Exercise increases TBC1D1 phosphorylation in human skeletal muscle

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Submitted 25 January 2011; accepted in final form 18 April 2011


First published April 19, 2011; doi:10.1152/ajpendo.00042.2011.

Exercise and weight loss are cornerstones in the treatment and prevention of type 2 diabetes, and both interventions function to increase insulin sensitivity and glucose uptake into skeletal muscle. Studies in rodents demonstrate that the underlying mechanism for glucose uptake in muscle involves site-specific phosphorylation of the Rab-GTPase-activating proteins AS160 (TBC1D4) and TBC1D1. Multiple kinases, including Akt and AMPK, phosphorylate TBC1D1 and AS160 on distinct residues, regulating their activity and allowing for GLUT4 translocation. In contrast to extensive rodent-based studies, the regulation of AS160 and TBC1D1 in human skeletal muscle is not well understood. In this study, we determined the effects of dietary intervention and a single bout of exercise on TBC1D1 and AS160 site-specific phosphorylation in human skeletal muscle. Ten obese (BMI 33.4 ± 2.4, M-value 4.3 ± 0.5) subjects were studied at baseline and a 2-wk dietary intervention. Muscle biopsies were obtained from the subjects in the resting (basal) state and immediately following a 30-min exercise bout (70% V˙O₂ max). Muscle lysates were obtained from the subjects in the resting (basal) state and immediately following a 30-min exercise bout (70% V˙O₂ max). Muscle lysates were analyzed for AMPK activity and Akt phosphorylation and for TBC1D1 and AS160 phosphorylation on known or putative AMPK and Akt sites as follows: AS160 Ser711 (AMPK), TBC1D1 Ser231 (AMPK), TBC1D1 Ser660 (AMPK), TBC1D1 Ser231 (AMPK), and TBC1D1 Thr590 (Akt). The diet intervention that consisted of a major shift in the macronutrient composition resulted in a 4.2 ± 0.4 kg weight loss (P < 0.001) and a significant increase in insulin sensitivity (M value 5.6 ± 0.6), but surprisingly, there was no effect on expression or phosphorylation of any of the muscle-signaling proteins. Exercise increased muscle AMPKα2 activity but did not increase Akt phosphorylation. Exercise increased phosphorylation on AS160 Ser711, TBC1D1 Ser231, and TBC1D1 Ser660 but had no effect on TBC1D1 Ser231. Exercise did not increase TBC1D1 Thr590 phosphorylation or TBC1D1/AS160 PAS phosphorylation, consistent with the lack of Akt activation. These data demonstrate that a single bout of exercise regulates TBC1D1 and AS160 phosphorylation on multiple sites in human skeletal muscle.

Glucose transport; adenosine 5′-monophosphate-activated protein kinase

With the dramatic increase in rates of type 2 diabetes throughout the world, there is a pressing need to understand the molecular mechanisms by which therapeutic interventions such as exercise and weight loss lead to improvements in glucose homeostasis and skeletal muscle glucose metabolism. Skeletal muscle is critical for glucose disposal, which is mediated by the transport of glucose into muscle cells through the glucose transporter protein GLUT4. In skeletal muscle, both contraction and insulin increase glucose transport via the stimulation of GLUT4 translocation from intracellular depots to the sarcolemma and transverse tubules (11, 17). GLUT4 translocation is regulated by complex signaling mechanisms (10, 13), and the Rab-GTPase-activating proteins (Rab-GAP) AS160 and TBC1D1 have recently been identified as distal components of this process (3, 8, 16, 26, 36). In the basal state, where glucose transport is low, AS160 and TBC1D1 are thought to retain GLUT4 in the intracellular vesicles by keeping target Rabs in an inactive, GDP-bound state (28). When the Rab-GAP activity is inhibited by phosphorylation, GLUT4 vesicles are released for translocation to the cell surface with increased transport of glucose across the cell membrane as a consequence (28).

