chronic inflammation.

Methods

Subjects

We used blood samples from an earlier report from our laboratory (Yamin et al. 2007) that used a standard eccentric protocol to induce muscle injury. Briefly, the study population included 70 healthy physical education students (42 males and 28 females; aged 22–32), who were healthy and were not receiving any medical treatment, and physically active but were not well-trained or competitive athletes. Subjects' exclusion criteria included an occupation that required heavy weight lifting, participating in a resistance-training program in the previous 6 months, baseline blood values outside of the normal range (men: 24–195 U/L, women: 24–170 U/L), known muscle disorders and any existing myopathy. Participants were all Israeli Caucasians, with an equivalent ratio of Ashkenazi and non-Ashkenazi descent. The study was approved by the Institution Review Board (Helsinki Committee) of the "Hillel-Yafe" Medical Center, and all participants gave written informed consent

before inclusion in the study and the start of any study-related procedures.

Eccentric exercise test

Details of the eccentric exercise protocol are given in our previous work (Yamin et al. 2007). Briefly, in that study subjects performed one set of 50 maximal eccentric movements of the elbow flexors at 120 deg/s (each contraction lasted 3 s with a 10-s rest between each contraction) of their nondominant arm, using the BIODEX dynamometer (BIODEX System 3). During each movement, subjects were verbally encouraged to produce a maximal effort to resist the ability of the dynamometer to extend the elbow. Subjects were instructed to drink water before the exercise session, and encouraged to maintain hydration and to monitor their urine color throughout the study. They were instructed to call the 24-h study phone to report whether their urine color changed from clear or yellow to a brownish color (no subject experienced darkened urine). Subjects were followed-up and instructed not to participate in any strenuous physical activity until their CK values had returned to near normal.

CK analysis

Blood samples were drawn from an antecubital vein by venipuncture for genotypic analysis and to determine whole blood CK activity pre- and 3, 24, 48, 72, 96, 120 and 168 h post-exercise. CK activity was determined by use of a commercially available Reflotron® CK assay using the Reflotron® system (Hørder et al. 1999).

Genetic analysis

Genomic DNA was extracted from peripheral blood leukocytes using a standard protocol (Sambrook et al. 1989). Subjects were genotyped for *TNFA* G-308A (rs361525) and *IL6* G-174C (rs13447445) promoter polymorphisms, using the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) approaches, as previously described (Wilson et al. 1992; Zheng et al. 2000). PCR fragments were amplified from ≈20 ng of each DNA sample used as a template in 20 µl polymerase chain reactions (PCR) containing 0.2 U Taq polymerase, 1× concentration of the supplied buffer, 0.2mmol/L concentration of each deoxynucleotide triphosphate, and 10 pmol of each of the following primers: 5′-AGGCAATAGGTTTTGAGGGCCAT-3′ (*TNFA*-sense) and 5′-TCCTCCCTGCTCCGATTCCG-3′ (*TNFA*-antisense) (Wilson et al. 1992), and 5′-TTGTCAAGACATGCCAAAGTG-3′ (*IL6*-sense) and 5′-TCAGACATCTCCAGTCCTATA-3′ (*IL6*-antisense) (Zheng et al. 2000). The initial denaturation at 95°C for 5 min was followed by 35 cycles of 94°C for 30 s, 60–61°C annealing for 30 s, and 65°C elongation for 45 s. *TNFA* PCR fragments (107-bp length) were digested with 5 U of restriction endonuclease *Nco* I in the supplied buffer for 2 h at 37°C. In the presence of the -308G allele, the 107-bp PCR product was cut into two fragments of 87 and 20 bp in length. A 300-bp fragment containing the *IL-6*-174 site was digested with the *Nla* III restriction (5 U) enzyme, yielding three fragments when the C allele was present (165 and 135 bp) and two fragments when allele G was present (54 and 246 bp). Experienced staff performed Genotyping. PCR scores by two independent investigators who were blind to subject data, correlated well ($R^2 = 0.991$).

Statistical analysis

The SPSS statistical package version 13.0 was used for statistical evaluation (SPSS Inc, Chicago, IL, USA). Pre- and post-exercise CK values were analyzed for their association with *TNFA* and *IL6* genotypes. A χ^2 test was used to confirm that observed genotype frequencies were in Hardy–Weinberg equilibrium. Normality of each quantitative variable was tested using the Shipiro–Wilk normality test. Genotype subtype comparisons were made by ANOVA and the Kruskal–Wallis test (asymmetrical data distribution). Continuous variables were compared according to genotype group by linear analysis of variance (ANOVA). Genotype distribution across levels of CK response was compared by χ^2 for linear trend. Physiological parameters and genotype data were used in multivariate analysis by the use of forward stepwise regression, in order to determine the model which best predicted CK response to eccentric exercise and to evaluate whether the number of *TNFA* and *IL6* alleles carried by each subject had statistical influence on laboratory parameters. To account for any possible effects of age, sex, or BMI, analyses were covaried for these parameters. Asymmetrically distributed variables were log transformed before regression analysis. Continuous data are presented as mean ± SD or as mean ± SEM square multiple correlation coefficients (R^2) were calculated. Two-tailed values of $P < 0.05$ were considered statistically significant.

Results

Individuals were 25 ± 3 years in age, 171 ± 8 cm in height, and 67 ± 10 kg in mass. Their physical characteristics, according to *TNFA* or *IL6* genotypes are summarized in Table 1. Age, height and body mass were not related to *TNFA*, or *IL6* genotype subtypes. Both studied gene variations had no influence on individuals' training status as measured by maximal VO_2 (VO_2max) and maximal voluntary isometric contraction (MVC). There were no differences

Table 1 Individuals' physical characteristics in relation to *TNFA* G-308A and *IL6* G-174C genotypes in study population ($n = 70$)

TNFA G-308A genotype	GG ($n = 58$)	GA ($n = 12$)	*P* value
Age (years)	25 ± 3	24 ± 2	0.33
Height (cm)	171 ± 7	169 ± 10	0.46
Weight (kg)	68 ± 10	65 ± 11	0.45
LBM (kg)	56 ± 10	52 ± 12	0.28
BMI (kg/m^2)	22.8 ± 1.8	22.4 ± 1.8	0.54
VO_2max [mL/(kg min)]	46.1 ± 7.7	42.3 ± 11.1	0.28
Maximal voluntary contraction (N m)	68.8 ± 23.3	60.6 ± 23.6	0.27
Baseline CK activity (U/L)	158 ± 12	158 ± 12	0.99

