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H55N polymorphism as a likely cause of variation in citrate synthase activity of mouse skeletal muscle

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Ratkevicius A, Carroll AM, Kikitevicius A, Venckunas T, McDermott KT, Gray SR, Wackerhage H, Lionikas A. H55N polymorphism as a likely cause of variation in citrate synthase activity of mouse skeletal muscle. *Physiol Genomics* 42A: 96–102, 2010. First published August 17, 2010; doi:10.1152/physiolgenomics.00066.2010.—Citrate synthase (CS) is an enzyme of the Krebs cycle that plays a key role in mitochondrial metabolism. The aim of this study was to investigate the mechanisms underlying low activity of citrate synthase (CS) in A/J mice compared with other inbred strains of mice. Enzyme activity, protein content, and mRNA levels of CS were studied in the quadriceps muscles of A/J, BALB/cByJ, C57BL/6J, C3H/HeJ, DBA/2J, and PWD/PhJ strains of mice. Cytochrome c protein content was also measured. The results of the study indicate that A/J mice have a 50–65% reduction in CS activity compared with other strains despite similar levels of Cs mRNA and lack of differences in CS and cytochrome c protein content. CS from A/J mice also showed lower Michaelis constant ($K_m$) for both acetyl CoA and oxaloacetate compared with the other strains of mice. In silico analysis of the genomic sequence identified a nonsynonymous single nucleotide polymorphism (SNP) (rs29358506, H55N) in Cs gene occurring near the site of the protein interacting with acetyl CoA. Allelic variants of the polymorphism segregated with the catalytic properties of CS enzyme among the strains. In summary, H55N polymorphism in Cs could be the underlying cause of low CS activity and its high affinity for substrates in A/J mice compared with other strains. This SNP might also play a role in resistance to obesity of A/J mice.

inbred strains; oxidative phosphorylation; polymorphisms; mitochondria

CITRATE SYNTHASE (CS) is a key enzyme of the mitochondrial Krebs cycle and has often been used as a mitochondrial marker in both animal and human studies (7, 16, 31). There is also evidence that citrate produced by CS plays an important role in the regulation of substrate oxidation by mitochondria (33). Poor performance of muscle mitochondria is a risk factor for the development of obesity and Type 2 diabetes (35, 39). It is thought that a low rate of fatty acid oxidation increases the accumulation of muscle lipids that activate pathways interfering with insulin signaling (17). On the other hand, high muscle oxidative capacity might promote efficient lipid oxidation and thus prevent insulin resistance (26). Habitual physical activity and regular exercise training can increase muscle oxidative capacity (8, 16). However, genetic factors might also play a role as the allelic variants of the genes can affect protein function.

Indeed, genetic variation is responsible for the predisposition to various metabolic conditions including obesity and Type 2 diabetes (29). In the mouse, naturally occurring allelic variants of genes have been captured and fixed in different inbred strains. Availability of the genomic sequence of a number of strains enables assessment of the role of allelic variation on protein function (5, 11).

Responses to high-fat diet vary among the inbred mouse strains (19, 21, 41). For instance, the A/J strain is more resistant to weight and fat gain when subjected to a high-fat diet than the C57BL/6J strain (19, 36). There is some evidence that high lipid oxidation in skeletal muscles might be of particular importance for resistance to obesity of A/J mice (19). However, A/J mice show reduced CS activity in the skeletal muscles compared with several other inbred strains (21). This finding suggests that A/J mice might have low mitochondrial content, contradicting a popular hypothesis about the importance of muscle oxidative capacity for resistance to obesity (17, 26).

The aim of the study was to investigate the mechanisms underlying the low activity of CS in A/J mice. We have compared enzyme activity, protein content, and mRNA levels of CS in the quadriceps muscles of A/J mice with BALB/cByJ (BALB), C57BL/6J (B6), C3H/HeJ (C3H), DBA/2J (D2), and PWD/PhJ (PWD) strains of mice. We have also assessed protein levels of another independent mitochondrial marker, cytochrome c, to examine if CS activity covaries with mitochondrial protein content (1). Our results suggest that low activity of CS in A/J mice is due to neither low mitochondrial content nor selective reduction in CS protein levels. Comparison of the genomic sequences between the six studied mouse strains suggests that the variation in CS functional properties could be an outcome of the nonsynonymous H55N polymorphism (rs29358506) in Cs gene.

