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The Effect of ACACB cis-Variants on Gene Expression and Metabolic Traits

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Abstract

Background: Acetyl Coenzyme A carboxylase β (ACACB) is the rate-limiting enzyme in fatty acid oxidation, and continuous fatty acid oxidation in Acacb knock-out mice increases insulin sensitivity. Systematic human studies have not been performed to evaluate whether ACACB variants regulate gene expression and insulin sensitivity in skeletal muscle and adipose tissues. We sought to determine whether ACACB transcribed variants were associated with ACACB gene expression and insulin sensitivity in non-diabetic African American (AA) and European American (EA) adults.

Methods: ACACB transcribed single nucleotide polymorphisms (SNPs) were genotyped in 105 EAs and 46 AAs whose body mass index (BMI), lipid profiles and ACACB gene expression in subcutaneous adipose and skeletal muscle had been measured. Allelic expression imbalance (AEI) was assessed in lymphoblast cell lines from heterozygous subjects in an additional EA sample (n = 95). Selected SNPs were further examined for association with insulin sensitivity in a cohort of 417 EAs and 153 AAs.

Results: ACACB transcribed SNP rs2075260 (A/G) was associated with adipose ACACB messenger RNA expression in EAs and AAs (p = 3.8 × 10−5, dominant model in meta-analysis, Stouffer method), with the (A) allele representing lower gene expression in adipose and higher insulin sensitivity in EAs (p = 0.04). In EAs, adipose ACACB expression was negatively associated with age and sex-adjusted BMI (r = −0.35, p = 0.0002).

Conclusions: Common variants within the ACACB locus appear to regulate adipose gene expression in humans. Body fat (represented by BMI) may further regulate adipose ACACB gene expression in the EA population.

Introduction

Acetyl-CoA carboxylase α and β (ACC1/ACACA and ACC2/ACACB) catalyze the synthesis of malonyl-CoA, the substrate for fatty acid synthesis and a regulator of fatty acid oxidation. Increased malonyl-CoA concentrations inhibit carnitine palmitoyltransferase-1 (CPT1) activity, decreasing the rate of fatty acid entry into mitochondria and subsequent fatty acid oxidation [1]. ACACB is the key regulator of the fatty acid oxidation pathway [2] and Acacb knock-out mice are reportedly protected against obesity and diabetes induced by high fat/high carbohydrate diets [3]. Continuous fatty acid oxidation in adipocytes of Acacb knock-out mice is one factor contributing to their high insulin sensitivity [4]. Human gene expression studies suggest that ACACB is abundantly expressed in both oxidative and lipogenic tissues [5,6].

When dietary intake exceeds the storage capacity of adipose tissue, excess lipid is delivered as ectopic fat to skeletal muscle, liver, pancreatic β-cells, and cardiac muscle. The resulting organ dysfunction, known as “lipotoxicity”, results in impaired insulin action and glucose homeostasis, and ultimately type 2 diabetes; however, the cellular mechanisms and intermediates are not fully understood [7–11]. Acacb knock-out mice [2,3,4] and rats treated with Acaca or Acacb anti-sense nucleotide inhibitors [12] suggested a potential therapeutic target for ACACB in insulin resistance, obesity, metabolic syndrome, and type 2 diabetes. Alternations in nutritional status may also regulate ACACA and ACACB expression [13,14].

ACACB single nucleotide polymorphism (SNP) rs4766587 [15,16] is associated with an increased risk of metabolic syndrome. Interestingly, a common SNP rs2268388 within ACACB is reproducibly associated with type 2 diabetes-related proteinuria.
and end-stage renal disease in non-African American (AA) populations [6,17]. Animal studies [3] suggest that the lack of strong association between ACACB variants and obesity/diabetes may be masked by the cross-regulation of ACACB expression and hormonal/nutritional status in insulin-sensitive tissues [18,19]. Gene-nutrient interactions may further influence the expression of functional variants in ACACB and their relationships with insulin sensitivity [16]. The ACACB cis regulatory SNPs rs2075259 and rs2075263 were significantly associated with ACACB messenger RNA levels in skeletal muscle (p = 3.0×10⁻² and p = 5.2×10⁻⁷, respectively) [20].