IL6 G-174C genotype	GG ($n = 44$)	GC ($n = 24$)	CC ($n = 2$)	*P* value
Age (years)	25 ± 3	25 ± 3	24 ± 1	0.87
Height (cm)	171 ± 7	172 ± 8	174 ± 14	0.79
Weight (kg)	67 ± 9	67 ± 7	72 ± 18	0.78
LBM (kg)	55 ± 10	55 ± 11	58 ± 15	0.89
BMI (kg/m^2)	22.8 ± 1.8	22.4 ± 1.9	23.6 ± 2.1	0.56
VO_2max [mL/(kg min)]	46.0 ± 8.5	44.9 ± 8.4	39.2 ± 4.5	0.51
Maximal voluntary contraction (N m)	65.5 ± 22.1	70.6 ± 26.3	67.8 ± 19.5	0.70
Baseline CK activity (U/L)	170 ± 14	138 ± 19	129 ± 65	0.35

Values are mean ± SD, or mean ± SE

in *TNFA*, or *IL6* genotypes between Ashkenazi and non-Ashkenazi descendants. The data on allele and genotype frequencies in the study population is shown in Table 2. Males [42 (60%)] and females [28 (40%)] did not differ by

Table 2 Genotype and allele frequencies of *TNFA* G-308A and *IL6* G-174C polymorphisms in study population ($n = 70$)

Genotype	*n* (%)			Allele frequencies
TNFA G-308A	GG	GA		f(G)/f(A)
All ($n = 70$)	58 (83)	12 (17)		0.91/0.09
Males ($n = 42$)	37 (88)[a]	5 (12)[a]		0.94/0.06[b]
Females ($n = 28$)	21 (75)[a]	7 (25)[a]		0.875/0.125[b]
IL6 G-174C	GG	GC	CC	f(G)/f(C)
All ($n = 70$)	44 (63)	24 (34)	2 (3)	0.80/0.20
Males ($n = 42$)	27 (64)[c]	14 (33)[c]	1 (3)[c]	0.81/0.19[d]
Females ($n = 28$)	17 (61)[c]	10 (36)[c]	1 (3)[c]	0.79/0.21[d]

Values are absolute and relative (in parentheses) frequencies

n no. of individuals; *TNFA* tumor necrosis factor-alpha gene; *GG* homozygous for the -308G allele; *GA* heterozygous; *IL6* interleukin-6 gene; *GG* homozygous for the -174G allele; *CC* homozygous for the -174C allele; *GC* heterozygous

[a] $X^2 = 1.21$, $df = 1$, $P = 0.27$ for *TNFA* G-308A genotype frequencies in males versus females

[b] $X^2 = 1.09$, $df = 1$, $P = 0.29$ for *TNFA* G-308A allele frequencies in males versus females

[c] $X^2 = 0.14$, $df = 2$, $P = 0.93$ for *IL6* G-174C genotype frequencies in males versus females

[d] $X^2 = 0.01$, $df = 1$, $P = 0.89$ for *IL6* G-174C allele frequencies in males versus females

TNFA, or *IL6* genotypes. All tested polymorphisms were in Hardy–Weinberg equilibrium (allele frequency *TNFA* -308 G/A = 0.91/0.09, expected genotype frequencies (%) GG/GA/AA = 83/16/1, $\chi^2 = 1.09$, $P = 0.57$; Allele frequency *IL6*-174 G/C = 0.8/0.2, expected genotype frequencies (%) GG/GC/CC = 64/32/4, $\chi^2 = 0.29$, $P = 0.86$).

The CK activity of the total study population in response to the eccentric exercise has been previously described (Yamin et al. 2007). Briefly, the average baseline and peak CK values were 157 ± 11 U/L (mean ± SE) and 4,480 ± 705 U/L (mean ± SE), respectively. Peak CK activity was noted in all subjects 96 h post-exercise. Gender had no influence on CK response. To determine genotype–phenotype correlations, we compared subjects' phenotypes among *TNFA* and *IL6* genotypes. As shown in Table 1, baseline CK values were not dependent on either *TNFA*, or *IL6* genotypes. However, the response to the eccentric exercise regimen as measured by peak CK activity and by the change in CK activity from baseline levels (delta CK activity = peak CK activity-baseline CK activity) was associated with both *TNFA* G-308A and *IL6* G-174C polymorphisms. The rare -174C allele of the *IL6* gene was associated with higher peak CK activity and with increased CK response (delta CK) as opposed to the -174G allele (Fig. 1a, $P = 0.001$ for ANOVA). This response was Dose-Dependent: individuals with two copies of the rare allele had higher post-exercise CK levels compared to heterozygous individuals, while individuals homozygous for the common allele had the lowest peak CK values and the

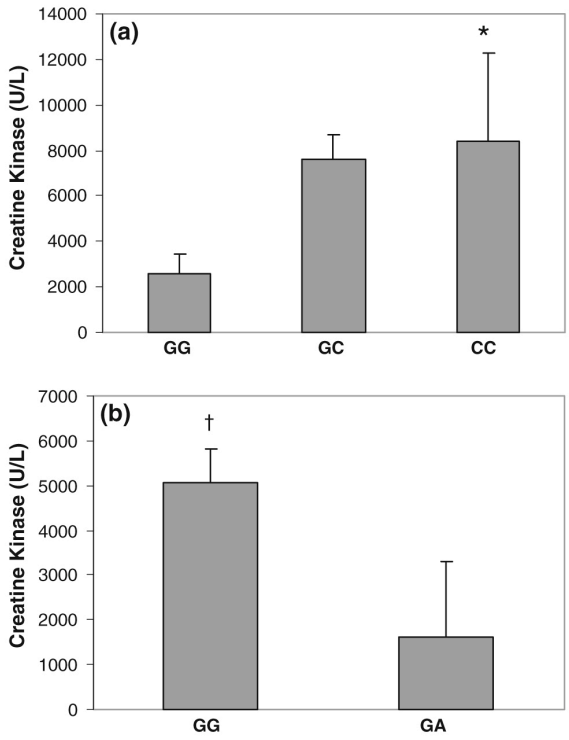

Fig. 1 Peak CK activity following eccentric exercise by *TNFA* G-308A (**a**) and *IL6* G-174C (**b**) genotypes (*TNFA* G-308A: GG = homozygous for the -308G allele, GA = heterozygous; *IL6* G-174C: GG = homozygous for the -174G allele, CC = homozygous for the -174C allele, GC = heterozygous). *ANOVA for GG/GC/CC $P = 0.001$; ANOVA for GC + CC versus GG $P = 0.0003$; $P = 0.0005$ for linear trend; †ANOVA for GG versus GA $P = 0.06$. Values are mean ± SE

lowest CK response (Fig. 1a, ANOVA $P = 0.0003$ for GG + GC genotypes versus CC genotype, $P = 0.0005$ for linear trend). As opposed to the -174C allele of the *IL6* gene, a milder association ($P = 0.06$) was noted between the rare *TNFA*-308A allele and CK activity. None of our individuals was homozygous for the -308A allele, but harboring one of its copies was enough to result in lower peak CK values and lower CK response to eccentric exercise (Fig. 1b). In a stepwise multivariate linear regression model, which included age, sex, BMI, and all genotype subtypes, peak CK activity was most associated with the *IL6*-174C homozygous genotype (adjusted odds ratio 3.29, 95% confidence interval 1.27–7.85, $P = 0.009$).

