ACTN3 and MLCK genotype associations with exertional muscle damage

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Submitted 2 February 2005; accepted in final form 31 March 2005

EXERTIONAL RHABDOMYOLYSIS is a condition in which unaccustomed, strenuous exercise results in damage to skeletal muscle. Typically, this damage is resolved without serious complications and presents as delayed-onset muscle soreness, pain, and stiffness (4, 6). However, in certain situations, such as with accompanying heat stress and dehydration, overexertion exercise results in a severe breakdown of skeletal muscle tissue that can become clinically relevant, leading to hospitalization, kidney failure, and even death for some individuals (14, 20, 21, 35).

Our laboratory has investigated exertional rhabdomyolysis in a controlled situation using an exercise model that consists of serial unilateral eccentric (muscle lengthening) contractions of the elbow flexor muscles (8). Eccentric contractions produce high levels of strain on muscle, resulting in damage that is manifested in prolonged losses in strength and range of motion, development of delayed onset muscle soreness, and increases in muscle proteins in the circulation (6, 8, 16, 26–29). Individual responses, however, to this standardized elbow flexion eccentric exercise are highly variable. For example, Sayers et al. (31) reported six case studies of subjects who had experienced exaggerated responses to eccentric exercise of the elbow flexors that included severe arm swelling, profound strength loss (60–84%), slow recovery of strength, and high creatine kinase (CK) activity in the blood (highest value of 38,517 U/l for one subject; normal values ~40–170 U/l). Why some individuals and not others incur severe muscle damage in response to a laboratory test or to “real-life” exercise is not known.

Ultrastructural abnormalities, such as Z-line streaming, have been reported, particularly in type 2 fibers of subjects who performed eccentric exercise (11). Some individuals may have more fragile or susceptible type 2 fibers than others, which could explain the large intersubject variability in response to eccentric exercise. Recently, our laboratory identified single nucleotide polymorphisms (SNPs) in two genes coding for proteins that relate to muscle fiber-type composition: α-actinin 3 (ACTN3; R577X) and myosin light chain kinase (MLCK; C37885A). The aim of this study was to determine whether variations in these genes were associated with 1) baseline blood CK activity, blood myoglobin (Mb) concentration, and strength, and 2) exercise-induced increases in CK activity and Mb concentration in the blood and/or strength loss and recovery after strenuous eccentric exercise. We had the opportunity to obtain blood samples from a large number of subjects who performed a standard elbow flexion exercise protocol in a controlled situation in which medical oversight ensured careful monitoring of the subjects.

ACTN3 R577X is one of three genes for α-actinin found in humans, which are important in binding and anchoring actin filaments (19, 24). ACTN3 is a myofibrillar protein localized to the Z disk, is highly conserved through evolution, and is a fast-twitch-specific isoform, expressed only in type II myofibers (1, 19, 25). A common polymorphism of ACTN3 was identified in humans that results in a stop codon and lack of detectable protein in homozygous individuals [C→T transversion at position 1,747 in exon 16, converting an arginine to a stop codon at residue 577 (R577X)] (24). Homozygotes for the...
577X allele are unable to produce ACTN3 protein in their muscle. Yang et al. (36) examined ACTN3 genotypes in 429 elite Australian athletes training at the Australian Institute of Sport and in 436 healthy Caucasian controls and found that male and female elite sprint athletes have higher frequencies of the 577R allele (and hence lower frequencies of the 577X allele) compared with controls. Furthermore, in a recent study, our laboratory found that women homozygous for the ACTN3 577X allele had the lowest baseline strength, and this was validated in ethnic subpopulations (3). These studies were critical for defining ACTN3 genotype as a quantitative trait locus for muscle function in humans. We now theorize that, in response to eccentric exercise, sarcomeres in type II myofibers would be more susceptible to Z-line disruption without the stabilizing influence of ACTN3 (10, 11).

MLCK functions to phosphorylate myosin’s regulatory light chain (RLC) (32). Although the binding of Ca$^{2+}$ to tropomyosin-troponin is the primary regulator of skeletal muscle contraction, RLC plays an important modulatory role in force development (33, 34). However, RLC is not readily phosphorylated in type I fibers and thus acts predominantly in type 2 fibers (12, 34). It is possible that MLCK polymorphisms alter the ability to phosphorylate RLC in type 2 fibers, thereby decreasing the ability to withstand strain during lengthening contractions (17).