We hypothesized that: (a) cis SNPs may regulate ACACB expression in insulin sensitive tissues and by consequence affect insulin sensitivity, and (b) body fat, represented by body mass index (BMI), may regulate ACACB expression in insulin responsive tissues. Therefore, we investigated the association between transcribed SNPs in ACACB and subcutaneous adipose tissue and skeletal muscle ACACB gene expression, as well as insulin sensitivity in European Americans (EAs) and AAs. We also tested for relationships between BMI and ACACB messenger RNA expression in adipose and skeletal muscle tissues.

Results

Demographic characteristics of study subjects are listed in Table 1.

ACACB transcribed SNPs are associated with adipose ACACB messenger RNA expression

Eleven ACACB SNPs were genotyped in 105 EA and 46 AA subjects who were non-diabetic at the time of adipose and skeletal muscle biopsies. The linkage disequilibrium (LD) plots are shown in Figure 1. The LD pattern in EAs was similar to HapMap Caucasians, but differed from AAs and HapMap Yoruba Africans (Figure S1). The patterns of ACACB LD in AAs reflected those reported in HapMap for Yoruba Africans, with the exceptions of rs2075260 and rs2075263 for which the observed level was midway between HapMap Caucasians and Yoruba.

In subcutaneous adipose tissue, SNPs rs7135947, rs2075259, rs2075260, and rs2075263 were nominally associated with ACACB messenger RNA levels from the combined sample of EAs and AAs, after adjusting for age, sex, BMI, and race (p = 0.02-0.05, additive model; Table 2). The association of rs2075260 with ACACB expression was seen in EAs and AAs with the same direction of effect (p = 0.0007 and 0.01 respectively after adjusting for age, sex, and BMI; dominant model) (Table 2). When combining the association p-values in the two races by meta-analysis, the best p-value for association of rs2075260 with ACACB gene expression reached 5.3×10⁻⁵ (dominant model, Fisher’s method) and 3.8×10⁻⁴ (dominant model, Stouffer’s method) (Figure 2). SNP rs7135947 was nominally associated with ACACB expression in AAs after adjusting for age, sex, and BMI (p = 0.04, additive model; Table 2). The association was not statistically significant in EAs, although the trend of expression versus genotype was consistent (Table 2). Since rs2075260 and rs7135947 are not in LD (r²=1 in both EAs and AAs), when accounting for the additive effect of the eQTL-increasing alleles (G for rs2075260 and C for rs7135947), the adjusted ACACB expression level was positively associated with the sum of eQTL-increasing alleles in AAs (p = 0.0046), as well as in EA and AA combined biopsy sample (p = 0.0001), but not in EAs alone (p = 0.11) (Figure S2; adjusted ACACB expression levels were controlled for age, sex, BMI, and ethnicity). Association between rs3742023 and ACACB expression was present in AAs, but not EAs (Table 2).

In skeletal muscle, rs2075260 and rs2075263 were nominally associated with ACACB messenger RNA levels in AAs, after adjusting for age, sex, and BMI (p = 0.03-0.003; additive and dominant/recessive models). However, association was not replicated in EAs and no evidence of association was observed in the combined biopsy sample for any of the tested SNPs (Table 2).

Allelic expression imbalance of ACACB transcribed SNPs in lymphoblast cell lines

To identify a potential regulatory effect of ACACB transcribed SNPs, we applied an independent method to test AEI for six of the eight coding variants in lymphoblast cell lines from 95 Utah EAs under controlled cell culture conditions. In this group, 18 subjects were heterozygous for rs2075260 (A/G), where the mean (± SE) normalized percentage of G allele expression in cDNA was 56.08±2.97%, significantly higher than observed in the genomic DNA background (49.96±0.34%; p = 0.048; see Figure 3). Other SNPs were either not significant or not consistent with adipose/skeletal muscle gene expression (data not shown).