Discussion

Unaccustomed eccentric exercise often results in muscle damage and repair. This study was encouraged by the relevant role of cytokines in inflammatory response to exercise. We specifically selected *IL6* G-174C and *TNFA* G-308A SNPs based on their associated biological activity and because they are known to influence the magnitude of inflammation under other physiological conditions. We hypothesized that blood indicators for exercise-induced muscle damage might be associated with *TNFA* and *IL6* genotypes. Data from the present study showed that both *IL6*-174C and *TNFA*-308G alleles were associated with remarkable increase in CK activity in response to an acute bout of eccentric exercise. Importantly, a linear trend was noted with the effect of *IL6* genotype on CK response, as individuals homozygous for the -174C allele experienced the highest CK values, heterozygous individuals displayed intermediate levels and individuals homozygous for the -174G allele had the lowest CK activity. Given the functionality of these SNPs, we suggest that homozygosity for the *IL6*-174C allele is a clinically important risk factor for exercise-induced muscle injury.

Both *IL6* G-174C and *TNFA* G-308A SNPs studied here have been linked to increased susceptibility to several conditions characterized by inflammation (Bennermo 2004; Cabrera et al. 1995; Hoppe et al. 2007; McGuire et al. 1994; Padovani et al. 2000; Sookoian et al. 2005; Swider et al. 1999; Wilson et al. 1997). To our knowledge, this study is the first to report on *IL6* G-174C and *TNFA* G-308A genotypes in relation to eccentric exercise-induced muscle injury.

Based on the premise that mutations positioned in the promoter regions could affect the binding of transcription factors, resulting in altered mRNA expression or protein levels, both *IL6* G-174C and *TNFA* G-308A polymorphisms have received a particularly large amount of attention due to their putative effects on *IL-6* and *TNF-α* expression. The position of the G-174C SNP at the promoter region of the *IL6* gene is close enough to the binding site of the glucocorticoid receptor and as such it has the ability to repress transcriptional activation. Furthermore, the change from a G to a C at this position creates a potential binding site for the transcriptional factor NF-1, which has been demonstrated to be a repressor of gene expression (Rein et al. 1995). Indeed, previous studies (Fishman et al. 1998; Terry et al. 2000) associated the G allele with increased transcription when stimulated by endotoxin and IL-1β as well as with increased plasma IL-6 response to a standardized inflammatory stimulus (Bennermo 2004). We found that individuals homozygous for this allele had significantly lower peak CK values and a milder CK response to exercise, suggesting that increased IL-6 response associated with the G allele is beneficial when humans are challenged by extremely intensive eccentric exercise. Interestingly, while the G allele has been shown to be associated with improved survival in septic patients, it turned

out to be harmful in chronic disease states such as NIDDM and atherosclerosis, indicating that increased IL-6 response protects against acute inflammatory stress (acute infection, exercise-induced muscle damage). However, IL-6 response to chronic repeated stimuli might be detrimental.

In addition to the strong *IL6* G-174C SNP association, a milder association of the *TNFA* G-308A variant was also reported in this study. The rare *TNFA*-308A has been shown to be a stronger inducer of reporter gene expression than the common allele (Wilson et al. 1997). Although the increased CK response to eccentric exercise associated with *TNFA* G-308A genotype did not reach significance, the direction of this association was similar to the results obtained with *IL6* G-174C genotype; higher TNF-α expression as marked by the -308A allele was related to lower CK levels and to milder CK responses to the exercise challenge.

The mechanism by which *TNFA* and *IL6* may affect CK response to eccentric exercise in unknown. However, several possible mechanisms could explain our findings. *TNFA* and *IL6* genotype may contribute the results by either affecting directly CK synthesis, or by influencing the magnitude of the reactive inflammatory process. Alternatively, the studied polymorphisms may be indirectly associated with the CK response via their relationship with the training status of the individuals. This is most unlikely given that in the present study genotype subtypes were not related to measures of fitness level. Thus, although the medical literature suggests that fitness level may be a significant confounder of CK response to exercise, individuals' training status may be of importance only when comparing sedentary individuals to well trained athletes. Our participants were of a comparable fitness level; all were physically active but none were well trained, engaged in a regular training program, or competitive athletes. Of note and in support of our results, in a recent study by Heled et al. (2007), fitness level had no effect on CK response. The studied *TNFA* and *IL6* gene promoter polymorphisms imply the regulation of transcriptional activity of both genes, which in turn affect the expression of proteins, or the inflammatory response. A plausible mechanistic explanation of the results with respect to their relevance for physiology may be that *TNFA* and *IL6* gene aberrations interfere with the synthesis of CK prior to its release with the damage. This could be supported by a fast (3 h) increase in circulating CK in response to the eccentric exercise. However, our findings showing rather a slower (over days) increase in circulating CK, argue against this possibility and suggest a role for de novo CK synthesis via an eventual effect on the inflammatory response.

Our findings relating *IL6* G-174C genotype with CK response to eccentric exercise are consistent with the prominent role of IL-6 in mediating inflammatory responses to exercise. Anti-inflammatory effects of IL-6 in skeletal muscle include inhibition of TNF-α production, and the induction in synthesis of other anti-inflammatory cytokines (Pedersen et al. 2003). The relationship between cytokine responses and muscle damage has been a point of debate for some time (Bruunsgaard et al. 1997; Helge et al. 2003; Hirose et al. 2004; Malm et al. 2004; Peake et al. 2006; Toft et al. 2002). Moreover, it is yet unknown whether inflammation itself is a cause or consequence of muscle damage after eccentric exercise. Studies in animal models have shown that TNF-α suppresses protein synthesis and induces catabolism of skeletal muscle (Ling et al. 1997). Aging-associated reduced skeletal muscle function has been linked to increased IL-6 expression (Pedersen et al. 2003). Moreover, both IL-6 and IL-1RA increase in the circulation following strenuous exercise (Pedersen and Toft 2000), suggesting that the coordinated regulation of cytokines is important for muscle repair. Thus, the inflammatory response appears to be required to initiate recovery from exercise. However, this response may need to be limited over time for optimal muscle adaptation to occur and it is probably the careful balance between pro-inflammatory factors and their negative regulators, which allows proper regeneration of skeletal muscle following tissue injury. Data suggest that there is a high degree of variability in the adaptive inflammatory process between healthy individuals (Dennis et al. 2004; Malm et al. 2000), which may contribute to the observed normal differences in CK response to a given exercise. Our data indicate that these differences may be attributable to inter-individual genetic variations and suggest that muscle injury is at least partially genetically regulated. Dennis et al. (2004) reported on coordinate regulation of IL-1β, IL-6 and TNF-α mRNAs in response to resistance exercise. In his study, inflammatory marker responses were influenced by *IL1B* gene variations, further supporting our findings.