Because of the roles of ACTN3 and MLCK in skeletal muscle function, particularly regarding type 2 fibers, we hypothesized that subjects homozygous for the ACTN3 577X allele, MLCK 37885A allele, and MLCK 49T allele would 1) demonstrate higher baseline CK and Mb and lower baseline allele, MLCK 37885A allele, and MLCK 49T allele would 2) show greater losses in muscle strength and greater increases in blood CK and Mb in response to eccentric exercise compared with the heterozygote or homozygous wild type.

**METHODS**

The blood samples used in this study were derived from a larger clinical trial from our laboratory that used a standard exercise protocol (8) to induce muscle soreness so that we could evaluate the efficacy of an analgesic to treat muscle pain. Briefly, in that study, one dosage of a topical nonsteroidal anti-inflammatory analgesic was applied to the elbow flexors ~12–14 h after the exercise. In the statistical tests for associations in the present study, we covaried for treatment groups and found that the treatment did not influence the results regarding the associations. Our laboratory carefully monitored kidney function (e.g., blood creatinine, blood urea nitrogen) in the days after the exercise and found that any changes in indicators of kidney function were small and not clinically significant (Clarkson PM, Kearns AK, Rouzier P, Rubin R, and Thompson PD, unpublished observations). We took an additional sample of blood for DNA sequencing. Subjects gave informed consent for this additional sample, and the protocol and informed consent document for both the clinical trial and the additional blood draw were approved by the University of Massachusetts Human Subjects Review Committee. Of the 208 subjects who participated in the clinical trial, 181 subjects agreed to have the additional blood sample taken for DNA testing. Viable DNA samples were isolated from 157 subjects: 78 men and 79 women. There were 115 Caucasians, 4 African-Americans, 6 Hispanics, 20 Asians, and 11 subjects who self-classified their race as “other.”

Subject inclusion criteria were agreeing to refrain from analgesic use, muscle treatments, strenuous or new physical activity, and alcohol during the study. 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 normal range, known muscle disorders, existing myopathy, diabetes mellitus, or hyperthyroidism. All subjects completed a physical exam by a physician to determine that they were healthy and eligible for the study.

Subjects performed 50 maximal eccentric (muscle lengthening) contractions of the elbow flexor muscles of their nondominant arm on a modified preacher curl bench (8). Two sets of 25 contractions were separated by a 5-min rest period. Each contraction was 3 s long, followed by 12 s of rest. The subject was instructed to maximally contract the elbow flexor muscles during the exercise, resisting the downward motion of the lever as an instructor moved the lever until the arm was fully extended. Subjects were instructed to drink water before and during the exercise visit and encouraged to maintain hydration throughout the study and monitor their urine color (no subject experienced darkened urine).

Before and immediately after the exercise, subjects performed three maximal voluntary isometric contractions (MVC) (3 s each with 1 min between trials). For this test, subjects were positioned on a preacher bench with their elbow angle at 90° and asked to pull against a force transducer. The average of the peak forces from each of the three trials was used as the maximal isometric strength value. Subjects returned to the laboratory 4, 7, and 10 days postexercise when strength testing was repeated.

Blood samples were taken before exercise and 4, 7, and 10 days postexercise to be analyzed for CK activity and Mb concentration. Blood samples were transported daily to Holyoke Hospital (Holyoke, MA) for CK and Mb analysis in a certified clinical laboratory. At the completion of the testing, another blood sample was drawn in EDTA-containing Vacutainer tubes and shipped deidentified to Children’s National Medical Center, Washington, DC, for DNA extraction and subsequent genotyping.

**Genotyping.** Genomic DNA was extracted from whole blood samples using the PUREGENE DNA Purification System (Gentra Systems, Minneapolis, MN) according to the manufacturer’s instructions. Of the 181 subject samples, viable DNA could only be obtained from 157 subjects.