The homology of AS160 and TBC1D1 is only ~50%, but the Rab-GAP domains are 79% identical, and the proteins may have some overlapping function (26). Whereas AS160 is ubiquitously expressed, TBC1D1 is almost exclusively expressed in skeletal muscles, suggesting that there may be a specialized function of TBC1D1 in muscle (31). Using adult mouse skeletal muscle, we and others have identified several phosphorylation sites on TBC1D1 using mass spectrometry (5, 31). Some of these phosphorylation sites are important functionally, since combined mutation of several sites impaired contraction-stimulated glucose transport in mouse skeletal muscle (3, 36). In contrast, single mutations of these phosphorylation sites had no effect on glucose transport (3, 36). To study the regulation of these different TBC1D1 phosphorylation sites by various stimuli, we have raised phosphospecific antibodies against four of these sites (36). Using these antibodies, we have determined that Ser231, Ser660, and Ser700 on TBC1D1 can be phosphorylated by AMP-activated protein kinase (AMPK) and that Thr590 is regulated by Akt in mouse skeletal muscle (36). In human skeletal muscle, it has been shown recently that exercise rapidly increases phosphorylation at Ser231 (equivalent to the Ser231 in mouse TBC1D1) (9). Whether exercise increases phosphorylation of these additional sites on TBC1D1 in human skeletal muscle has not been investigated.

Similar to TBC1D1, AS160 contains multiple phosphorylation sites (28), and the regulation of these sites has been studied in both mouse (15, 16, 34) and human skeletal muscle (33, 37). In human vastus lateralis skeletal muscle, insulin stimulation was first shown to increase Thr442 phosphorylation (14), and more recently insulin has been reported to regulate Ser341, Ser388, Ser660, and Ser751 (33, 37). Phosphorylation of AS160 on Ser711, a site regulated by both AMPK and insulin in mouse muscle (35), is also increased by exercise in human skeletal muscle (35).
Diet intervention and weight loss have beneficial effects on skeletal muscle insulin sensitivity (1) and can also increase contraction-stimulated glucose uptake (23). The underlying mechanisms are unknown but may involve alterations in glycogen concentrations (25). Reductions in carbohydrate intake are known to reduce muscle glycogen concentrations, which can lead to enhanced glucose uptake both in the basal state and after exercise (23, 25). This raises the possibility that the effects of diet intervention could be mediated through alterations in TBC1D1 and AS160 expression and/or phosphorylation in skeletal muscle.

The overall goal of the current study was to test the hypotheses that 1) a single bout of exercise increases TBC1D1 and AS160 phosphorylation in human skeletal muscle and 2) exercise-induced phosphorylation of TBC1D1 and AS160 is enhanced after a dietary intervention. For this purpose we studied obese nondiabetic subjects, a population at high risk for developing type 2 diabetes. We found that dietary intervention had no effect on TBC1D1 and AS160 expression or phosphorylation. However, a single bout of moderate-intensity exercise significantly increased site-specific phosphorylation of these Rab-GAP proteins, suggesting that TBC1D1 and AS160 may be important in the regulation of exercise-stimulated glucose transport in skeletal muscle.

MATERIALS AND METHODS

Subjects. Ten obese but otherwise healthy subjects (7 women, 3 men) without any ongoing pharmacological treatment except for oral contraceptives were recruited for the study. Subject characteristics and predicted food intake are given in Table 1. All potential participants underwent a comprehensive medical examination as well as routine blood sample tests to exclude any unknown medical conditions, including type 2 diabetes. None of the subjects were performing any strenuous physical exercise on a regular basis. The study was approved by the regional ethics committee in Stockholm, Sweden, and all participants gave their written informed consent after being informed about the purpose and nature of the study.

Study protocol. All subjects underwent a 2-wk period of dietary intervention, as described below. Before and after the diet intervention, subjects underwent assessment of maximal oxygen uptake (V\textsubscript{O\textsubscript{2}}\text{max}), body composition (dual-energy X-ray absorption), and insulin sensitivity (hyperinsulinemic clamp). On a separate occasion before and after the dietary intervention, a muscle specimen was obtained from the vastus lateralis muscle at basal and after a 30-min period of cycle ergometer exercise at a workload corresponding to 70% of V\textsubscript{O\textsubscript{2}}\text{max}.