MATERIALS AND METHODS

Animals and muscles. All procedures involving mice were approved by the Lithuanian Republic Alimentary and Veterinary Public Office, and the study was carried out at the Lithuanian Academy of Physical Education. Breeding nuclei consisting of two male and two female mice for each of the six laboratory mouse strains were obtained from the Jackson Laboratory (Bar Harbor, ME). We studied 12 males from each of the A/J, BALB, B6, C3H, D2, and PWD strains. These particular strains were selected for the following rea-
sons: firstly, they provide genetically diverse sample of strains from the laboratory mouse family tree (30); secondly, a large number of polymorphisms were identified among these strains (11); thirdly, available panels of the recombinant inbred (25) and chromosome substitution strains (12, 36) can facilitate analyses of the genetic influences. The animals were kept in the same room at a temperature of 22–24°C and 40–60% humidity. The normal 12/12-h light/dark cycle was reversed. Mice were housed in standard mouse cages, one to three mice per cage. Animals were fed chow diet and received tap water ad libitum. At 117 ± 2 days of age mice were euthanized by cervical dislocation. Quadriceps muscle from left hindlimb was excised and snap frozen in isopentane precooled by liquid nitrogen and stored at −80°C. The muscle was then subdivided into three approximately equal portions by the cross sectional cuts. These muscle samples were weighed (electronic balance KERN ABS 80-4), and these ~60 mg samples were randomly assigned for measurement of CS activity, Cs mRNA quantification, and protein analysis by immunoblotting, respectively. All reagents were from Sigma-Aldrich (Poole, UK) unless otherwise indicated.

**CS activity.** Muscle samples were placed in 10 volumes of ice-cold lysis buffer [50 mM Tris·HCl, 100 mM KHPO4, 2 mM EDTA, 0.2% (wt/vol) bovine serum albumin, pH was adjusted to 7.0], cut in small pieces with scissors, and homogenized with an ULTRA-TURRAX homogenizer (Rose Scientific, Edmonton, Canada). Homogenates were shaken for 60 min and centrifuged at 13,000 g for 10 min. The supernatants were taken, and the protein concentration was measured using the Bradford assay (Bio-Rad, Hertfordshire, UK) for GENESYS 10 Bio UV-Vis Spectrophotometer (Thermo Fisher Scientific, Waltham, MA). We used the same spectrophotometer for assessment of CS activity, applying similar methods as in our previous study (15). The molar extinction coefficient used was 13,600 l·mol⁻¹·cm⁻¹ for coenzyme A-DTNB at 412 nm. The CS reaction reagent consisted of 100 mM triethanolamine-HCl, DTNB (100 µM), Triton-X (0.25% vol/vol), oxaloacetate (0.5 mM), acetyl CoA (0.31 mM) with pH adjusted to 8.0. The reaction reagent (1,000 µl) included also 10 µl of muscle homogenate that was added to start the reaction. The absorbance changes were measured every 15 s over 3 min for determination of maximum CS activity. Initial velocity (V₀) of CS was also determined at varied substrate concentrations by measuring absorbance changes every 5 s. All assays were carried out at room temperature of 21°C. CS from porcine heart was used as a standard (C3260 –200UN, Sigma-Aldridge, UK) for assay calibration. Coefficient of variation changes every 5 s. All assays were carried out at room temperature of 21°C. CS from porcine heart was used as a standard (C3260 –200UN, Sigma-Aldridge, UK) for assay calibration. Coefficient of variation