Genotypes for all SNPs in this paper were obtained with the use of a TaqMan allelic discrimination assay that employs the $5'$ nuclelease activity of Taq polymerase to detect a fluorescent reporter signal generated during PCR reactions. The PCR reactions for each SNP contained 20 ng DNA, 900 nM primers, 200 nM probes, and TaqMan Universal PCR Master Mix, and No AmpErase UNG (Applied Biosystems, Foster City, CA) in a final volume of 15 µl. PCR was performed on a MJ Research Tetrad thermal cycier (Waltham, MA). For each SNP, the primers and probes are located in Table 1. The PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>SNP</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
<th>WT Allele Probe (5' VIC)</th>
<th>MT Allele Probe (5' FAM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MLCK</td>
<td>C49T</td>
<td>TCCAGCAAGCAACTTCGAGGCTTT</td>
<td>GCCAGTGAGGACGAGGAAGG</td>
<td>CTGAAAGCTCGGCTGG</td>
<td>CTGAAGCTCTGACGG</td>
</tr>
<tr>
<td>MLCK</td>
<td>C37885A</td>
<td>ACAATTTCAGCAAAAGCTCGCTGGA</td>
<td>GGTGGCTCTTCTTTTCTGTGCA</td>
<td>CTTCAGGAGAGCTCAC</td>
<td>CTTCAGGAGGCTCAC</td>
</tr>
<tr>
<td>ACTN3</td>
<td>R577X</td>
<td>AGATACAGTTCCAGAGAACACT</td>
<td>AGCTGGAAATGCGAGATGAG</td>
<td>TGCTCTCAGAAGG</td>
<td>GGCTCTCAGAAGG</td>
</tr>
</tbody>
</table>

SNP, single nucleotide polymorphism; WT, wild type; MT, mutant type.
RESULTS

Table 3 presents the significant association of baseline CK phenotype with the ACTN3 R577X polymorphism. Contrary to our hypothesis, baseline CK activity was lower in the homozygote ACTN3 577X allele compared with the heterozygote (RX) ($P < 0.05$), but subjects with the wild-type genotype (RR) did not differ from the other two groups. This association was verified in the subpopulation of men ($P = 0.04$). We found that ACTN3 R577X polymorphism explained 3.5% of all variation within baseline CK activity. We could not test the association with Mb baseline or with 7 or 10 days postexercise because the assay was not sensitive below 27 μg/l (many subjects had values of 27 μg/l at these time points). ACTN3 R577X was not associated with baseline strength or the change in strength or blood proteins after exercise.

In support of our hypothesis, MLCK C49T was strongly associated with the increase in CK activity and Mb concentra-

SNPs, and there was no difference in sex distribution among the genotypes. Compared with the total sample composed of 13% Asians, there were 17% Asians homozygous for the ACTN3 577X allele and 33% Asians homozygous for the MLCK 49T allele. Of the six subjects homozygous for the MLCK 49T allele, one classified himself as Middle Eastern, three were Caucasians, and two were Asian. The one subject homozygous for the MLCK 37885A allele was Asian. Thus, there was a disproportionate number of Asians who were homozygous for the variant alleles. The responses of the total subject population for strength, CK, and Mb to the eccentric exercise regimen are depicted in Figs. 1 and 2. Table 2 presents the genotype and allele frequencies for ACTN3 and MLCK SNPs.

The 157 subjects from which viable DNA could be isolated were 24.1 ± 5.2 (mean ± SD) yr of age, 170.8 ± 9.9 cm in height, and 73.3 ± 17.0 kg in mass. There was no relationship of age or body mass with any of the genotypes for the tested SNPs, and there was no difference in sex distribution among the genotypes. Compared with the total sample composed of 13% Asians, there were 17% Asians homozygous for the ACTN3 577X allele and 33% Asians homozygous for the MLCK 49T allele. Of the six subjects homozygous for the MLCK 49T allele, one classified himself as Middle Eastern, three were Caucasians, and two were Asian. The one subject homozygous for the MLCK 37885A allele was Asian. Thus, there was a disproportionate number of Asians who were homozygous for the variant alleles. The responses of the total subject population for strength, CK, and Mb to the eccentric exercise regimen are depicted in Figs. 1 and 2. Table 2 presents the genotype and allele frequencies for ACTN3 and MLCK SNPs.

