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PAPER

Calpain 3 gene expression in skeletal muscle is associated with body fat content and measures of insulin resistance

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OBJECTIVE: To investigate whether skeletal muscle gene expression of calpain 3 is related to obesity and insulin resistance.

DESIGN: Cross-sectional studies in 27 non-diabetic human subjects and in Psammomys obesus, a polygenic animal model of obesity and type 2 diabetes.

MEASUREMENTS: Expression of CAPN3 in skeletal muscle was measured using Taqman fluorogenic PCR. In the human subjects, body composition was assessed by DEXA and insulin sensitivity was measured by euglycemic–hyperinsulimemic clamp. In Psammomys obesus, body composition was determined by carcass analysis, and substrate oxidation rates, physical activity and energy expenditure were measured by whole-body indirect calorimetry.

RESULTS: In human subjects, calpain 3 gene expression was negatively correlated with total (P = 0.022) and central abdominal fat mass (P = 0.034), and with blood glucose concentration in non-obese subjects (P = 0.017). In Psammomys obesus, calpain 3 gene expression was negatively correlated with circulating glucose (P = 0.013) and insulin (P = 0.034), and with body fat mass (P = 0.049). Indirect calorimetry revealed associations between calpain 3 gene expression and carbohydrate oxidation (P = 0.009) and energy expenditure (P = 0.013).

CONCLUSION/INTERPRETATION: Lower levels of expression of calpain 3 in skeletal muscle were associated with reduced carbohydrate oxidation and elevated circulating glucose and insulin concentrations, and also with increased body fat and in particular abdominal fat. Therefore, reduced expression of calpain 3 in both humans and Psammomys obesus was associated with phenotypes related to obesity and insulin resistance.


Keywords: calpain 3; Psammomys obesus; obesity; insulin resistance; type 2 diabetes

Introduction

While type 2 diabetes is clearly a polygenic disorder, few of the genes involved are known. A number of genome-wide scans in different populations have been performed, and susceptibility genes for type 2 diabetes have been localized to various regions of the genome.1–3 One linkage study in Mexican Americans localized a major susceptibility locus for type 2 diabetes, NIDDM1, to chromosome 2q37.3.1

Recently, the evidence for linkage to type 2 diabetes was found to be associated with a G-to-A polymorphism within intron 3 of the CAPN10 gene.6 CAPN10 encodes calpain 10, a ubiquitously expressed member of the calpain family of calcium-activated nonsosomal cysteine proteases. The polymorphism is involved in the regulation of CAPN10 expression.6 A study in Pima Indians7 found that those individuals with normal glucose tolerance who had a G/G genotype at UCSNP-43 in CAPN10 had decreased rates of postabsorptive and insulin-stimulated glucose turnover that appeared to result from decreased rates of carbohydrate oxidation. Additionally, G/G homozygotes were found to have reduced CAPN10 mRNA expression in their skeletal muscle.7

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A region on chromosome 15 has been shown to contain a locus that interacts with CAPN10 to increase susceptibility to diabetes in Mexican Americans.\(^9\) CAPN3, encoding calpain 3, is located in this region suggesting other calpain genes may also affect susceptibility to diabetes.\(^9\) Mutations in CAPN3, resulting in loss of proteolysis of substrates by this protease, are responsible for Limb Girdle Muscular Dystrophy type 2A,\(^10,11\) indicating that the function of calpain 3 is vital to skeletal muscle integrity.

In this study we measured the expression of the gene encoding calpain 3 in skeletal muscle of a group of 24 non-diabetic subjects, and in Psmummys obesus, a polygenic animal model of obesity and type 2 diabetes.\(^12,13\) The aim of this study was to determine whether CAPN3 gene expression in skeletal muscle was related to diabetes- or obesity-related phenotypes.