Exercise protocol and muscle biopsies. Maximal oxygen consumption (V\textsubscript{O\textsubscript{2}}\text{max}) was determined on a cycle ergometer, using a stepwise load increase protocol. O\textsubscript{2} uptake, ventilatory volume, and CO\textsubscript{2} production were measured on line (Sensormedics V\text{max} 229), and the leveling-off criterion was used to define V\textsubscript{O\textsubscript{2}}\text{max} (38).

In the morning on the day of the exercise protocol, a basal muscle specimen was obtained after a 30-min period of rest. A 5- to 7-mm incision was made using local anesthesia and sterile conditions 12–15 cm proximal to the superior border of the patella, and a muscle specimen was obtained from the superficial border of the vastus lateralis muscle using the Bergstrom needle. This was followed by a 30-min period of exercise on the cycle ergometer at a workload corresponding to 70% of determined V\textsubscript{O\textsubscript{2}}\text{max} after which another muscle specimen was obtained from the contralateral leg following the same procedure. The muscle tissue was immediately dissected free from fat and connective tissue and placed in liquid nitrogen.

Hyperinsulinemic normoglycemic clamp. The subjects reported to the laboratory after an overnight fast. After basal blood sampling, a hyperinsulinemic normoglycemic glucose clamp was started. Repeated blood sampling was performed during the steady-state period of the clamp, as described below.

Normoglycemic hyperinsulinemia was created by continuous infusion of insulin (Actrapid; NovoNordisk, Bagsvaerd, Denmark), which was given at a rate of 1.0 mU·kg\textsuperscript{-1}·min\textsuperscript{-1}, aiming to yield a standardized plasma insulin concentration. The blood glucose concentration was kept at normoglycemic levels (as close as possible to 4.5 mM) by controlling blood glucose levels at least every 10 min by adjusting the flow rate of a variable 200 mg/ml glucose infusion (Braun, Melsungen, Germany). After insulin had been infused for ~60 min, steady-state glucose concentrations were achieved, and the amount of glucose infused during the following 60 min was used to calculate whole body insulin sensitivity (mg glucose/kg body wt\textsuperscript{-1}·min\textsuperscript{-1}), as described earlier (6).

Body composition, including body fat and fat-free mass, was measured using dual-energy X-ray absorptiometry (Prodigy Lunar, GE Lunar, Madison, WI).

Dietary intervention. The subjects were instructed to consume a low-carbohydrate, high-protein, high-fat diet. Each subject met individually before, after 1 wk, and after the dietary intervention period with a registered dietitian to perform a 24-h dietary recall and to review the central features of the diet. The macronutrient content of the diet was calculated using Dietist XP (Bromma, Sweden) software. The diet consisted of limiting carbohydrate intake without restricting consumption of fat and protein. In brief, subjects were instructed to reduce carbohydrate intake to 21 g/day but were allowed to eat protein and fat as much and as often as they wanted. This included fresh fish, eggs, beef, turkey, chicken breasts without skin, fresh ham slices, raw or steamed vegetables, and butter. Only limited amounts of cheese and cream cheese were allowed. In addition, specific brands of salad dressings and snack foods with limited carbohydrate content were allowed, whereas sauces, gravies, or other ingredients that contain carbohydrates were not recommended. The complete diet instructions can be found in the supplemental data. Subjects were instructed to maintain their regular degree of physical exercise during the intervention period.

Polymerase chain reaction. Total RNA was isolated from human muscle and was reverse-transcribed to cDNA. TBC1D1 was amplified by PCR with splice exon-flanking primers. Primers utilized were as

Table 1. Subject characteristics and diet composition before and at the end of the 2-wk diet intervention