**Immunoblotting for cytochrome c and CS.** Similar methods were used as described previously (2). Muscle samples were homogenized in 10 volumes of ice-cold lysis buffer [50 mM Tris·HCl, 1 mM EDTA, 1 mM EGTA, 1% (vol/vol) Triton X-100, 0.1% (vol/vol) 2-mercaptoethanol, pH was adjusted to 7.5] supplemented with protease inhibitor cocktail, 10 mM β-glycerophosphate, 50 mM NaF, and 0.5 mM Na3VO4. Protein concentration was determined using the Bradford assay (Bio-Rad). For immunoblotting, 50 µg of protein was loaded on 10% polyacrylamide gel, separated using SDS-PAGE, and then transferred to polyvinylidene fluoride membrane. We assessed the quality of transfer and protein loading by staining the membranes with Ponceau S. Then membranes were washed with Tris-buffered saline (TBS) containing 0.1% (vol/vol) Tween-20 (TBS-T buffer) before being blocked with 5% (wt/vol) nonfat milk in TBS-T buffer. Afterward, membranes were incubated overnight at 4°C with the primary antibody against cytochrome c (1:1,000 dilution, #4272; Cell Signaling, Beverly, MA) or citrate synthase (1:1,000 dilution, #CIS11-A; Alpha Diagnostic, San Antonio, TX) followed by 2-h exposure to horseradish peroxidase-conjugated secondary antibodies (1:2,000 dilution, #7071; Cell Signaling), and detection using ECL detection reagent (Amersham Biosciences, Buckinghamshire, UK) and Bio-Rad Imager for immunoblotting (Bio-Rad). Two control samples of protein mix from all six strains of mice were loaded onto each gel for in-between gel comparison. All blots were analyzed using ImageJ.
software (28). The blot density was corrected for protein loading as assessed by Ponceau S and normalized to the average values of the two control samples in the same gel.

Real-time RT-PCR for CS. Similar methods were used as described previously (40). Firstly, total RNA was extracted. Muscle sample was homogenized in 1 ml of ice cold TRIZOL Reagent (Invitrogen, Paisley, UK) and 200 μl of chloroform was added. The mix was shaken, incubated at room temperature for 5 min, and centrifuged at 10,000 g for 15 min. For RNA precipitation, supernatant was taken, mixed with 0.5 ml isopropanol, incubated at room temperature for 10 min, and then centrifuged at 10,000 g for 10 min. Afterward, excess isopropanol was removed, 1 ml of ethanol added, and the mix was centrifuged again at 10,000 g for 5 min. The pellet was then dried in air and dissolved in 50 μl of RNase-free water (DEPC-treated water, Ambion). The samples did not show any signs of RNA fragmentation as verified by sample separation on the agarose gel. Then RNA concentration was measured (Nanodrop 2000; Thermo Scientific, Loughborough, UK), and 2 μg of RNA was taken for cDNA synthesis using a standard procedure (Invitrogen, Warrington, UK). The total reaction volume was 20 μl and included 50 mM Tris·HCl (pH 8.3), 75 mM KCl, 3 mM MgCl₂, 0.5 mM dNTP Mix (0.5 mM each dATP, dGTP, dCTP, and dTTP), 5 mM DTT, 150 ng of random primers, and 200 units of SuperScript III reverse transcriptase. The reaction was carried out at 42°C for 50 min and terminated by incubation at 95°C for 5 min. Quantification of mRNA was performed by real-time PCR using Roche Lightcycler 480 II (Roche Diagnostics, Sussex, UK). Multiplex Taqman assays were performed for Cs as a target gene and Gapdh as a reference gene in the same sample. Universal probe library (UPL, Roche) was used for selection of intron spanning Cs primers (accession number: NM 026444; forward primer: 5′-GGAAG-GCTAAGAACCCTTGGA-3′ and reverse primer: 5′-TCATCTCCGT-CATGCCCATAGT-3′), and the corresponding UPL probe (UPL probe #100, Roche Diagnostics). The 20 μl of amplification mix contained 3 μl of cDNA solution, 1.0 μl of TaqMan Rodent GAPDH Control Reagent (Applied Biosystems, Warrington, UK), 0.2 μl of UPL probe (UPL probe: #100, Roche Diagnostics), 2× Lightcycler probes mastermix, and 0.2 μM Cs primers. PCR was started with 10 min at 95°C for denaturation. The program continued with 40 cycles of 10 s at 95°C and 30 s at 60°C. Each assay included cDNA samples from studied mice, no-template control, and four dilutions of cDNA mix from all six strains of mice to calculate the corresponding amplification efficiency \[E=10^{-1/b}−1; \text{b is regression coefficient}\]. The threshold cycle (Ct) was the cycle number at which fluorescence intensity exceeded a fixed threshold. Relative Cs mRNA expression was calculated using the following formula: \[\text{Cs mRNA} = \left(1 + E_{\text{Gapdh}}\right)^{-1/b} (1 + E_{\text{Gapdh}})^{-1} \text{Cs mRNA}\].