**Methods**

**Human study**

**Subjects.** The study was approved by the St Vincent's Hospital Human Research and Ethics Committee (Sydney, Australia) and all subjects gave their written informed consent to participate. Subjects with a history of any major illness including diabetes were excluded. Nineteen adult Caucasian subjects (eight male/11 female) underwent a series of metabolic assessments, including determination of body composition (by dual energy X-ray absorptiometry (DEXA)) and measurement of whole body insulin sensitivity (via euglycemic–hyperinsulinemic clamp). Data were also available for an additional eight female subjects who did not undergo a clamp. All of the subjects had normal glucose tolerance. For all metabolic determinations subjects were studied after an overnight fast.

**Anthropometry and body composition.** Participants wore light clothing, with footwear removed. Weight was measured to the nearest 0.1 kg using a digital scale. Height was measured to the nearest 0.1 cm using an anthropometer. Body mass index (BMI) was calculated as weight divided by height squared (kg/m\(^2\)). Whole body DEXA (Lunar DPX-Lunar Radiation Corporation, Madison, WI, USA; software version 1.35.9) was used to analyze body composition according to the method of Bergstrom.**\(^6\)** In the subset of subjects for whom biopsies were collected during elective knee repair surgery, the euglycemic clamp and DEXA determinations were made 4 weeks prior to surgery and under the same conditions as the other subjects. For all biopsy samples, total RNA was extracted and purified from approximately 50 mg of muscle, using a guanidium thiocyanate–phenol technique (Tri-Reagent, Sigma, MO, USA), according to the manufacturer's instructions. Following extraction, RNA samples were treated with RNase-free DNase (Promega, WI, USA). Total RNA yields were determined and standardized to 0.2 mg/ml by correlation to a standard RNA concentration curve, using a SYBR Green II (Molecular Probes, OR, USA) RNA standard assay.**\(^17\)** First-strand cDNA was generated from 0.2 \(\mu\)g of RNA, in a 10 \(\mu\)l volume using random hexamers as primers (SuperScript, Gibco BRL, MD, USA).

**Tagman PCR.** Taqman PCR was performed as described above. Beta-actin was used as an endogenous control to standardize the amount of cDNA added to a reaction. Primer sequences were as follows: CAPN3 forward, 5'-TGC TCT GCA GTG TGA CAA GCT TCT-3'; CAPN3 reverse, 5'-GCT ACT GAG GGT TGG TCG AGA A-3'; beta-actin forward, 5'-GAC AGG ATG CAG AGG AAG ATT ACT-3'; beta-actin reverse, 5'-TGA TCC ACA TCT GCT GGA AGG T-3'; Fluorogenic probe sequences were 5'-TGT CTG TGA AGG GGC GCT G-3' for the calpain 3 gene, and 5'-ATC ATT GCT CCT CCT GAG CGC AAC TAC TC-3' for the beta-actin gene.
Animal studies

Psammomys obesus colony. A Psammomys obesus colony is maintained at Deakin University, with the breeding pairs fed ad libitum a diet of lucerne and standard laboratory chow. Study animals were weaned at 4 weeks of age and sustained on a diet of standard laboratory chow from which 12% of energy was derived from fat, 63% from carbohydrate and 25% from protein (Barastoc, Pakenham, Australia). Animals were housed in a humidity and temperature controlled room (22 ± 1°C) with a 12-12 h light-dark cycle. All experiments were carried out following the Australian NHMRC principles of laboratory animal care and approved by the Deakin University Animal Ethics Committee.

Experimental animals

Part 1. A total of 33 animals were selected to represent three groups according to previously published criteria: group A—normal glucose tolerance; group B—impaired glucose tolerance; group C—type 2 diabetes. All animals were male and aged 18 weeks. The animals were killed and the tissues immediately removed, frozen in liquid nitrogen and stored at −80°C. Fat mass was calculated as the combined weight of mesenteric, suprascapular, perirenal, epididymal and intramuscular (from between the heads of gastrocnemius) fat pads. Classification as obese or lean was based on previously published criteria.