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tion at 4 days postexercise, which was the time point of the peak response (Fig. 3, Table 4). Both the homozygous wild type (CC) and the heterozygous group showed significantly lower CK activity and Mb concentration than the homozygous rare allele (TT). In Fig. 3, it can be observed that the TT genotype had the largest changes in CK and Mb, and, although not statistically significant, there appeared to be a dose-response association. MLCK C49T explained 15.2% of the variation in the CK increase and 8.0% of the Mb increase after exercise. MLCK C49T was also associated with baseline strength (Table 4), and this association was verified in the subpopulation of men (P = 0.05). However, the association was contrary to our hypothesis that subjects homozygous for the rare allele would demonstrate the lowest baseline strength, when indeed they showed the highest baseline strength.

MLCK C37885A was associated with CK on day 7 postexercise (P < 0.05), with the heterozygote group (5,289 ± 12,172 U/l) having higher values than the homozygous wild type (2,354 ± 3,095 U/l), and on day 10 postexercise (785 ± 1,310 and 406 ± 442 U/l, respectively) (P < 0.05). Adjusting for baseline strength resulted in nonsignificant C37885A genotype associations with CK for day 7. Although not significant, there was a trend for the heterozygous MLCK C37885A to have a higher Mb level at 4 days postexercise compared with the homozygous wild type (494 and 342 ng/ml, respectively). MLCK C37885A was also associated with postexercise strength loss (P = 0.023); the heterozygotes demonstrated greater strength loss (~57.0, ± 17.9%) compared with the homozygous wild type (~49.7, ± 20.3%). Note from Fig. 1 that the average strength loss for subjects possessing the rare MLCK C37885A allele was greater than the average strength loss for the entire subject population, but the strength loss for the wild-type genotype was the same as for the entire subject population.

**DISCUSSION**

This study determined whether ACTN3 and MLCK polymorphisms were associated with circulating levels of CK and Mb and isometric elbow flexor strength (MVC) before and after an eccentric exercise designed to induce nonclinically relevant rhabdomyolysis. The major findings of this investigation were that 1) baseline CK activity was associated with ACTN3 genotype, 2) baseline muscle strength was associated with MLCK C49T genotype, and 3) increases in blood CK and Mb after eccentric exercise were associated with MLCK C49T and the increase in CK after exercise was associated with MLCK C37885A. Three and a half percent of the variability in baseline CK activity was attributable to ACTN3 R577X, 1.2% of the variability in baseline muscle strength was attributed to MLCK C49T, and 15.2% of the variability in the CK response and 8.0% of the variability in the Mb response to eccentric exercise were attributed to the MLCK C49T genotype (Table 4).

ACTN3. Homozygotes for the 577X allele are unable to produce any ACTN3 protein in their muscle. Yang et al. (36) found that the ACTN3 R577X genotype was related to performance in 429 elite Australian athletes training at the Australian Institute of Sport, such that fewer than expected sprint athletes were homozygous for the ACTN3 577X allele. In a recent study, our laboratory found that women homozygous for the

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**Table 2. Genotypes and allele frequencies of SNPs tested**

<table>
<thead>
<tr>
<th>Gene</th>
<th>SNP</th>
<th>Genotype</th>
<th>N</th>
<th>Baseline CK, U/L</th>
<th>% Variation Attributable to ACTN3 Genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>MLCK</td>
<td>C37885A</td>
<td>Wild-type homozygote</td>
<td>121 (77.1%)</td>
<td>106.2 ± 9.9</td>
<td></td>
</tr>
<tr>
<td>MLCK</td>
<td>C37885A</td>
<td>Heterozygote</td>
<td>35 (22.3%)</td>
<td>126.6 ± 6.6</td>
<td></td>
</tr>
<tr>
<td>MLCK</td>
<td>C37885A</td>
<td>Mutant homozygote</td>
<td>1 (0.6%)</td>
<td>99.1 ± 9.1*</td>
<td></td>
</tr>
<tr>
<td>ACTN3</td>
<td>R577X</td>
<td>Wild type</td>
<td>35 (22.7%)</td>
<td>106.2 ± 9.9</td>
<td></td>
</tr>
<tr>
<td>ACTN3</td>
<td>R577X</td>
<td>Heterozygote</td>
<td>78 (50.6%)</td>
<td>126.6 ± 6.6</td>
<td></td>
</tr>
<tr>
<td>ACTN3</td>
<td>R577X</td>
<td>Mutant homozygote</td>
<td>41 (26.6%)</td>
<td>99.1 ± 9.1*</td>
<td></td>
</tr>
</tbody>
</table>