Association of insulin sensitivity with ACACB cis transcribed SNPs in the metabolic sample

SNP rs2075260, which showed the strongest association between adipose ACACB expression for EAs and AAs in the biopsy sample, together with rs2075259 and rs2075263, which

<p>| Table 1. Demographic and laboratory characteristics of study sample. |
| --- | --- | --- | --- | --- | --- | --- |</p>
<table>
<thead>
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<th>Sample</th>
<th>Race</th>
<th>n (M/F)</th>
<th>Age (yr)</th>
<th>BMI (kg/m²)</th>
<th>TG (mg/dl)</th>
<th>TC (mg/dl)</th>
<th>HDL-C (mg/dl)</th>
<th>S1 (×10⁻⁴ min⁻¹[U/ml]⁻¹)</th>
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<td>28.9±5.9</td>
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<td>AA</td>
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</tbody>
</table>

Data expressed as mean±SD. EA, European American; AA, African American; AR, Arkansas; UT, Utah; M, male; F, female; NA, not available; TG, triglyceride; TC, total cholesterol; HDL-C, HDL cholesterol; S1, insulin sensitivity.
doi:10.1371/journal.pone.0023860.t001
showed strong association with insulin sensitivity in 62 previously evaluated non-diabetic subjects (40 EA and 22 AA)\(^{(20)}\), were further genotyped in the metabolic sample (416 EA and 153 AA). Rs7135947 was also genotyped in this sample due to significant association with adipose ACACB expression in AAs from the biopsy study sample. The association results with insulin sensitivity are shown in Table 3. Only rs2075260 was nominally associated with insulin sensitivity in EAs after adjusting for age, sex, BMI, sibship, and cohort (Utah or Arkansas)\(^{\leftarrow p = 0.04\text{, additive model}\}}\). Allele G, associated with higher ACACB expression in the adipose biopsy sample (Figure 2) and AEI (Figure 3), was also associated with lower insulin sensitivity in EAs after appropriate adjustment (Figure 4). No significant association of this SNP was seen in AAs (additive\(^{\leftarrow p = 0.71; \text{ Table 3}\}}\).

Adipose ACACB gene expression correlates with BMI in EA

Adipose ACACB messenger RNA expression level was negatively associated with age and sex-adjusted BMI in EAs \((r = -0.35, p = 0.0002; \text{ Figure 5a})\), but not in AAs (Figure 5b). Significant differences in BMI or \(S_I\) were not found between EAs and AAs in

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**Figure 1.** Linkage Disequilibrium (LD) plot of studied ACACB SNPs. 1a: Linkage Disequilibrium (LD) plot of studied ACACB SNPs in European Americans. 1b: Linkage Disequilibrium (LD) plot of studied ACACB SNPs in African Americans.
doi:10.1371/journal.pone.0023860.g001

**108020kb-108200kb (NCBI Build 36)**
Table 2. *ACACB* messenger expression vs. genotype in adipose and muscle tissues in EAs and AAs.

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<th>12 N</th>
<th>22 exp</th>
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<th>p add</th>
<th>p dom</th>
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**EA**, European American; **AA**, African American. P values were adjusted by age, sex, BMI. p add, p value under dominant model; p dom, p value under dominant model; p rec, p value under recessive model; p add_c, p value of combining EA and AA by additional adjustment of race.

11 exp, 12 exp, 22 exp: *ACACB* relative expression levels in subjects with genotype 11, 12, and 22 respectively. Freq1: Frequency of allele 1; SE: standard error; N: number of subjects.

doi:10.1371/journal.pone.0023860.t002
the biopsy sample (Table 4). However, adipose ACACB expression was significantly lower in EAs than AAs in the biopsy sample (Table 4).

Association of BMI, triglycerides (TG), total cholesterol (TC), and HDL cholesterol (HDL-C) with ACACB cis transcribed SNPs in the metabolic sample

No significant associations were identified with BMI, TG, TC or HDL-C for the selected SNPs in the metabolic sample. SNP rs2075260 showed the lowest p-values for association with BMI in EAs and AAs (dominant \( p = 0.19 \) and \( 0.10 \), respectively; Table S1); however, significant association was not observed in the combined EA and AA sample. No association was identified with TG, TC, and HDL-C (data not shown).