Part 2. To further investigate the relationship between calpain 3 gene expression in skeletal muscle and metabolism, a separate group of 22 non-diabetic Psammomys obesus was studied. At 18 weeks of age the animals were placed in an indirect calorimeter for 24 h (Oxycon, Columbus Instruments, OH, USA). Oxygen consumption and carbon dioxide production were recorded at 15 min intervals, and physical activity was recorded using an infrared beam system (OptoVarimex Mini-InfraRed Animal Activity Monitor System, Columbus Instruments, OH, USA). Metabolic parameters (carbohydrate and fat oxidation, respiratory quotient, physical activity and total energy expenditure) were calculated from these measurements as previously described. Immediately following removal from the calorimeter, the animals were weighed and blood was collected from the tail vein. The animals were then killed and the red gastrocnemius muscle dissected and removed for gene expression analysis.

RNA extraction and reverse transcription

RNA was extracted from the red gastrocnemius muscle using TriZol (Life Technologies, Rockville, MD, USA) according to the manufacturer’s recommendations for skeletal muscle. The RNA was quantitated by spectrophotometry (Beckman Instruments, Fullerton, CA, USA) and 2 μg electrophoresed through a 1% glyoxal agarose gel (Ambion, Austin, TX, USA) to ensure RNA integrity. The RNA was then reverse transcribed using AMV reverse transcriptase with oligo(dT) primers (Promega, Madison, WI, USA).

TaQman PCR

Gene expression in each cDNA sample was quantitated using Taqman PCR technology on an ABI Prism 7700 sequence detector (PE Applied Biosystems, Foster City, CA, USA). Cyclophilin was used as an endogenous control to standardize the amount of cDNA added to a reaction. Primer sequences were as follows: CAPN3 forward, 5'-CGC CCC TGG TAC AGA AAG T-3'; CAPN3 reverse, 5'-GGC GGT TGG TCC AGA AAC T-3'; cyclophilin forward, 5'-CCC ACC GTG TTC TTC GAC AT-3'; cyclophilin reverse, 5'-CCA GTG CTC AGA GCA CGA AA-3'. Fluorogenic probe sequences were 5'-CAG CGG CCC TCG TTC ACA GAC A-3' for the calpain 3 gene, and 5'-CGG GCC TTC TTC GAC ATG TGT GC-3' for the cyclophilin gene. Both probes had the reporter dye FAM attached to the 5' end and the quencher dye TAMRA attached to the 3' end. Primers and probes were designed based on the Psammomys obesus calpain 3 and cyclophilin gene sequences, which were obtained using standard methods. PCR conditions were 50°C for 2 min, 95°C for 10 min followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. All samples were assayed in duplicate.

Statistical analysis

All data are expressed as mean ± s.e.m. Variables were transformed where appropriate to approximate a normal distribution, which was assessed using a Kolmogorov–Smirnov test. A one-way analysis of variance in combination with post hoc LSD or Games–Howell tests (depending on homogeneity of variance in groups) were used to compare means between groups, and an independent samples t-test was used where appropriate. A two-tailed Pearson or Spearman correlation was performed to analyze linear relationships between gene expression and phenotypes. Differences were considered significant at \( P < 0.05 \), and \( P \)-values were not corrected for multiple comparisons.

Results

Human study

The characteristics of the subjects included in this study are detailed in Table 1. CAPN3 expression in skeletal muscle was negatively related to fat mass in human subjects (\( r = -0.44, \ P = 0.022 \); Figure 1). This relationship was
Table 1  Characteristics of the human subjects included in this study

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Mean ± s.e.m.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>45 ± 3</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>27.7 ± 1.0</td>
</tr>
<tr>
<td>Fat mass (kg)</td>
<td>29.1 ± 2.4</td>
</tr>
<tr>
<td>Abdominal fat mass (kg)</td>
<td>1.85 ± 0.16</td>
</tr>
<tr>
<td>Fasting glucose (mmol/l)</td>
<td>4.69 ± 0.19</td>
</tr>
<tr>
<td>Fasting insulin (mU/l)</td>
<td>7.67 ± 1.39</td>
</tr>
</tbody>
</table>