Values are means ± SE; means were adjusted for gender, body weight, and treatment. CK, creatine kinase. *RX > XX, P = 0.035.
ACTN3 577X allele had the lowest baseline MVC strength, and this was validated in ethnic subpopulations (3). It is estimated that ~18% of the population is homozygous for this loss-of-function polymorphism (19). Our sample, however, shows a higher percentage (26.6%) homozygous for the 577X allele, which is similar to the 27.2% found in our laboratory’s previous investigation (3).

Similar to the results of the previous study (3), we did find a trend that the null homozygotes had lower baseline strength compared with the heterozygotes (39.4 ± 17.4 kg) and the homozygote wild types (38.4 ± 18.8 kg), but this was not significant. We also found that subjects homozygous for the ACTN3 577X allele demonstrated significantly lower resting CK activity than the heterozygotes (P = 0.03), contrary to our expectations. There was no association of ACTN3 R577X with the increase in CK and Mb in response to the eccentric exercise, despite the significant association of ACTN3 R577X with baseline CK. The finding of no association of ACTN3 R577X with increased CK and Mb is contrary to our belief that structural differences in the ACTN3-deficient fibers would influence muscle damage, in which case we would expect the null mutation group to demonstrate greater increases in CK and Mb in response to eccentric exercise.

The relationship of ACTN3 R577X with baseline CK (XX < RX) could be a chance finding because there was no statistically significant difference between RR and RX. However, if it is not a chance finding, then the lower CK for subjects with the homozygous ACTN3 577X allele must be attributable to factors that influence resting CK activity in healthy individuals, such as sex, age, ethnicity, muscle mass, and physical activity level (18, 30). Baseline CK activity is known to differ between sexes such that women have lower resting CK values than men (5, 9), but there were not more women who were homozygous mutants for ACTN3 577X. Also, the age, body weight, and height distribution was not different between the XX and RX group. Because we did not assess muscle mass, it is possible that the XX group had lower muscle mass, which could explain the lower resting serum CK activity. There were more Asians homozygous for the 577X allele [17% (7/41)] than heterozygous [6% (9/78)], suggesting that ethnicity could be a factor in explaining the lower CKs in the XX group compared with the RR group; however, the samples size of Asians is too small to make any firm conclusions.

Resting serum CK is also related to physical activity level, which itself may be related to amount of muscle mass. Resting CK is known to be higher in athletes than nonathletes, reflecting higher muscle activity and larger muscle mass (15, 22, 23).

Table 4. Exercise-induced changes in CK, Mb, and baseline strength among MLCK C49T genotypes

<table>
<thead>
<tr>
<th>SNP</th>
<th>Genotype</th>
<th>N</th>
<th>CK 4 Days Postexercise, U/L</th>
<th>N</th>
<th>CK 4 Days Postexercise Minus Baseline, U/L</th>
<th>N</th>
<th>Mb 4 Days Postexercise, ng/ml</th>
<th>N</th>
<th>Baseline Strength, kg</th>
</tr>
</thead>
<tbody>
<tr>
<td>MLCK C49T</td>
<td>CC</td>
<td>96</td>
<td>5.606.1 ± 1.086.4</td>
<td>95</td>
<td>5.548.7 ± 1.086.6</td>
<td>97</td>
<td>316.7 ± 43.5</td>
<td>100</td>
<td>35.8 ± 1.0</td>
</tr>
<tr>
<td></td>
<td>CT</td>
<td>50</td>
<td>8.476.8 ± 1.479.9</td>
<td>50</td>
<td>8.354.7 ± 1.480.2</td>
<td>50</td>
<td>433.4 ± 59.5</td>
<td>51</td>
<td>40.8 ± 1.4</td>
</tr>
<tr>
<td></td>
<td>TT</td>
<td>6</td>
<td>2.9063.5 ± 4.305.7*</td>
<td>6</td>
<td>2.8934.1 ± 4.306.5*</td>
<td>6</td>
<td>937.1 ± 173.2†</td>
<td>6</td>
<td>41.3 ± 4.1†</td>
</tr>
</tbody>
</table>

% Variation attributable to MLCK C49T

15.2% 15.2% 8.0% 1.2%

Values are means ± SE; means were adjusted for gender, body weight, and treatment. *CC < TT, P < 0.001; CT < TT, P < 0.001, not significant when adjusted for baseline strength; †CC < TT, P = 0.003; CT < TT, P = 0.019, not significant when adjusted for baseline strength; ‡CC < CT, P = 0.019.