Discussion

ACACB is a key rate limiting enzyme in mitochondrial fatty acid oxidation. In this study, we found that the ACACB transcribed SNP rs2075260 was associated with adipose tissue ACACB gene expression in EAs and AAs and with insulin sensitivity in EAs; the allele associated with lower gene expression was also associated with higher insulin sensitivity. This is consistent with the mouse model, which demonstrated that inhibition of Acacb improved insulin sensitivity [3]. These data suggest that ACACB cis-acting SNPs may regulate gene expression in humans, potentially altering insulin sensitivity independent of BMI. Although the association of rs2075260 with insulin sensitivity was not statistically significant in the AA sample, the effect of this allele was similar in magnitude and in the same direction as that observed in EAs. This result is similar to that seen in an independent German Sorb population sample whose LD pattern was similar to HapMap CEU with the association between rs2075263 (G/T) and homeostasis model assessment of insulin resistance (HOMA-IR; \( p = 0.22, \) beta = 0.06, \( n = 793 \)) [personal communication: Stumvoll M, 2011] [21], as well as in Diabetes Genetics Initiative (DGI) for HOMA-IR (\( p = 0.03, \) beta = 0.11, \( n = 1393 \)), where the meta-p value of these two cohorts reached 0.01 and 0.02 for Stouffer and Fisher’s methods respectively with minor allele C indicating lower insulin sensitivity, consistent with rs2075260 in Table 3 where minor allele G represented lower insulin sensitivity in the AR and UT Caucasian metabolic sample . Our inability to detect association at this locus in AAs may reflect low statistical power in this small sample. Concerns about multiple testing are relevant in genetic studies. The number of SNPs to account for in this study is impacted by the fact that rs2075259, rs2075260, and rs2075263 are in high genotypic concordance in Caucasians. Given this, we presented the raw \( p \) values without adjustment for multiple comparisons.

The non-synonymous SNP rs2075260 (G/A) encodes an amino acid substitution Val2141Ile [NM_001093]. We are unable to predict ACACB protein structural changes caused by this amino acid substitution (http://snpeffect.vib.be/snp_main.php?id = 34162381). We scanned this SNP using MatInspector (http://www.genomatix.de ) and TFSEARCH (http://www.cbrc.jp/research/db/TFSEARCH.html) and have not identified potential anchoring loci for a transcription factor binding site at rs2075260. Therefore, we are unable to determine whether this SNP is a functional variant. However, rs2075259 (G/A), in high genotypic concordance with rs2075260, forms activating protein (AP1)/ v-maf musculoaponeurotic fibrosarcoma oncogene homolog (MAF) anchoring sites for allele G (matrix similarity = 0.96-0.98), while allele A abolished these potential binding sites. As we demonstrated, the G allele of rs2075259 is associated with lower ACACB gene expression in adipose tissue (Table 2). AP1/MAF may act as a suppressor, or impact other repressors, of gene expression [22,23]. This is supported by the strong negative correlation between messenger level of ACACB and MAF/AP1S2 (AP1 subunit 2) in our adipose gene global expression study in non-diabetic subjects (unpublished data).
No SNP was associated with BMI in the metabolic sample (Table S1). However, adipose $ACACB$ expression was negatively associated with age and sex-adjusted BMI in EAs (Figure 5a). This correlation cannot be explained by the known regulatory function of $ACACB$ on body weight and insulin sensitivity in the mouse model [2,3,4]. We suspect that BMI may regulate adipose $ACACB$ expression in EAs. Although this cross-sectional study cannot provide direct evidence, this hypothesis is supported by our observation that HepG2 cell $ACACB$ expression was downregulated after treatment with 1 mM palmitate [13]. It is conceivable that nutritional stress (e.g., diet-induced obesity and free fatty acid exposure) regulates $ACACB$ expression. Downregulation of $Acacb$ was observed in visceral fat tissue in rats fed a high-fat diet, while *Juniperus chinensis* extract significantly reduced this effect [14]. AMPK (AMP activated protein kinase) may be the link between $ACACB$ gene expression and nutritional status [24]. $ACACB$ activity [25] can be directly inhibited by AMPK. This may constitute a physiologic link between higher BMI and lower $ACACB$ gene expression and supports the metabolic adaptation model proposed by Weyer et al based on energy expenditure, fat oxidation, and body weight regulation in Pima Indians [26]. The lack of association between adipose $ACACB$ gene expression and BMI in AAs may be due to the smaller sample size or imprecision of BMI as a measure of adiposity. Alternatively, AAs may be less sensitive to nutritional stress in terms of regulating adipose $ACACB$ expression. Adipose $ACACB$ expression levels are significantly higher in AAs than EAs (Table 4). However, BMI and insulin sensitivity were nearly equivalent in EAs and AAs in the biopsy study sample (Table 4). This finding may reflect racial differences in $ACACB$ expression in response to similar body fat. Different genetic or environmental factors may affect $ACACB$ expression between EAs and AAs.