Several studies reported on CK response and the reactive inflammatory process associated with different types of eccentric exercise (Bruunsgaard 1997; Hirose et al. 2004; Malm et al. 2000; Peake et al. 2005; Peake et al. 2006; Steinberg et al. 2007). The accumulated data suggest that changes in inflammatory markers are dependent on the muscle groups used, or the amount of muscle mass recruited during the exercise (reviewed in Peake et al. 2006). In the present study, we preferred to choose an eccentric exercise protocol using elbow flexion rather than the knee extensors based on data suggesting that the magnitude of muscle damage is greater and the recovery of muscle function is slower following the use of a small muscle mass (arm elbow flexors) than a large muscle mass (lower limbs). This is most probably because this muscle group is exposed to eccentric muscle strain less often than leg muscles (Jamurtas et al. 2005).

A few limitations of our study should be noted. First, our study population was relatively small and homogenous in terms of age and ethnicity. We found the population frequency of the *IL6* G-174C to be 63, 24 and 3% for GG, GC and CC, respectively. Considerable interethnic variation in the frequencies of this polymorphism has been demonstrated (Fishman et al. 1998), with the -174C allele being rarer in Gujarati Indians and Afro-Caribbeans compared with UK Caucasians, which is consistent with our findings. The population frequency of the *TNFA* G-308A was 83 and 17% for GG and GA, respectively. This genotype distribution showed a similarity to the respective frequencies reported in dbSNP using European, African American, and Asian populations (CCR samples), or a Caucasian group. Despite being within the expected frequencies, the small number of homozygous *IL6*-174C individuals ($n = 2$) may limit the ability to assess the *IL6* G-174C genotype association with CK response. However, given that heterozygous *IL6* G-174C genotype displays a remarkable increased CK response compared to homozygous wild-type individuals (mean ± SE U/L: 7,592 ± 1,111 vs. 2,604 ± 821, respectively), we do believe that our results represent true associations. Second, although we hypothesized that the *TNFA* G-308A and *IL6* G-174C SNPs themselves possess bona fide effects on CK response, we cannot rule out the possibility that other markers in linkage disequilibrium (LD) with these SNPs are causative (Terry et al. 2000). However, our study was not designed primarily to study the effects of haplotypes and therefore cannot refute or confirm such effects. Further studies using haplotype analyses are needed to determine whether it is indeed the *TNFA* and *IL6* locus or other genes in LD that are responsible for these associations.

In summary, the results of the present study clearly indicate a positive association between *IL6* G-174C SNP and systemic CK response to strenuous exercise. Taking into account the central role of IL-6 in inflammatory process and muscle repair, the present finding might be of major relevance. Further mechanistic studies would augment our findings to determine whether the gene products regulated by *IL6* G-174C and *TNFA* G-308A promoter polymorphisms are involved in the pathways leading to exercise-induced muscle damage. Nevertheless, these polymorphisms may prove informative for the prediction of genetic risk for exertional rhabdomyolysis and thereby may contribute to primary prevention in specific populations.

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Paper III

CK-MM gene polymorphism does not influence the blood creatine kinase activity after exhaustive eccentric exercise.

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CK-MM Gene Polymorphism Does not Influence the Blood CK Activity Levels After Exhaustive Eccentric Exercise

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Key words
- Quartiles
- genotype
- inter-individual variability
- muscle damage

Abstract

Gene variants, such as creatine kinase (CK) polymorphisms, have been suggested to explain the inter-individual blood CK response to eccentric exercise. However, since this association is still doubtful, the purpose of this study was to analyse the relationship between the magnitudes of the CK response to exercise with the occurrence of muscle *CK-MM* NcoI polymorphism in young healthy subjects. Blood CK activity was assessed in 70 subjects immediately before and 3, 24, 48, 72, 96, 120, 168 h after strenuous eccentric exercise. Based on the amount of CK release by each subject, the sample was distributed in quartiles and the genotype and allele frequency distribution was compared among quartiles. Despite the inter-individual variability of CK response observed between subjects, there were no differences in genotype and allele frequencies among quartiles. The results allowed us to conclude that CK response after exhaustive eccentric exercise is not associated with *CK-MM* NcoI polymorphism.

Introduction

Exercise-induced muscle damage is a common human condition observed after the practice of unusual and/or exhaustive exercise, especially when a high percentage of eccentric contractions is required, such as in military training, weight lifting or marathon running [22]. Exercise-induced muscle damage is associated with the enhanced permeability of the muscle cell membranes promoting the increased blood levels of specific proteins usually localized within the muscle fibers [6,18]. Among these proteins, creatine kinase (CK) is one of the most used indicators of muscle damage [4,15] since it is found almost exclusively in the skeletal and cardiac muscles.

Markedly high blood levels of CK (300–10 174 U/L) are often seen following prolonged weight carriage activities (e.g. 2–20 h marching or running), and particularly following short exercises that involve intense resistive eccentric contractions (e.g. push-ups, squats, etc.) [4,8]. Nevertheless, there is a wide inter-individual variability in the magnitude and time course of plasma CK response related with exercise-induced muscle damage the subjects usually being classified as low, medium and high CK responders [5]. The reasons underlying this great inter-individual variability remain unclear, but there is growing evidence for a genetic contribution to the exertional muscle damage vulnerability [7,8]. In fact, it is known that several inherited genetic muscular disorders, such as McArdle's disease and Duchenne's muscular dystrophy, may predispose individuals to develop exercise-induced muscle damage [29]. Additionally, it has been suggested that healthy subjects who were exposed to similar resistive exercise load and environmental conditions presented different susceptibility to develop clinical symptoms and signs of muscle damage [29]. Moreover, it is also accepted that the wide variability in the CK response induced by exercise cannot just be predicted solely based on age [14,17], race [21,22], body composition [20] and the individual's level of physical activity or inactivity [25]. Therefore, other factors, particularly genetic factors, should also be considered as contributors for the development and severity of exercise-induced muscle damage [27,28].