Database analysis. The mouse phenome database was used to carry out the search for nonsynonymous polymorphisms (5, 38). The PolyPhen analysis was utilized to predict possible effect of the amino acid substitution on the function of CS protein (14, 27). It relies on the sequence, phylogenetic, and structural information characterizing the substitution.

Statistical analysis. All data analysis was performed using Prism 3.0 software. The nonlinear fits were generated to estimate Michaelis constant \((K_m)\) of CS for both acetyl CoA and oxaloacetate. The
and CS protein content between the A/J and five other strains guided our analysis to examination of the coding sequence of Cs as a potential cause of altered catalytic properties of CS in A/J mice. The results of in silico analysis of the Cs gene are presented in Table 1. We found two coding single nucleotide polymorphisms (SNPs) with allelic variants segregating among the six strains, rs29358506 and rs36437163. The rs29358506 results in H55N substitution of histidine with asparagine in the A/J strain only. This substitution occurs in evolutionarily conserved region (see Table 2) proximal to the coenzyme A binding site (27) and in PolyPhen screening is predicted to alter protein function. Location of the polymorphisms and the fact that its segregation pattern coincides with the pattern of variation in enzyme activity among the strains are consistent with a causative role of the H55N substitution on catalytic properties of CS. The rs36437163 separates the PWD strain from five other strains. However, it is synonymous in its nature and the PWD strain did not differ significantly from other strains in CS enzyme kinetics.

**DISCUSSION**

The main aim of the study was to identify the mechanism underlying low activity of CS in the skeletal muscles of A/J mice compared with other inbred mice. The results of our study suggest that the H55N polymorphism could be the underlying cause of this phenomenon. Firstly, neither mRNA nor protein levels of CS differed between A/J mice and other studied strains, suggesting that variation in Cs gene expression and protein content is not involved. Secondly, A/J mice had the same levels of cytochrome c protein as mice from other strains, suggesting that mitochondrial content is not reduced in A/J mice. Thirdly, CS from A/J mice showed higher affinity for substrates (lower \( K_m \)) compared with D2 and C3H strains. Fourthly, the H55N polymorphism occurred in the evolutionary conserved region of the gene and is predicted to affect protein function.

Exercise training studies show that CS activity of skeletal muscles can vary depending on levels of motor activity in mice (9, 10, 16). Therefore, it can be argued that the difference in CS activity is secondary to the variation in the home cage ambulation (22, 24). However, when the levels of spontaneous cage activity were assessed over a 4 h period of the dark cycle, the strains involved in our study ranked from the most to least active as follows: BALB > DBA >> B6 > A/J >> C3H (38). This ranking does not correspond with our data on CS enzyme activity. For example, CS activity in the most active strain, BALB, is the same as in DBA or B6 strains, whereas the A/J mice showed significantly lower CS activity compared with C3H mice despite of a similar level of spontaneous cage activity.