$ACACB$ variants have not been shown to be associated with BMI or diabetes using GWAS. However, functional variants (e.g., transcribed SNPs) may still regulate gene expression and impact insulin sensitivity. Our biopsy sample was modest and generated

![Figure 3. Allelic Expression of G of rs2075260 in both genomic and cDNAs of Utah EA lymphoblast cell lines (n = 18). doi:10.1371/journal.pone.0023860.g003](image)

Table 3. Association between selected transcribed SNPs and insulin sensitivity (SI) in the metabolic sample.

<table>
<thead>
<tr>
<th>SNP</th>
<th>Allele (1/2)</th>
<th>Race</th>
<th>S_i vs.Genotype/n</th>
<th>genetic power*</th>
<th>P_add</th>
<th>P_dom</th>
<th>P_rec</th>
<th>P_add_comb</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>EA</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>rs7135947</td>
<td>C/T</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>CT</td>
<td>6.37±0.56</td>
<td>6.79±0.37</td>
<td>7.16±0.79</td>
<td>0.66</td>
<td>0.72</td>
<td>0.76</td>
</tr>
<tr>
<td></td>
<td></td>
<td>TT</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>rs2075259</td>
<td>A/G</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>CT</td>
<td>6.39±0.72</td>
<td>6.69±0.46</td>
<td>6.94±0.45</td>
<td>0.83</td>
<td>1.00</td>
<td>0.33</td>
</tr>
<tr>
<td></td>
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<td></td>
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<td></td>
</tr>
<tr>
<td>rs2075260</td>
<td>A/G</td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>CT</td>
<td>6.80±0.44</td>
<td>6.50±0.47</td>
<td>5.87±0.51</td>
<td>0.04</td>
<td>0.87</td>
<td>0.046</td>
</tr>
<tr>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>rs2075263</td>
<td>C/T</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>CT</td>
<td>6.52±0.66</td>
<td>6.59±0.47</td>
<td>6.99±0.43</td>
<td>0.58</td>
<td>0.57</td>
<td>0.94</td>
</tr>
<tr>
<td></td>
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</tr>
</tbody>
</table>

EA European American; AA African American.

Data are presented as least squares mean±SE, after controlling for age, sex, and BMI. Location (Arkansas/Utah) is additionally adjusted for in EA.

P_add reflects p-value for the additive model; P_dom reflects p-value for the dominant model; P_rec reflects p-value for recessive model. P_add_comb is additionally adjusted for race when EA and AA are combined for analysis.