Indeed, a close association between the inter-individual variability of blood CK response to exercise and a *CK* polymorphism was recently suggested affecting the CK-MM isoenzyme [11], the most prominent CK isoform found in skeletal muscle (98 %) [9]. In the study of Heled et al. [11] the authors concluded that participants with the

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CK-MM AA genotype had a six-fold higher risk of being high-responders as compared to GG and AG genotypes. However, some weaknesses related with the data analysis, the reduced aggressiveness of the performed exercise and the cutoff point used to define high CK responders might have compromised this conclusion. Indeed, it may be argued that in the work of Heled et al. [11] the magnitude of muscle damage induced by exercise was extremely reduced as expressed by the cutoff point used to define subjects as "high CK responders" (above the 90th percentile or presented CK activity higher than 230 U/L). This response, however, is extremely reduced when compared to that usually described in literature after eccentric exercise, where plasma CK levels can be ~10 fold higher reaching values above 10 000 U/L [28]. Additionally, as a result of the data analysis employed by Heled et al. [11] mixing homozygote (GG) and heterozygote (GA) subjects, it was not clear if there is or is not a gene dose effect on the reported CK response.

Therefore, since from our point of view the association between the *CK-MM* Nco I genotype (National Center for Biotechnology Information rs no. 1803285) and the CK response to eccentric exercise-induced muscle damage is still uncertain, the main purpose of this study was to utilize more demanding eccentric contractions to induce indubitable muscle damage in healthy young individuals, associating the magnitude of plasma CK response with the occurrence of *CK-MM* Nco I gene polymorphism. Based on the results of Heled et al. [11], even using a different approach, we hypothesized that the *CK-MM* NcoI polymorphism may contribute to explain the inter-individual variability of CK response following exercise.

Materials and Methods

Subjects

Seventy physically active students (42 males and 28 females; aged 25 ± 3 years; height 171 ± 8 cm; and weight 67 ± 10 kg) volunteered for the study. Participants were healthy, non-smokers, and not receiving any medical treatment nor involved in competitive sports. Moreover, subjects had not been committed with heavy weight lifting or resistance training programs in the previous 6 months, and presented baseline blood values of CK activity within the normal range (males: 24–195 U/L, females: 24–170 U/L). Participants were all Israeli Caucasians, with an equivalent ratio of Ashkenazi and Non-Ashkenazi descent. The study was approved by the Institution Review Board (Helsinki Committee) of the "Hillel-Yafe" Medical Center, and all participants gave written informed consent before inclusion in the study and the start of any study related procedures. The study has moreover been conducted in accordance with the ethical standards of the IJSM [10].

Exhaustive eccentric exercise protocol

Based on the maximal elbow flexors eccentric contraction assessed 2 months before the beginning of the experimental protocol, subjects performed one set of 50 maximal eccentric contractions of the elbow flexor muscles of their non-dominant arm at 120 °s^{-1} (each contraction lasted 3 s), starting with the elbow flexed at 50 ° and ending at an angle of 170 °, using the BIODEX dynamometer (BIODEX System 3). This protocol had been used in earlier studies [27,28] and was sufficient to produce reasonable muscle damage. Subjects were seated with the arm supported and were stabilized at the waist and the chest. During each movement, subjects were verbally encouraged to produce a maximal effort to resist the ability of the dynamometer to extend the elbow. Subjects were given a 10 s rest between each contraction, while the dynamometer arm returned passively to the starting position. Subjects were instructed to drink water before the exercise session, and were encouraged to maintain hydration and to monitor their urine colour throughout the study. They were also instructed to call the 24 h study phone to report whether their urine changed from clear or yellow to a brownish colour (no subject experienced darkened urine). Subjects were followed-up and instructed not to participate in any strenuous physical activity until their CK values had returned to near normal.

Analysis of CK activity and genotyping for CK-MM NcoI polymorphism

Blood samples were drawn from an antecubital vein by venipuncture before and 3, 24, 48, 72, 96, 120 and 168 h post-exercise to determine whole blood CK activity, which was determined with a commercially available Reflotron@CK assay using the Reflotron@system [12]. Blood samples obtained before exercise were also used for genotypic analysis. Genomic DNA was extracted from peripheral blood leukocytes using a standard protocol [19]. We used a PCR amplification that was conducted to amplify 1 170 base pair (bp) DNA fragment localized in the gene followed by restriction digestion using the NcoI restriction endonuclease to distinguish between the A and G alleles [16]. The allele without the NcoI restriction site was designated as the G allele (1 170 bp) whereas the allele with the polymorphic NcoI site was designated as the A allele (985 bp + 185 bp). This DNA fragment was amplified using the specific primer pairs: 5′-GTGCGGTGGACACAGCTGCCG-3′ (Forward) and 5′-CAGCTTGGTCAAAGACATTGAGG-3′ (Reverse). PCR fragments were amplified from 20 ng of each DNA sample used as a template in 20 µl polymerase chain reactions (PCR) containing 0.2U Taq polymerase, 1× reaction buffer, 0.2 mmol/L concentration of each deoxynucleotide triphosphate, and 10 pmol of each primer: The initial denaturation at 95 °C for 5 min was followed by 35 cycles of 94 °C for 30 s, 58 °C annealing for 30 s, and 65 °C elongation for 45 s.

Data analysis

The SPSS statistical package version 15.0 was used for statistical evaluation (SPSS Inc, Chicago, IL, USA). Data are presented as mean ± standard deviation (SD) or as otherwise stated. Normality of each quantitative variable was tested using the Shapiro-Wilk normality test. CK values were analysed across time of the experimental protocol, and CK peak was identified for each subject as the highest value attained after exercise. Individual "maximal CK response" (Δ_{CK}) was determined by the difference between CK peak and CK value recorded at baseline (before exercise). The Δ_{CK} of males and females was compared using the Man-Whitney U test (asymmetrical data distribution). Taking into account the huge interindividual variability of maximal CK response in both genders, samples were separated by gender and further divided into quartiles of Δ_{CK}. Subjects located in the 1st quartile were the lowest CK responders while those placed in the 4th quartile comprised the highest responders. For each quartile the allele frequencies were determined by gene counting. A χ^2 test was used to confirm that the observed genotype frequencies were in Hardy-Weinberg equilibrium, and to compare alleles and genotype frequencies between quartiles. The significance level was set at $p < 0.05$.

Results

The individual absolute CK values for each gender along the experimental protocol are depicted in **Fig. 1**, which shows a great interindividual variability of CK levels following eccentric exercise. For men and women the average values of CK at baseline were 119.9±39.7 U/L and 109.9±32.4 U/L, respectively, while the Δ_{CK} was 4,859.7±6,809.9 U/L and 3,515.5±4,163.5 U/L. No significant differences were found between genders for both baseline ($p=0.31$) and Δ_{CK} ($p=0.95$).

The quartile distribution of Δ_{CK} for males and females is presented in **Fig. 2**. Despite the different quartile cutpoints, a similar pattern of Δ_{CK} can be observed in both genders. Consequently, further analysis regarding the genotype and allele frequency distributions between Δ_{CK} quartiles were performed joining all subjects of each quartile from both genders.

The genotype and the allele frequency for all samples and for each gender are shown in **Table 1**. Males [42 (60%)] and females [28 (40%)] did not differ in *CK-MM* NcoI genotype and allele frequencies.

