ACE ID genotype affects blood Creatine Kinase response to eccentric exercise

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Running Head:
ACE genotype and CK response
ABSTRACT

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 post-exercise CK activity was determined. ACE genotype was significantly associated with post-exercise 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 ± S.E.M. U/L: II, 8882 ± 2362; ID, 4454 ± 1105; DD, 2937 ± 753, ANOVA, P = 0.02; P = 0.009 for linear trend). Multivariate stepwise regression analysis, which included age, sex, BMI, 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, while the DD genotype may have protective effects. These findings support the role of local RAS in the regulation of exertional muscle injury.

Keywords: Genetics, ACE, Insertion/deletion, Renin-Angiotensin System, eccentric exercise
INTRODUCTION

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 males and 28 females; aged 22-32) volunteered for the study. Participants were physically active but none were well trained or competitive athletes. All were healthy, non-smokers, 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 months, baseline blood values outside of the normal range (males: 24-195U/L, females: 24-170U/L), known muscle disorders, 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⁻¹ (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 post-exercise. 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 20µl polymerase chain reactions (PCR) containing 0.2U Taq polymerase, 1× reaction buffer, 0.2mmol/L 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 minutes was followed by 35 cycles of 94°C for 30 seconds, 58°C annealing for 30
seconds, and 65°C elongation for 45 seconds. In order 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 (r²=0.991).

Data 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 ACE genotypes. A X² 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 chi-square 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 ACE
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 ± S.D. or as mean ± S.E.M. Square
multiple correlation coefficients ($R^2$) were calculated. Two-tailed values of $P < 0.05$ were considered statistically significant.

RESULTS

Subjects were 25±3 years 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 % $II/ID/DD = 14%/47%/39%$, $X^2 = 0.03$, $P = 0.98$). 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 males and 28 (40%) were females, 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 Figure 1. Briefly, the baseline CK values were 157 ± 11 U/L (mean ± S.E.M.), and the average peak CK activity which was noted 96h post-exercise (in all subjects) was 4480 ± 705 U/L (mean ± S.E.M.). 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 which 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 as 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 over-load-induced skeletal muscle hypertrophy (16). Moreover, Ang II has been shown to regulate oxygen consumption and affect muscle energy expenditure (7), and higher VO\textsubscript{2}max levels have been associated with 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 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\(_2\) (BDKRB2) 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-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 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 to our average delta CK levels (1048 U/L vs. 4480 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 level 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 to 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 non-coding 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 which 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.
REFERENCES


FIGURE LEGEND

Figure 1: 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 post exercise.
Table 1. ACE ID allele and genotype frequencies in the study group with subjects’ physical characteristics.

<table>
<thead>
<tr>
<th></th>
<th>ACE II</th>
<th>ACE ID</th>
<th>ACE DD</th>
<th>Allele I</th>
<th>Allele D</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>All, n=70</td>
<td>10 (0.14)</td>
<td>32 (0.46)</td>
<td>28 (0.40)</td>
<td>52 (0.37)</td>
<td>88 (0.63)</td>
<td></td>
</tr>
<tr>
<td>Male, n=42</td>
<td>5 (0.12)*</td>
<td>21 (0.50)*</td>
<td>16 (0.38)*</td>
<td>31 (0.37)*</td>
<td>53 (0.63)*</td>
<td>*</td>
</tr>
<tr>
<td>Female, n=28</td>
<td>5 (0.18)*</td>
<td>11 (0.39)*</td>
<td>12 (0.43)*</td>
<td>21 (0.37)*</td>
<td>35 (0.63)*</td>
<td>#</td>
</tr>
<tr>
<td>Age, years</td>
<td>24 ± 3</td>
<td>25 ± 3</td>
<td>25 ± 3</td>
<td>0.18</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Height (cm)</td>
<td>171± 6</td>
<td>171± 9</td>
<td>171± 7</td>
<td>0.91</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>67 ± 9</td>
<td>67 ± 10</td>
<td>67 ± 10</td>
<td>0.98</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LBM (kg)</td>
<td>55 ± 11</td>
<td>55 ± 11</td>
<td>54 ± 11</td>
<td>0.93</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>22.6 ± 1.6</td>
<td>22.7 ± 1.6</td>
<td>22.8 ± 9</td>
<td>0.96</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Values are absolute and relative (in parentheses) frequencies, mean ± SD for continuous variables; n, no. of individuals. ACE, angiotensin-converting enzyme; I, insertion allele; D, deletion allele; DD, homozygotes for the deletion allele; II, homozygotes for the insertion allele; ID, heterozygotes. *X² = 0.93, df = 2, P = 0.62 for genotype frequencies in males vs. females. #X² = 0.01, df = 1, P = 0.91 for allele frequencies in males vs. females.
Table 2. **Subjects’ phenotypes in relation to ACE genotype**

<table>
<thead>
<tr>
<th></th>
<th>ACE II n=10</th>
<th>ACE ID n=32</th>
<th>ACE ID+II n=42</th>
<th>ACE DD n=28</th>
<th>ANOVA P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline CK activity (U/L)</td>
<td>147 ± 27</td>
<td>158 ± 15</td>
<td>155 ± 13</td>
<td>161 ± 19</td>
<td>0.92/0.82* 0.71#</td>
</tr>
<tr>
<td>Peak CK activity (U/L)</td>
<td>8882 ± 2361</td>
<td>4454 ± 1105</td>
<td>5508 ± 1040</td>
<td>2937 ± 753</td>
<td>0.02/0.07* 0.009#</td>
</tr>
<tr>
<td>Delta CK activity (U/L)</td>
<td>8735 ± 2352</td>
<td>4296 ± 1108</td>
<td>5353 ± 1041</td>
<td>2778 ± 757</td>
<td>0.02/0.07* 0.009#</td>
</tr>
</tbody>
</table>

Values are reported as mean ± S.E.M. n, no. of individuals. ACE, angiotensin-converting enzyme; I, insertion allele; D, deletion allele; DD, homozygotes for the deletion allele; II, homozygotes for the insertion allele; ID, heterozygotes. *P value for CK activity in ACE II+ID vs ACE DD. #P value for linear trend.
Figure 1

![Graph showing CK activity (U/L) over time post-exercise (h)]