*Genetic power was estimated to detect 10% of the variation in insulin sensitivity and other metabolic traits in the metabolic sample (assuming a type I error rate = 0.0005).

doi:10.1371/journal.pone.0023860.t003
adjusted for age, sex, BMI, cohort, and sibship (GEE model). Olson’s model of explanations for the phenotypic differences observed between resistant to obesity, diabetes and insulin resistance) published by lower insulin sensitivity in mice fed high-fat diets [29]. It suggests that ACACA [27]. This may be supported by the effects of soraphen, might potentially have “dominant negative” activity toward the mRNA in frame, resulting in a mutated but otherwise intact motif [2]. RNA splicing across the targeting cassette might leave strategies were employed. The targeting strategy employed in the binding site was deleted in both models, different targeting gene expression. It is noteworthy that this result was based on insulin sensitivity. As in the present study, association between ACACB represented by BMI, may serve as a “negative feed-back” signal down-regulating ACACB expression in adipose tissue, possibly as a mechanism of metabolic adaptation. Longitudinal intervention studies will assist in interpreting whether high ACACB expression is a risk factor for obesity and type 2 diabetes. Gene*gene interaction studies in larger cohorts will be helpful to identify any undetected diabetes or obesity genes that interact with ACACB. Analysis of alternative splicing of ACACB in adipose tissue and skeletal muscle may be helpful to address tissue-specific transcripts and regulatory SNPs involved in gene expression.

**Materials and Methods**

**Subjects and phenotypes**

The “biopsy sample” consisted of 105 EAs and 46 AAs from Little Rock, Arkansas who lacked diabetes based on ADA diagnostic criteria 2010 [32]. All participants underwent a screening visit during which height, weight, fasting blood lipids, and blood insulin and glucose concentrations (fasting, 30, 60 and 120 minutes after a standard 75-g oral glucose load) were measured, and adipose and skeletal muscle biopsies were performed using a Bergstrom needle under local (lidocaine) anesthesia at University of Arkansas for Medical Sciences (UAMS), Little Rock, Arkansas. Biopsy samples were immediately rinsed in normal saline, cut, and snap frozen in liquid nitrogen. Premenopausal women were studied in the follicular phase of the menstrual cycle.

The “metabolic sample” consisted of 440 non-diabetic EAs (417 with measures of insulin sensitivity) and 163 non-diabetic AAs (153 with measures of insulin sensitivity). AAs were recruited in Arkansas, whereas 293 EAs were recruited from Arkansas and 124 were siblings from 62 nuclear families of Northern European descent ascertained in Utah. Subjects in the previously described “biopsy sample” were a subset of the Arkansas “metabolic sample”. An insulin-modified (0.04 U/kg), frequently sampled
intravenous glucose tolerance test (FSIGT) was performed, as reported [33]. Insulin sensitivity ($S_I$) was calculated from the FSIGT using either the MinMod (Utah sample) or MinMod Millenium (Arkansas Sample) programs [34,35]. These programs use the same algorithms and provide nearly identical estimates of $S_I$. 

Figure 5. Adipose ACACB expression vs. adjusted BMI. Figure 5a Adipose ACACB expression vs. adjusted BMI in EA. Figure 5b Adipose ACACB expression vs. adjusted BMI in AA. doi:10.1371/journal.pone.0023860.g005
**Table 4.** Comparison of ACACB expression, Si, and BMI between EAs and AAs in the biopsy. Sample.

<table>
<thead>
<tr>
<th></th>
<th>EA (n = 105)</th>
<th>AA (n = 46)</th>
<th>P value</th>
<th>Adjustment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adipose ACACB expression</td>
<td>1.68±0.10</td>
<td>2.33±0.15</td>
<td>0.0004</td>
<td>age, sex, BMI</td>
</tr>
<tr>
<td>Adipose ACACB expression</td>
<td>1.76±0.10</td>
<td>2.37±0.17</td>
<td>0.002</td>
<td>age, sex, SI</td>
</tr>
<tr>
<td>SM ACACB expression</td>
<td>0.83±0.03</td>
<td>0.93±0.06</td>
<td>0.15</td>
<td>age, sex</td>
</tr>
<tr>
<td>SM ACACB expression</td>
<td>0.83±0.03</td>
<td>0.93±0.06</td>
<td>0.15</td>
<td>age, sex, BMI</td>
</tr>
<tr>
<td>SM ACACB expression</td>
<td>0.80±0.04</td>
<td>0.90±0.06</td>
<td>0.18</td>
<td>age, sex, SI</td>
</tr>
<tr>
<td>S(10^-4) min^-1 [uU/mg]</td>
<td>3.65±0.19</td>
<td>3.60±0.30</td>
<td>0.88</td>
<td>age, gender, BMI</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>28.1±0.54</td>
<td>29.6±0.86</td>
<td>0.15</td>
<td>age, gender</td>
</tr>
</tbody>
</table>

Data are least squares mean±SE. The controlled covariates are listed in the adjustment column. SM: skeletal muscle; EA: European American; AA: African American.