There was no deviation from the Hardy-Weinberg equilibrium (allele frequency *CK-MM* NcoI = 0.27 (A)/0.73 (G), expected genotype frequencies % GG/AG/AA = 7%/40%/53%, $X^2=0.25$, $p=0.61$). The genotype and the allele frequency for Δ_{CK} quartiles are shown in **Table 2**.

Not any significant differences between genotype/allele frequencies and the different prototypes of CK release were found after exercise defined by quartiles. In particular, there were no differences in genotype/allele frequencies between the lowest CK responders (subjects of 1st quartile) and the highest CK responders (4th quartile) ($X^2=1.91$, df = 2, $p=0.38$), or between the highest responders and the remaining sample ($X^2=2.79$, df = 2, $p=0.25$), or even between the lowest responders and the remaining sample ($X^2=4.05$, df = 2, $p=0.13$).

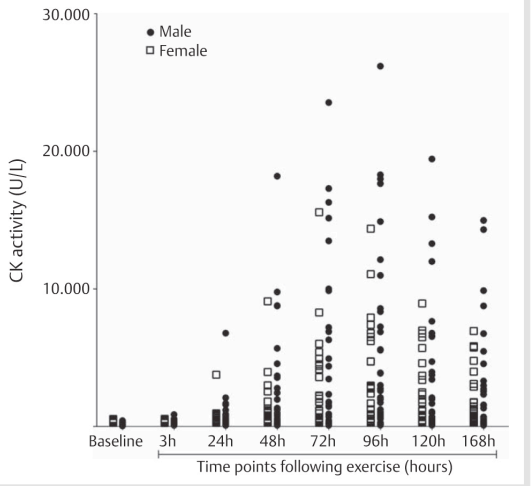

Fig. 1 Distribution in males and females of individual CK (creatine kinase) activity at baseline (before exercise) and along different time points (hours) following exercise.

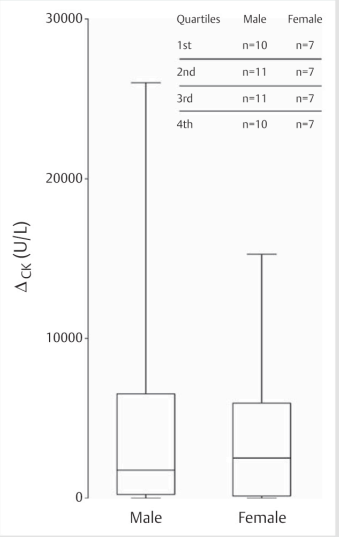

Fig. 2 Quartiles of maximal CK response (Δ_{CK}) to exhaustive eccentric exercise in males and females. The sample size within each quartile is also presented.

Discussion

The present study clearly shows a marked increase of CK activity in blood following demanding eccentric exercise, with peak values reaching as high as ≈26 000 U/L in males. Nevertheless, as expected, the blood CK activity showed a huge inter-individual variability, with several subjects presenting post-exercise CK levels identical to those registered at baseline. Despite the apparently lower peak CK values observed in females compared to males, the distribution pattern of blood CK activity was similar

Table 1 Genotype and allele frequencies of *CK-MM* NcoI polymorphisms in the studied population (n = 70). Values are absolute and relative (in parenthesis) frequencies.

		Genotype[a]			Allele frequencies[b]	
		AA	GA	GG	AlleleA	AlleleG
All	n = 70	5(7)	28(40)	37(53)	38(0.27)	102(0.73)
Male	n = 42	21(50)	18(43)	3(7)	60(0.71)	24(0.29)
Female	n = 28	16(57)	10(36)	2(7)	42(0.75)	14(0.25)

GG (rare genotype) homozygous for the *CK-MM* NcoI; AA homozygous and GA heterozygous for the *CK-MM* NcoI allele.

[a] – $X^2=0.376$, df = 2, $p=0.83$ for *CK-MM* NcoI genotype frequencies in males vs. females.

[b] – $X^2=0.07$, df = 1, $p=0.79$ for *CK-MM* NcoI allele frequencies in males vs. females

Table 2 Genotype and allele frequencies of *CK-MM* NcoI polymorphisms in each quartile of CK response. Values are absolute and relative (in parentheses) frequencies.

		Genotype[a]			Allele frequencies[b]	
Δ_{CK}	Sample	AA	GA	GG	AlleleA	AlleleG
1st quartile	n = 17	3(17.6)	7(41.2)	7(41.2)	13(0.38)	21(0.62)
2nd quartile	n = 18	0(0)	10(55.6)	8(44.4)	10(0.28)	26(0.72)
3rd quartile	n = 18	0(0)	7(38.9)	11(61.1)	7(0.19)	29(0.81)
4th quartile	n = 17	2(11.8)	4(23.5)	11(64.7)	8(0.24)	26(0.76)

GG (rare genotype) homozygous for the *CK-MM* NcoI; AA homozygous and GA heterozygous for the *CK-MM* NcoI allele.

[a] – $X^2=9.32$, df = 6, $p=0.15$ for *CK-MM* NcoI genotype frequencies between quartiles.

[b] – $X^2=3.42$, df = 3, $p=0.33$ for *CK-MM* NcoI allele frequencies between quartiles

in both genders along time post-exercise. Surprisingly, in contrast to that previously reported in the literature [11] our data does not support a role of *CK-MM* NcoI polymorphism to explain the CK variability between subjects.

Exercise-induced muscle damage is characterized by the breakdown of skeletal muscle cells accompanied by the leakage of muscular components into the circulation, which is explained by the occurrence of intrinsic and extrinsic degenerative processes to the fibers, triggered by mechanical and/or metabolic overload [2]. As a result, an elevated CK activity is normally observed in blood, which is frequently used to assess the severity of muscle damage [29]. In comparison with men, a lower CK activity level following strenuous exercise is widely assumed in women, which is traditionally explained by the role of estrogens in membranes stabilization and in the attenuation of inflammatory reaction [23]. Indeed, despite the absence of significant differences in Δ_{CK}, the scatter plot data depicted in **Fig. 1** shows a general trend of women to present lower peak CK values as compared to men. However, it is important to note that even presenting apparently lower CK values, women showed a pattern of CK responses along the time post exercise identical to male. These findings are in agreement with a previous study, which showed an identical time course post exercise in both genders, despite the lower CK levels observed in females [24]. Independent of the influence of the hormonal environment, the CK response showed a considerable interindividual variability in both genders in our study as already reported in the literature [8]. Since CK variability cannot be predicted just by age, race, body composition, and physical activity or inactivity of subjects [14,17,20–22,25], several authors have suggested a genetic influence to explain why subjects submitted to the same degree of effort may release different amounts of CK [27,28]. Several genes might be proposed as potential candidates to explain the above-referred variability, such as those encoding metabolic enzymes, structural proteins of the cytoskeleton or of the T–Tubules structure as well as those involved in the control of muscle proteolysis or inflammatory reaction.