**Table 1. SNPs in mouse Cs gene from A/J, BALB, B6, D2, C3H, and PWD strains**

<table>
<thead>
<tr>
<th>Chromosomal location, Mb (Build 37)</th>
<th>A/J</th>
<th>Balb</th>
<th>B6</th>
<th>D2</th>
<th>C3H</th>
<th>PWD</th>
<th>dbSNP rs</th>
</tr>
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<tbody>
<tr>
<td>10:127.78558</td>
<td>A</td>
<td>C</td>
<td>C</td>
<td>C</td>
<td>C</td>
<td>C</td>
<td>rs29358506</td>
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<tr>
<td>10:127.788302</td>
<td>G</td>
<td>G</td>
<td>G</td>
<td>G</td>
<td>G</td>
<td>A</td>
<td>rs36437163</td>
</tr>
</tbody>
</table>

SNP, single nucleotide polymorphism; Cs, citrate synthase.
activity. Thus, 50–65% lower CS activity in A/J mice compared with other strains is not consistent with being secondary to the variation in motor activity among the strains. This conclusion is further supported by the fact that levels of CS and cytochrome c protein were not reduced in A/J mice compared with the other strains. There is a significant body of evidence suggesting that muscle exercise and other interventions stimulating mitochondrial biogenesis induce a coordinated increase in cytochrome c and CS protein as well as CS activity of skeletal muscles (1, 3, 31).

The relationship between mitochondrial protein content and CS activity was distorted in A/J mice compared with other strains. This phenomenon led us to testing of the catalytic properties of CS enzyme in mice. We investigated CS kinetics in greater detail by assessing Vmax at varied substrate concentrations. These measurements showed that CS from A/J has a higher affinity for both acetyl-CoA and oxaloacetate compared with the other strains that showed very similar enzyme kinetics. CS can be inhibited by ATP, CoA, succinyl-CoA, propionyl-CoA, and citrate (37). We measured the enzyme activity in muscle homogenates, and the concentration of some metabolites might have been slightly different between the muscle samples. However, it seems unlikely that such a large difference in CS kinetics between A/J mice and other strains could stem from variation in metabolite concentration of muscle homogenates. It is more likely that A/J mice express a particular variant of CS differing in kinetic properties from CS in other mice strains.

Comparison of the genomic sequences between the six mice strains suggested that low CS activity of A/J mice could be an outcome of the H55N polymorphism (rs29358506) in exon 3 of the Cs gene. This was the only nonsynonymous SNP that segregated with the differences in CS activity among the tested strains. The substitution occurred in the evolutionarily conserved region and will alter the functional properties of the protein as predicted by the PolyPhen analysis, which relies on multiple sequence alignments of homologous proteins (32).

Mutations in Cs gene can have a significant impact on catalytic properties of CS enzyme. For example, substitution of one amino acid in the active site for oxaloacetate binding produced a 600-fold decrease in catalytic activity of CS enzyme from the pig heart (18). The H55N substitution occurs in a proximity to the CS site interacting with acetyl-CoA (27). Thus effects of H55N substitution on CS activity are expected to be much less pronounced compared with mutations in the active site itself.

It might seem surprising that A/J mice do not show any apparent health problems and can be as active as other strains of mice (24). We could not identify any signs of compensation for the reduced CS activity, as CS and cytochrome c protein content in quadriceps muscle of A/J mice did not differ from other strains. A comparison of gene expression profile in skeletal muscles between A/J and B6 mice also does not reveal any marked differences that could be linked to CS activity (19). This apparent paradox can be explained by the fact that CS capacity to produce citrate exceeds significantly the rate of substrate flux through the Krebs cycle and a ~50% reduction in CS activity might not limit mitochondrial ATP production even in the metabolically active tissues (4, 20). This is highlighted by the findings that maximal aerobic performance of A/J mice did not differ significantly from B6, D2, and C3H (23).

It is important to stress that physiological consequences of the uncoupled CS activity from mitochondrial respiration have not been studied yet. Indeed, the CS activity was lower in skeletal muscles of diabetic patients compared with obese and healthy volunteers, but this was a reflection of reduced mitochondrial content rather than a selective depression of CS, since all the mitochondrial markers were reduced in parallel with CS activity (35). A selective suppression of CS activity might provide an interesting model for studying the effect of mitochondrial metabolism in health and disease. We have examined only the quadriceps muscle. However, similar differences between CS in A/J mice and other strains have been reported for the gastrocnemius muscle (21). These findings would also be relevant for other tissues of A/J mice expressing CS as the described changes in CS catalytic properties are likely to be due to rs29358506 in Cs gene.