<table>
<thead>
<tr>
<th></th>
<th>Prediet</th>
<th>Postdiet</th>
<th>Paired t-test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight, kg</td>
<td>96.8 ± 2.5</td>
<td>92.6 ± 2.4</td>
<td>P &lt; 0.001</td>
</tr>
<tr>
<td>Fat mass, kg</td>
<td>43.6 ± 1.4</td>
<td>42.4 ± 1.6</td>
<td>P &lt; 0.05</td>
</tr>
<tr>
<td>Lean body mass, kg</td>
<td>52.4 ± 3.0</td>
<td>50.1 ± 2.8</td>
<td>P &lt; 0.001</td>
</tr>
<tr>
<td>Food intake, kJ·kg\textsuperscript{-1}·day\textsuperscript{-1}</td>
<td>78.7 ± 4.7</td>
<td>72.5 ± 2.7</td>
<td>P = 0.30</td>
</tr>
<tr>
<td>Energy from fat, %</td>
<td>31.4 ± 2.5</td>
<td>59.2 ± 1.5</td>
<td>P &lt; 0.001</td>
</tr>
<tr>
<td>Energy from protein, %</td>
<td>18.9 ± 1.7</td>
<td>35.9 ± 1.7</td>
<td>P &lt; 0.001</td>
</tr>
<tr>
<td>Energy from carbohydrates, %</td>
<td>47.8 ± 2.6</td>
<td>4.6 ± 0.3</td>
<td>P &lt; 0.001</td>
</tr>
<tr>
<td>M value, mg·kg\textsuperscript{-1}·min\textsuperscript{-1}</td>
<td>4.3 ± 0.5</td>
<td>5.6 ± 0.6</td>
<td>P &lt; 0.001</td>
</tr>
<tr>
<td>Total cholesterol, mM</td>
<td>5.4 ± 0.3</td>
<td>5.1 ± 0.3</td>
<td>P = 0.11</td>
</tr>
<tr>
<td>HDL cholesterol, mM</td>
<td>1.3 ± 0.1</td>
<td>1.3 ± 0.1</td>
<td>P = 0.17</td>
</tr>
<tr>
<td>LDL cholesterol, mM</td>
<td>3.5 ± 0.2</td>
<td>3.5 ± 0.3</td>
<td>P = 0.85</td>
</tr>
<tr>
<td>Triglyceride, mM</td>
<td>1.25 ± 0.1</td>
<td>0.92 ± 0.1</td>
<td>P &lt; 0.05</td>
</tr>
<tr>
<td>Blood glucose, mM</td>
<td>4.6 ± 0.2</td>
<td>4.3 ± 0.2</td>
<td>P = 0.001</td>
</tr>
<tr>
<td>Insulin, pM</td>
<td>100 ± 33</td>
<td>83 ± 59</td>
<td>P = 0.11</td>
</tr>
<tr>
<td>Steady-state glucose, mM</td>
<td>4.3 ± 0.2</td>
<td>4.4 ± 0.4</td>
<td>P = 0.93</td>
</tr>
<tr>
<td>Steady-state insulin, pM</td>
<td>590 ± 142</td>
<td>511 ± 162</td>
<td>P = 0.16</td>
</tr>
</tbody>
</table>

Data are presented as means ± SE; 10 subjects (7 women and 3 men) were studied before and at the end of the 2-wk diet intervention. All blood samples, except for steady-state samples, were drawn after an overnight fast. Steady-state glucose and insulin were drawn during the last 60 min of a hyperinsulinemic normoglycemic glucose clamp.
follows: first set, forward 5′-CCATGTTGAGTCTTGG-3′ and reverse 5′-CATCTTACACACTGATGTC-3′; second set, forward 5′-TACCGGAGCGCAGTATT-3′ and reverse 5′-CACAGTCTTCACCGAAG-3′. Amplicons were separated by agarose gel electrophoresis and imaged with ethidium bromide staining under UV light.

Measurements of intracellular signaling. Muscle biopsies were homogenized in a buffer containing 20 mM Tris, 50 mM NaCl, 5 mM Na2PO4, 50 mM NaF, 250 mM sucrose, 2 mM DTT, 1% Triton-X 100, 2 μg/ml aprotinin, 5 μg/ml leupeptin, 0.5 μg/ml pepstatin, 10 μg/ml antipain, 1.5 mg/ml benzamidine, and 500 μM/ml phenylmethylsulfonylfluoride, pH 7.4. Muscle lysates (200 μg) were immunoprecipitated with specific antibodies to the α1 and α2 catalytic subunits of AMPK and protein A beads (Sigma). The reaction was performed using the synthetic SAMS peptide as substrate, as described previously (20, 39).