One of those candidates was recently proposed by the study of Heled et al. [11] reporting that *CK-MM* NcoI polymorphism is related with individual differences in blood CK after moderate exercise. The authors found that subjects with the AA genotype had a six-fold higher risk to be high responders than those with the GG and AG genotype. Obviously in contrast with the results reported by Heled et al. [11], the present study showed no differences in *CK-MM* NcoI genotypes and alleles frequency between high vs. low CK releasers.

This kind of disagreement is not uncommon in population-association studies [27] and may be attributable to different experimental designs and/or genetic characteristics of the studied population. For instance, in the study by Heled et al. [11], subjects performed alternating submaximal concentric-eccentric contractions within a given time, while we had requested our subjects to perform 50 maximal eccentric contractions. As a result of higher overload imposed to skeletal muscle in the present study, the average increase in peak serum CK in high responders was almost 18 times higher in our study than that of study by Heled et al. [11] Therefore, based on the assumption that CK is a marker of muscle damage, it is reasonable to question the severity or even the occurrence of muscle damage in the former study. On the other hand, using a more demanding exercise, the present work raises little doubts about the real existence of muscle damage, making the interindividual variability more obvious and, consequently, allowing a clear distinction between low and high CK responders. Furthermore, another non-negligible methodological issue in the study of Heled et al. [11] was the procedure used to compare the CK response between groups of genotype (AA vs. AG+GG), which precluded the demonstration of the existence of an additive genetic effect of the *CK-MM* NcoI polymorphism on CK response, as presumably required in this type of report. In contrast, using a different methodological approach based on the level of CK response, we have analysed the genotype and allele frequency distribution among groups with a wide range of CK response. Assuming a polygenetic influence on exercise-induced muscle damage [7,8,27,28], the possibility cannot be excluded that other genes differently expressed in the populations of the two studies could have contributed to the divergence of our results and those reported by Heled et al. [11].

Nevertheless, apart from the unsettled issue regarding the association of *CK-MM* NcoI polymorphism with CK response, another conceptual problem is yet to be solved: is CK blood level a reliable marker of exercise-induced muscle damage? Do CK blood levels really parallel the degree of muscle damage? Although a favourable response to these questions has been provided by several authors [1,3], others have established that differences in CK release after exercise do not consistently reproduce differences in the degree of histological muscle damage [24]. Thus, while doubt still persists, one should not consider the use of CK activity alone as marker of muscle damage. Consequently, in order to ascertain the real influence of gene polymorphisms on exercise-induced muscle damage, future studies should consider the use of more rigorous methodologies to assess the true degree of muscle damage.

In summary, we did not find any association of *CK-MM* NcoI polymorphisms with the behaviour of CK response. However, since CK is not consensually accepted as a good and reliable marker of muscle damage, our results cannot safely exclude the hypothetical influence of *CK-MM* NcoI polymorphism on the degree of muscle damage induced by exercise.

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Overall Discussion

The major findings of our experimental work revealed an apparent influence of genetic factors on exercise-induced muscle damage.

In the first study, we observed a positive association of the *ACE ID* genotype with CK response to strenuous exercise. Accordingly, it has been suggested that the *II* genotype imposes increased risk for developing muscle damage, while the *DD* genotype may have protective effects.

Several potential mechanisms may explain how the *ACE* genotype influences individuals' CK response to strenuous exercise. The increased ACE activity associated with the *DD* genotype may lead to enhanced production of Ang II, which is the predominant biological product of RAS mediating many of the local effects of ACE on skeletal muscle. Ang II is a necessary factor in mediating vascular smooth muscle growth and capillary density in skeletal muscle (Jones et al. 2003). Ang II has a direct hypertrophic effect on skeletal muscle, and AT1R-mediated Ang II is crucial for optimal overload-induced skeletal muscle hypertrophy (Gordon et al., 2001). Moreover, Ang II has been shown to regulate oxygen consumption and affect muscle energy expenditure (Cassis et al., 2002). Indeed, higher VO_2max levels have been associated with the ACE D allele (Zhao et al., 2003), indicating an improved oxidative capacity, although most of the previous studies have associated the ACE I allele (lower ACE activity, high kinin ligand generation, and increased half-life of bradykinin) with increased skeletal muscle metabolic efficiency and perhaps improved contractile function (Shen et al., 1995; Henriksen et al., 1999; Shiuchi et al., 2001; Boushel et al., 2002). Local RAS activity in skeletal muscle seems to be much more complex. ACE is not only involved in Ang II production and bradykinin degradation, but also regulates the levels of Ang (1-7) peptide, which is known to cause vasodilatation (Jones et al., 2003). Thus it is possible that the protective effects of the *ACE D* allele against exercise-induced skeletal muscle damage are mainly mediated through the fine-tuning that regulates the levels of Ang II and Ang (1-7). Another explanation for our findings may rely on the

mechanisms underlying the process of muscle damage. In exertional muscle damage, the initial inciting event is associated with an uncontrolled movement of Ca^{+2} into the sarcoplasm, triggering the next stage in the damage process (Proske and Allen, 2005). The activation of Bradykinin receptor B2 (BDKRB2) might lead to transient rises in inositol 1,4,5-triposphate which is involved in excitation-contraction coupling via increases in cytoplasmic Ca^{+2} (Lopez and Parra, 1991). Bradykinin stimulates glucose uptake in the presence of insulin, a process related to alteration in intracellular Ca^{+2} concentration (Kudoh and Matsuki, 2000). Moreover, data suggest that this process is enhanced by the inhibition of ACE (Kudoh et al., 2000). Interestingly, there is evidence that Ang II can affect both sympathetic and neuromuscular transmission (Jones et al. 2003). Thus, it is conceivable that ACE genotype affects CK response through involvement in the regulation of the excitation coupling process.

In the second study it was found that the *IL6 G-174C* genotype was associated with CK activity in a dose-dependent fashion. Subjects with one or more of the *-174C* alleles had a greater increase and higher peak of CK values than subjects homozygous for the *G* allele and for *GG + GC* genotypes versus *CC* genotype. The *IL6-174CC* genotype was associated with a greater than threefold increased risk of massive CK response. A milder association was noted between the *TNF-α G-308A* genotype and CK activity, which can be explained by several mechanisms. The *TNF-α* and *IL-6* genotype may contribute to the obtained results either by directly affecting CK content or by influencing the magnitude of the reactive inflammatory process.