Taken together with our laboratory’s previous finding (3), subjects homozygous for the 577X allele have the lowest muscle strength and the lowest resting CK activity, which may indicate that these individuals are less active and/or have lower muscle mass.

MLCK C37885A and C49T. MLCK C49T is a novel SNP discovered in our previous study, but we did not find associations with muscle size or strength or their response to resistance training (13). MLCK C37885A is another novel SNP that our laboratory discovered but had not tested for associations with muscle phenotypes. In the present study, subjects homozygous for the MLCK 49T allele showed significantly greater increase in CK activity and Mb concentration (P ≤ 0.019) in response to exercise and higher baseline strength (P < 0.05) than the other genotypes. Subjects heterozygous for MLCK C37885A showed higher CK (P < 0.05) in the blood after eccentric exercise compared with wild types. MLCK functions to phosphorylate myosin’s RLC (32), which plays an important modulatory role in force development (33, 34). It is possible that these polymorphisms increase the ability to phosphorylate RLC. This would generate more tension during the stretch of the eccentric contraction (strain) and more consequent damage, because damage is related to strain on myofibrils (17).

Childers and McDonald (2) tested the hypothesis that phosphorylation of RLC increases eccentric contraction-induced injury in skinned type II fibers of rat psoas muscle. When incubated with MLCK, fibers showed a significant increase in Ca²⁺ sensitivity, an increased isometric force and peak power, and a greater force deficit after a series of stretches to induce injury. The authors concluded that the greater force deficits indicated greater injury to the fiber, likely because of increased number of force-generating cross bridges during the contraction. They also noted the possibility that MLCK influenced susceptibility to contraction-induced damage by direct or indirect (via RLC phosphorylation) modulation of the structural integrity of the myofibrillar lattice. Our results showing an association of MLCK C49T with baseline strength, albeit weak, support the conclusion that the number of force-generating cross bridges during the contractions led to more damage and hence more CK and Mb release. Furthermore, when we adjusted for baseline strength in the analysis, most of the significant associations of genotype with increases in CK and/or Mb were no longer significant.

Several studies have noted that prolonged strength loss in the days after eccentric exercise is not related to the increase in blood CK activity (6), indicating different mechanisms responsible for these two changes. Here we show that the increase in
release of proteins (CK and Mb) from damaged muscle was strongly associated with MLCK C49T genotype, but that prolonged relative strength loss was not. It should be noted that there were only six subjects who were homozygous for the MLCK 49T allele, and therefore the significance of the findings should be evaluated in a larger population. Furthermore, it is of interest that, of the six subjects who were homozygous for the MLCK 49T allele, two were Asians (33 vs. 13% in the total subject population) and five were men. The one subject who was homozygous for the MLCK 3788A allele was an Asian male.

In conclusion, the most important finding in this study is that subjects homozygous for the MLCK 49T rare allele demonstrated greater increases in CK and Mb in response to eccentric exercise, and subjects heterozygous for the MLCK 3788A rare allele (there was only 1 subject homozygous for this rare allele) demonstrated greater increases in CK activity and a trend for greater increases in Mb compared with the wild-type genotype. The greatest risk associated with exertional rhabdomyolysis is the development of acute renal failure because of increased circulating Mb that can precipitate in the kidneys in environmental conditions of heat stress and dehydration. The association of MLCK C49T and MLCK C3788A genotypes with increases in CK and Mb may be an important clinical finding because subjects possessing the rare alleles may show an exaggerated increase in Mb in response to exercise stress. Individuals possessing rare alleles for MLCK C49T and MLCK C3788A may be at higher risk for rhabdomyolysis and acute renal failure, especially in situations of heat stress and dehydration.

GRANTS

This study was supported in part by a grant from the United States Army Research Institute of Environmental Medicine.

REFERENCES