Subjects provided written, informed consent under protocols approved by either the Institutional Review Board of the University of Utah Health Sciences Center or UAMS. Information from these subjects was de-identified and samples transferred to the Wake Forest University School of Medicine (WFUSM). This study was approved by the WFUSM Institutional Review Board.

Laboratory measurements

Insulin levels were measured using an immunoenzymometric assay (Molecular Light Technology, Wales, UK) and plasma glucose by a glucose oxidase assay. Standard clinical assays (lipids, glucose) were performed at LabCorp (Burlington, NC).

Genotyping

Eight common transcribed ACACB SNPs were genotyped in the biopsy sample (rs2878960, rs4766516, rs11065772, rs2300455, rs7135947, rs2241220, rs3742023, rs2075260), all with minor allele frequencies >0.05 in Caucasians and Yoruba Africans based on HapMap data (http://hapmap.ncbi.nlm.nih.gov/). Additionally, rs2268388 (significantly associated with diabetic nephropathy) [6,17] and rs2075259/rs2075263 (two top SNPs for ACACB expression quantitative trait loci [eQTL] in skeletal muscle) [20] were genotyped. Genotyping of the biopsy sample was performed on a PSQ 96 Pyrosequencer (Biotage, Uppsala, Sweden). Additional genotyping in metabolic sample participants was performed by pyrosequencing or the ABI TaqMan assay (Applied Biosystems, Foster City, CA). Genotype distributions for all variants met Hardy-Weinberg expectations (p>0.01). The overall genotype call rate was above 98%. Sixty-nine duplicated QC samples were randomly distributed across genotyping plates of the metabolic sample to assure 100% reproducibility. For the biopsy sample, in addition to the fact that pyrosequencing is a highly reliable genotyping method which visualizes and quantifies both alleles of target SNPs, AEI requires genotyping of both genomic and cDNAs, which virtually serve as duplicates to assure accuracy.

Linkage disequilibrium plots (D’ and r²) were generated using the HaploView program (http://www.broadinstitute.org/haplovew).

Gene expression

Total RNA was isolated from subcutaneous adipose tissue using the RNAeasy Lipid Tissue Mini Kit (QIAGEN, Valencia, CA) and from skeletal muscle using the Ultraspec RNA kit (Biotex Laboratories, Houston, TX). The quantity and quality of isolated RNA were determined by ultraviolet spectrophotometry and electrophoresis, respectively, using the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA), and 1 μg was reverse transcribed using random hexamer primers with Qiagen reverse transcription reagents (QIAGEN, Valencia, CA). All RNA samples from a single study population and tissue were reverse transcribed using the same kit on the same day. The standard curves were generated using pooled RNA from assayed samples. Primers were designed to capture most known splice variants where the amplicon spanned an intron. ACACB expression was measured by real time PCR (SybrGreen) on an ABI 7500-Fast Real time-PCR system (Applied Biosystems, Foster City, CA) using 18S ribosomal RNA as a normalization standard. Primer sequences were as follows: forward 18S: ATCAACTTTCGATGGTAGTCG; reverse: TCTCTGGATGGTGATGCC. ACACB—Forward: GGCGCTTCTGCCTCCTCACA; ACACB—Reverse: CGTTCTCTCCTGGTCACACGA [36].