The IL-6 response to exercise depends on the type of polymorphism (*-174 G/C)* within the *IL-6* promoter (*G*-allele or *C*-allele). In healthy subjects, polymorphism has been shown to influence IL-6 transcription and circulating IL-6 levels (Schotte et al., 2001). A functional *G/C* polymorphism at position *-174* of the *IL-6* promotor in the 5' flanking region has been demonstrated in which the luciferase reporter vector assay, *a-174 G* construct, showed significantly higher expression than the corresponding *-174C* construct. In addition, the *G* allele

was associated with a higher level of plasma IL-6 in healthy adults (Fishman et al., 1998).

The indirect increase of IL-6 activity associated with the *GG* genotype reduces inflammation, and may restrict inflammation by stimulating the production of anti inflammatory cytokines including IL-1ra, IL-10, Cortisol, and soluble TNF-α receptors (Petersen and Pedersen, 2005), and inhibiting the production of pro- inflammatory cytokines including tumor necrosis factor α and IL-1 (Aderka et al., 1989; Gabay et al., 1997; Xing et al., 1998; Di Poi and Ferraccioli, 1999). Suppressed pro-inflammatory cytokines by the anti-inflammatory cytokines would decrease plasma CK activity (Hirose et al., 2004).

The increased production of IL-6 by skeletal muscle after acute exercise (Petersen and Pedersen, 2005) can improve glucose handling and sustain the energy demand of muscle by enhancing the activity of adenosine monophosphate-activated protein kinase. Thus, it is thought that the improved glucose dynamics seen after a bout of exercise might be mediated by the exercise induced production of IL-6 (Carey et al., 2006).

The fact that IL-6 can inhibit TNF-α response demonstrates another potential benefit of muscle-derived IL-6. TNF-α has been linked to insulin resistance by inhibiting glucose uptake in skeletal muscle (Penkowa et al., 2003). IL-6 appears to play an integral role in the repair process following muscle damage (Hibi et al., 1996).

Thus, it is possible that the protective effects of the *IL-6 -174 G* genotype against exercise-induced skeletal muscle damage are caused mainly by increasing glucose uptake to the skeletal muscle and restricting inflammation, by stimulating the production of anti-inflammatory cytokines, inhibiting the production of pro-inflammatory cytokines, which directly or indirectly influence the muscle's ability to repair.

The rare *TNF-α G-308A* has been shown to be a stronger inducer of C-reactive protein levels than the common allele (Wilson et al., 1995). Although

the increased CK response to eccentric exercise associated with *TNF-α G-308A* genotype did not reach significance, the direction of this association was similar to the results obtained with the *IL-6 G-174C* genotype; the higher TNF-α expression marked by the *-308A* allele was related to lower CK levels and to milder CK responses to the exercise challenge. The lack of significance may be related with the sample size used in this study.

The protective effect of *TNF-α* gene promoter polymorphisms implies regulation of the transcriptional activity of the gene, which in turn affects the expression of proteins, or the inflammatory response. A plausible mechanistic explanation of the results with respect to their relevance for physiology may be that *TNF-α* gene aberrations interfere with the synthesis of CK prior to its release with cell damage. This could be supported by a fast (3 h) increase in circulating CK in response to eccentric exercise (Brancaccio et al., 2007). However, our findings, which show rather a slower (over several days) increase in circulating CK, argue against this possibility, and suggest an eventual effect on the inflammatory response.

Regarding the third study, although Heled et al. (2007) reported that the *CK-MM NcoI* polymorphism is associated with a risk of exaggerated CK response to exercise, we did not find any association between *CK-MM NcoI* polymorphism and maximal eccentric exercise in our work.

The differences between our results and those of Heled et al. (2007) may be due to different study designs. In the study by Heled et al. (2007) subjects performed alternating sub maximal resistive concentric-eccentric exercise within a given time, while we requested that our subjects perform 50 maximal eccentric contractions. As a result, the average increase in serum CK in high responders was almost 18 times higher in our study than in the study by Heled et al. (2007) following the same criteria (90^{th} percentile: 18656.6 ± 4390.9 U\L and 1048 ± 421 U\L, respectively). In addition, Heled et al. (2007) has not demonstrated any additive genetic effect of the *CK-MM NcoI* polymorphism on CK response.

This discrepancy may also lie in the linkage between *CK-MM NcoI* polymorphism and the gene encoding the Ryanodine receptor 1 (Wappler et al., 2001). The *RYR1* gene is located in the same chromosome region as the *CK-MM* gene (19q13.3). Mutations in *RYR1* has been associated with susceptibility to exertional muscle damage (Wappler et al., 2001). Therefore, the possible association between *CK-MM NcoI* polymorphism to CK response to exercise in the study of Heled et al. (2007) could be due to mutations in the *RYR1* gene which are related to exertional muscle damage and imply a possible functional linkage that may influence muscle function during both mechanical work and metabolic stress and not the *CK-MM NcoI* polymorphism.

Study limitations

The present work tried to associate SNPs with the magnitude of CK response after exercise without testing any cause / effect relationship. Therefore, in order to better understand these cause/effect relationships further studies through gene and protein expression are needed. Another conceptual problem is yet to be solved: is CK blood level a reliable marker of exercise-induced muscle damage? Do CK blood levels really parallel the degree of muscle damage? Although a favorable response to these questions has been provided by several authors (Brancaccio et al., 2007; Cannon et al., 1990; Houmard et al., 1990; Khan, 2009; Umegaki et al., 1998), others have established that differences in CK release after exercise do not consistently reproduce differences in the degree of histological muscle damage (Van der Meulen et al., 1991). Consequently, in order to ascertain the real influence of gene polymorphisms on exercise-induced muscle damage, future studies should consider the use of more rigorous methodologies to assess the true degree of muscle damage.

Conclusions

Based on our experimental data, it is possible to conclude that at least some SNPs are associated with the intensity of muscle damage evaluated by the amount of blood CK response. This general conclusion is based on the following observations:

1. A positive association was found between the *ACE* genotype and CK response to repetitive eccentric contractions, which supports a hypothetical influence of local RAS on the magnitude of exertional muscle injury.

2. A positive association was also observed between *IL6 G-174C* SNP and systemic CK response to strenuous exercise, giving emphasis to the role of inflammatory response in the physiopathology of exercise-induced muscle damage.

3. No association was found between *CK-MM NcoI* polymorphisms and the magnitude of CK response.

With the exception for the *CK-MM NcoI* polymorphisms, the remaining hypotheses of our investigation were confirmed.

Clinical implications and directions for future research

The above results have important implications for medicine, as the presence of gene variants that are associated with exertional muscle damage may help to identify healthy individuals who are at high risk for developing dangerous complications.

Further mechanistic studies are needed to determine whether the gene products regulated by *IL6 G-174C* and *TNFA G-308A* promoter polymorphisms are involved in the pathways leading to exercise-induced muscle damage. Since CK is not consensually accepted as a good and reliable marker of muscle damage, other studies using muscle biopsies will be necessary to support our results.

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