Reduction in CS activity may not have a significant effect on mitochondrial ATP production but is likely to affect regulation of cell metabolism. Mitochondrial citrate can cross the mitochondrial membrane via antiport with malate and inhibit cytosolic phosphofructokinase-1, contributing to postprandial insulin resistance (13, 33). Cytosolic citrate also participates in regulation of fatty acid oxidation as it can be converted to acetyl-CoA, the substrate for acetyl-CoA carboxylase (ACC) in the synthesis of malonyl-CoA (33). Malonyl-CoA is a potent inhibitor of carnitine palmitoyltransferase-I that is responsible for long chain fatty acid transport into mitochondria. Citrate acts as an allosteric activator of ACC, which is of particular importance for fat metabolism in liver and muscle cells (39). Thus reduction in citrate synthesis might promote fatty acid uptake and oxidation in mitochondria.

A characteristic feature of the A/J mice is their resistance to fat and weight gain when exposed to a high-fat diet, which is in contrast to the obesity-prone B6 strain (19, 34). The mechanisms underlying these phenotypic differences are unknown, but several studies have implicated the region of chromosome 10, where the Cs gene resides (6, 36). For instance, the chromosome substitution strain, which carries chromosome 10 substituted from the A/J strain onto a B6 background was obesity resistant (36). A congenic strain carrying the A/J allele in the region of Cs gene also showed resistance to obesity (6, 34). Among ~200 genes in this congenic region, 17 genes are polymorphic (missense polymorphisms) between the A/J and B6 strains. However, only three of those 17 genes are known to be implicated in metabolism. These are serin hydroxymeth-

### Table 2. Species alignment of amino acid sequence of CS

<table>
<thead>
<tr>
<th>Species</th>
<th>Amino Acid Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mus musculus</td>
<td>MALLTAAARLLGAKKNSCLVLAAHASSTNLKDVLSNLIPKEQAR1KTFRQQHKTVVQGTIQTVDMMY</td>
</tr>
<tr>
<td>Rattus norvegicus</td>
<td>MALLTAAARLLGAKKNSCLVLAAHASSTNLKDVLSNLIPKEQAR1KTFRQQHKTVVQGTIQTVDMMY</td>
</tr>
<tr>
<td>Sus scrofa</td>
<td>MALLTAAARLLGAKKNSCLVLAAHASSTNLKDVLSNLIPKEQAR1KTFRQQHKTVVQGTIQTVDMMY</td>
</tr>
<tr>
<td>Homo sapiens</td>
<td>MALLTAAARLLGAKKNSCLVLAAHASSTNLKDVLSNLIPKEQAR1KTFRQQHKTVVQGTIQTVDMMY</td>
</tr>
<tr>
<td>Bos taurus</td>
<td>MALLTAAARLLGAKKNSCLVLAAHASSTNLKDVLSNLIPKEQAR1KTFRQQHKTVVQGTIQTVDMMY</td>
</tr>
</tbody>
</table>

The underlined region contains histidine (H) that is substituted by asparagine (N) in the A/J strain.
ytransferase 2, glutaminase 2, and Cs, but only the H55N polymorphism of the Cs gene occurs in the evolutionarily conserved region and is likely to alter protein function. Thus, it is conceivable that CS might play a role in resistance to obesity of A/J mice. It is likely that this polymorphism is a recent mutation that perhaps occurred and became fixed in the A/J strain. Among 110 strains and substrains with available genotypes of rs29358506 only the A/J strain carries the allele (38).

In summary, the results of our study suggest that the allelic variation in the Cs gene could be the underlying cause of low CS activity in A/J mice. CS from A/J mice did show different enzyme kinetics compared with other mouse strains. We hypothesize that the allelic variant of Cs gene is contributing to obesity resistance of A/J strain.

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DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the author(s).

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