Allelic expression imbalance (AEI)

Transformed lymphocytes were cultured from an independent sample of 95 unrelated HapMap Utah Caucasians (EAs) as reported [30]. Total RNA was extracted using the RNAeasy mini kit (QIAGEN, Valancia, CA), quantity and quality was assessed on an Agilent 2100 Bioanalyzer (Agilent Technologies, Inc, Santa Clara, CA). Unequal expression of ACACB alleles was sought as evidence for cis acting regulatory variants by comparing peak heights in individuals heterozygous for the synonymous coding SNPs rs2878960, rs4766516, rs2300455, rs7135947, rs2241220, and rs2075260 [37]. Rs11065772 and rs3742023 failed in assay design. Briefly, total RNA was reverse transcribed using random hexamers. Allelic specific quantification of both cDNA and genomic DNA samples was determined using the same assay for pyrosequencing on a PSQ 96 Pyrosequencer (Biotage, Uppsala, Sweden) with peak height quantified using Allele Quantification software (Biotage, Uppsala, Sweden).

Statistical analysis

S_1 was estimated from the insulin and glucose data using either the MinMod (Utah sample) or MinMod Millenium (Arkansas sample) programs. Gene expression levels were normalized to 18S RNA and the ratio was used in all calculations. Statistical analyses were performed using the SAS 9.1 software of the SAS Institute (Cary, NC). Correlations between ACACB expression and BMI or Si were assessed using a general linear model after controlling potential confounders (age, race, sex, BMI). To approximate normality, the logarithm of ACACB expression was used in all analyses. Allelic specific expression was assessed by comparing the percentage of normalized genomic and cDNA expression on observed alleles using the method of Fagarty et al [38]. For the biopsy samples, a generalized linear model (GENMOD) was used to assess the association between genotype and ACACB gene.
expression after adjusting for age, sex, and BMI, although age and sex were not obvious confounders (Table S2). For analysis under an additive model, homozygotes for the allele (1/1), heterozygotes (1/2), and homozygotes for the allele (2/2) were coded to a continuous variable (0, 1, and 2). The dominant model was defined as contrasting genotypic groups 1/1 vs. 1/2 vs. 2/2. The recessive model was defined as contrasting genotypic groups 1/1 vs. 1/2 vs. 2/2. The generalized estimating equations (GEE) procedure was used to account for sibships in the metabolic sample in addition to age, sex, BMI, and cohort, since Utah subjects were family-based. We analyzed each SNP using the same genetic model for all analyses, irrespective of the study sample. A meta-analysis was performed using Stouffer [39] and Fisher’s method [40]. P-values < 0.05 were considered to represent a nominal level of statistical significance.

**Supporting Information**

**Figure S1** Linkage Disequilibrium (LD) plot of studied ACACB SNPs in HapMap. S1a: Linkage Disequilibrium (LD) plot of studied ACACB SNPs in HapMap Caucasians. S1b: Linkage Disequilibrium (LD) plot of studied ACACB SNPs Yoruba Africans. (TIF)

**Figure S2** Adjusted ACACB expression by sum of eQTL-increasing alleles in adipose. Adjusted ACACB expression levels were controlled for age, sex, BMI, and ethnicity. eQTL-increasing alleles: G for rs2075260; C for 7135947. P values were obtained under general linear model (GLM). EA: European American; AA: African American; All: EA and AA combined. (TIF)

**Table S1** Association between selected transcribed SNPs and BMI in the metabolic sample. Data are least squares mean±SE, after controlling age and sex. Cohort location (Arkansas/Utah) is additionally adjusted for in EA. (XLS)

**Table S2** Summary of Type III testing for age and sex as individual coefficients of ACACB expression in the biopsy sample. EA: European American; AA: African American; DF: degree of freedom. (XLS)

**Acknowledgments**

The authors are grateful to DGI investigators for generously making their data publicly available, and thank Dr. Clifton Bogardus for his critical reading of this manuscript. This work is dedicated to the memory of the late Dr. Steven C. Elbein.

**Author Contributions**

Conceived and designed the experiments: SCE BIF LM PAA MM. Performed the experiments: LM AKM NKS KAL. Analyzed the data: LM PWF BIF. Contributed reagents/materials/analysis tools: BIF PWK SKD JSP PAA. Wrote the paper: LM. Contributed data: AT PK MS. In-depth review and editing: BIF PWK SKD JSP PAA. Supervised the study: BIF SCE.

**References**