Table 2  Phenotypic data of Psammomys obesus used in Part 1 of the study

<table>
<thead>
<tr>
<th>nGT*</th>
<th>IGTb</th>
<th>Type 2 diabetes</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>11</td>
<td>11</td>
</tr>
<tr>
<td>Body weight (g)</td>
<td>187 ± 6</td>
<td>240 ± 5&lt;sup&gt;a,b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Percentage body fat</td>
<td>3.1 ± 0.4</td>
<td>6.2 ± 0.3&lt;sup&gt;a,c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Glucose (mmol/l)</td>
<td>4.1 ± 0.2</td>
<td>4.8 ± 0.2</td>
</tr>
<tr>
<td>Insulin (µU/ml)</td>
<td>68 ± 10</td>
<td>347 ± 53&lt;sup&gt;a,c&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

*Normal glucose tolerance; †Impaired glucose tolerance.
<sup>a</sup>Significantly different to nGT (P<0.05; one-way ANOVA with post hoc LSD (for body weight and percentage body fat) or Games–Howell (for glucose and insulin) test).
<sup>b</sup>Significantly different to nGT and IGT (P<0.05; one-way ANOVA with post hoc LSD (for body weight and percentage body fat) or Games–Howell (for glucose and insulin) test).

Figure 1  Correlation between calpain 3 gene expression and fat mass in human subjects (n=27, r=-0.44, P=0.022).

Figure 2  Relationship between calpain 3 gene expression and blood glucose concentration in non-obese human subjects (n=18, r=-0.65, P=0.017).

Figure 3  expression was negatively associated with both fasting plasma glucose (n=8, r=-0.83, P=0.042) and serum insulin concentration (n=8, r=-0.79, P=0.036) in male subjects. The glucose infusion rate during the hyperinsulinemic–euglycemic clamp was not associated with CAPN3 expression.

Expression was negatively associated with both fasting plasma glucose (n=8, r=-0.83, P=0.042) and serum insulin concentration (n=8, r=-0.79, P=0.036) in male subjects. The glucose infusion rate during the hyperinsulinemic–euglycemic clamp was not associated with CAPN3 expression.

Animal studies: Part 1
The phenotypic data for the animals used in this study are given in Table 2. There was no significant difference in calpain 3 gene expression in skeletal muscle between the three groups of animals, although expression levels tended to be lower in the diabetic animals (ANOVA, P=0.10; Figure 3). However, CAPN3 expression was negatively correlated with blood glucose concentration (Spearman r=-0.43, P=0.013) and plasma insulin concentration (Pearson r=-0.37, P=0.034; Figure 4). The negative association
between CAPN3 expression and blood glucose remained significant when diabetic animals were removed from the analysis (n = 22, Spearman r = -0.52, P = 0.014).

The expression of CAPN3 in skeletal muscle was significantly reduced in obese animals (P = 0.040). In addition, CAPN3 expression was negatively correlated with fat mass (Pearson r = -0.35, P = 0.049; Figure 5), and there was a tendency for a negative relationship with body weight (Pearson r = -0.33, P = 0.061). The expression of CAPN3 was also negatively associated with the mass of the mesenteric (Pearson r = -0.38, P = 0.030) and epididymal (Pearson r = -0.36, P = 0.037) fat pads, but not with the supraspinular, perirenal or intramuscular fat pad weights.

Multiple linear regression analysis suggested that only blood glucose concentration was independently associated with the expression of CAPN3 in skeletal muscle (P = 0.031; covariates included were glucose, insulin and fat mass).

Animal studies: part 2
In non-diabetic Psammomys obesus, the expression of CAPN3 in skeletal muscle was associated with the rate of carbohy-

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Figure 4 Association between calpain 3 gene expression in skeletal muscle and blood glucose (n = 33, r = -0.43, P = 0.013) and plasma insulin concentration (n = 33, r = -0.37, P = 0.034) in Psammomys obesus.

Figure 5 Relationship between calpain 3 gene expression and body fat mass in Psammomys obesus (n = 33, r = -0.35, P = 0.049).

Figure 6 Association between carbohydrate oxidation and calpain 3 gene expression in Psammomys obesus (n = 22, r = 0.57, P = 0.009).

Figure 7 Association between total energy expenditure and calpain 3 gene expression in Psammomys obesus (n = 22, r = 0.54, P = 0.013).
rate oxidation (Pearson r = 0.57, P = 0.009; Figure 6). This relationship remained significant after adjustment of carbohydrate oxidation rate for body weight by linear regression analysis. CAPN3 expression was also correlated with the total energy expenditure of the animals (Pearson r = 0.54, P = 0.013; Figure 7). CAPN3 expression in the red gastrocnemius muscle was not associated with the rate of fat oxidation, respiratory quotient or physical activity levels in Psammomys obesus.

**Discussion**

In this study we have shown that reduced expression of CAPN3 was associated with several metabolic parameters indicative of insulin resistance and type 2 diabetes, including reduced carbohydrate oxidation and elevated circulating glucose and insulin concentrations. In addition, lower levels of CAPN3 expression were associated with increased body fat, particularly in the abdominal region. With the exception of carbohydrate oxidation, which was not measured in the human subjects, all of these relationships were seen in both Psammomys obesus and human subjects.

Two recent studies have implicated CAPN10 in the development of type 2 diabetes, and the results of this study suggest that CAPN3 may also be involved. The expression of CAPN3 was negatively correlated with blood glucose concentrations in Psammomys obesus (Figure 4), as well as in non-obese, non-diabetic human subjects. CAPN3 expression was also inversely associated with plasma insulin levels in Psammomys obesus and male, but not female, human subjects. These data suggest that reductions in CAPN3 expression in skeletal muscle are associated with the development of insulin resistance. This is consistent with the recent suggestion that CAPN3 may interact with CAPN10 to influence susceptibility to type 2 diabetes.

It should be noted that the mRNA levels measured in this study could represent differences in the expression of CAPN3 and/or stability of the transcript. In particular, CAPN3 mRNA stability could be affected by metabolic changes in insulin resistant muscle cells. In either case, we have identified relationships between measured levels of CAPN3 mRNA and variables related to obesity and insulin resistance, and will continue to use the term 'CAPN3 expression' when referring to mRNA levels for simplicity. In addition, it must be considered that the differences in mRNA levels observed need to be extended by further studies measuring CAPN3 protein levels and activity in skeletal muscle to confirm the relationships suggested by the expression data.

This study has also demonstrated a positive relationship between CAPN3 expression in skeletal muscle and whole-body carbohydrate oxidation (Figure 6). This relationship was remarkably similar to that shown between CAPN10 expression and carbohydrate oxidation in Pima Indians, and suggests that substrates of calpain 3 may be important in the regulation of substrate utilization in muscle. The mechanism by which CAPN3 affects carbohydrate oxidation is unknown at this time, although it is possible that CAPN3 cleaves and deactivates a substrate that inhibits insulin action, for example protein-tyrosine phosphatase 1B (PTP-1B). PTP-1B is known to undergo proteolytic cleavage by calpains, although there is no direct evidence of an interaction between calpain 3 and PTP-1B. Calpain 3 has numerous known substrates, and is thought to play a crucial role in skeletal muscle structure and function. Apart from direct effects on key enzymes in various metabolic pathways, calpain 3 is thought to cleave a number of cytoskeletal proteins that could have profound effects on intracellular signaling mechanisms. Therefore it is plausible that insufficient production of calpain 3 in skeletal muscle could have a significant impact on insulin action.

The data presented here are cross-sectional in nature and do not enable distinction between cause and effect regarding the relationship between CAPN3 gene expression and insulin resistance related phenotypic variables. Therefore it is unclear whether reduced expression of CAPN3 in skeletal muscle contributes to or results from the development of insulin resistance. In this regard, an interesting recent study showed that inhibition of calpain activity in both adipocytes and soleus muscle strips from rats dramatically reduced 2-deoxyglucose uptake in response to insulin, as well as glycogen synthesis. These results are consistent with the hypothesis that reduced calpain activity in skeletal muscle could be a contributing factor in the development of insulin resistance. Further studies are required to identify genetic variants in the region of the CAPN3 gene that may affect its expression, and to test these for association with phenotypes related to insulin resistance.

The lack of association between the GIR during the hyperinsulinemic-euglycemic clamp and CAPN3 expression is not consistent with other findings in this study that suggest a relationship between CAPN3 gene expression and insulin resistance related phenotypes. The clamp results show no apparent relationship between CAPN3 expression in skeletal muscle and maximally stimulated, whole-body glucose uptake. However, other results from this study suggest that CAPN3 expression is related to basal whole-body carbohydrate oxidation rate and fasting plasma glucose and insulin levels. Therefore, it appears that the skeletal muscle expression of CAPN3 is associated with measurements of insulin action under basal conditions, but not during maximal stimulation.

We also found an inverse relationship between CAPN3 expression in skeletal muscle and body fat mass in both Psammomys obesus and human subjects (Figures 1 and 5). In addition, CAPN3 expression was negatively correlated with central abdominal fat in humans, and with the mass of two abdominal (mesenteric and epididymal) fat pads in Psammomys obesus. Fat mass, and in particular abdominal fat mass, is known to be strongly linked to insulin resistance, thus forming two of the key components of the Metabolic Syndrome. Indirect calorimetry in Psammomys obesus identified an association between...
CAPN3 expression and total energy expenditure, but not with physical activity levels. Given the well-established relationship between visceral adiposity and insulin resistance, it is now necessary to identify factors that affect CAPN3 expression in skeletal muscle. Such factors may include metabolites such as glucose and free fatty acids (FFA), or humoral factors such as insulin and glucocorticoids. Preliminary experiments in our laboratory suggest that linoleic acid impaired CAPN3 expression in C2C12 myotubes in vitro (data not shown). While further studies are required, this raises the possibility that inhibition of CAPN3 activity by elevated circulating FFA levels in obesity could provide a mechanistic link between obesity and insulin resistance.

One striking aspect of this study is the marked similarity between results obtained in Psammomys obesus and human subjects, including correlations between CAPN3 expression and glucose and insulin concentrations, fat mass and abdominal fat mass. We have investigated the development of obesity and type 2 diabetes in Psammomys obesus for several years, and the results of this study add to our contention that these rodents are an excellent polygenic animal model of obesity and type 2 diabetes. Our investigations in Psammomys obesus now include concerted attempts to identify skeletal muscle substrates of CAPN3 that may be involved in the development of obesity and type 2 diabetes.

There also appears to be a number of similarities between the results described here for CAPN3 and those recently reported for CAPN10. In both cases, calpain expression in skeletal muscle was correlated with carbohydrate oxidation rate. In addition, the G/G genotype at UCSCNP-43 in CAPN10 was associated with reduced CAPN10 expression, elevated fasting glucose and 2h insulin concentration during oral glucose tolerance test (OGTT), and reduced sleeping metabolic rate. These results are comparable to our findings that CAPN3 expression was negatively correlated with circulating glucose and insulin concentrations, and positively associated with total energy expenditure but not physical activity levels. The consistency of the results obtained in two different human population samples, as well as in Psammomys obesus, strongly suggest an involvement of the calpain family of proteases in the development of insulin resistance, type 2 diabetes, and possibly obesity. While the results of this study are cross-sectional in nature, there is genetic evidence to suggest that an interaction between CAPN3 (or a nearby gene) and CAPN10 affects susceptibility to type 2 diabetes.

Our results, together with those of Baier and colleagues, add weight to this contention by demonstrating that altered expression of these genes is associated with a metabolic profile consistent with the development of insulin resistance and type 2 diabetes.

In summary, we have shown that in both Psammomys obesus and human subjects, reduced expression of CAPN3 was associated with elevations in circulating glucose and insulin concentrations, decreased carbohydrate oxidation and the accumulation of body fat, particularly in the abdominal region. We suggest that altered expression of CAPN3 contributes to the development of insulin resistance, obesity and type 2 diabetes.

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References