Western blot analyses were used to assess protein and phosphorylation levels of various proteins. Antibodies to AMPKα1 and phosphospecific (Ser231)-acetyl-CoA carboxylase (ACC) antibody were from Millipore (Billerica, MA). GLUT4 antibody was from Chemicon (Temecula, CA). Phosphospecific AMPK (Thr172), Akt, phosphospecific Akt (Ser737 and Thr928), TBC1D1, and AS160 antibodies were from Cell Signaling Technology (Beverly, CA). ACC expression was assessed using HRP-conjugated streptavidin (Pierce Chemical, Rockford, IL). Antibody to AMPKα2 was generated as described previously (20). Phosphospecific TBC1D1 (Ser231, Ser660, Ser700, Thr591) and TCB1D4 Ser711 were generated as described previously (35, 36).

Glycogen concentrations. Muscle samples were hydrolyzed in 2 N HCl at 100°C for 2 h followed by neutralization with 2 N NaOH, and glycogen content was measured by the hexokinase enzymatic method using a glucose HK reagent (Eagle Diagnostics, Desoto, TX).

Statistical analysis. Data are expressed as means ± SE. Normality of the data was tested with the Kolmogorov-Smirnov test of normal distribution. Where P > 0.20, the data was considered to be normally distributed. All normally distributed data were compared using Student’s t-test or one- or two-way ANOVA. The differences between groups were considered significant when P < 0.05.

RESULTS

Clinical and metabolic characteristics of the subjects. The effects of the diet intervention on subject characteristics and food intake are summarized in Table 1. There was a major shift in macronutrient intake during the diet intervention. The percent energy intake from carbohydrates was reduced from 48 ± 8 to 5 ± 1, a 90% reduction. Fat and protein increased from 32 ± 8 and 19 ± 5 to 59 ± 5 and 36 ± 5, respectively (P < 0.05 for all). The caloric intake during the diet intervention was not statistically significant from the prediet. As has been shown in previous studies, this diet causes a rapid weight loss (2). The average weight loss after diet intervention was 4% (4.2 ± 0.4 kg), and this was associated with an increase in insulin sensitivity as indicated by a ~30% increase in the M value and a small but significant decrease in fasting blood glucose concentrations. The weight loss was composed of a 4% loss of lean body mass and 3% loss of fat mass. There was also a significant decrease in plasma triglyceride concentrations, whereas cholesterol concentrations were unaffected.

Human skeletal muscle expresses multiple splice isoforms of TBC1D1. Mouse skeletal muscles express the short and long splice isoform of TBC1D1, with the long form predominant (31). Interestingly, only the long form contains the Ser660 and Ser700 phosphorylation sites, whereas the Ser231 and Thr590 sites are expressed in both splice variants (3). It is not known whether multiple splice variants of TBC1D1 are expressed in human skeletal muscle, and therefore, we determined the relative expression of the long and short TBC1D1 splice variants in human skeletal muscle by amplifying TBC1D1 by PCR with splice exon-flanking primers. The amplicons were separated by agarose gel electrophoresis. Two sets of primers each yielded three products (Amplicon DNA level: 1:0.41: 0.28) (Supplemental Fig. S1; Supplemental Material for this article is available online at the AJP-Endocrinology and Metabolism website). Sequencing results confirmed that all three amplicons are splice variants of TBC1D1. The short-form TBC1D1 is missing the entire splice exon (SE) domain, whereas the medium form lacks only the NH2-terminal part of the SE domain in TBC1D1. Our results suggest that three splice variants of TBC1D1 are expressed in human skeletal muscle. The weight of the short form is predicted to be ~140 kDa, the medium form 146–148 kDa, and the long form ~155 kDa. As shown in Fig. 1A, TBC1D1 could be detected by a clear band just above the 150-kDa marker, and at longer exposures two faint bands below the 150-kDa marker could be observed. These data suggest that the long form of TBC1D1 is the predominant splice variant expressed in human skeletal muscle. We quantified the predominant TBC1D1 band (Fig. 1B) and AS160 (Fig. 1C) and did not see an effect of diet intervention on protein expression of TBC1D1 and AS160.

Exercise increases site-specific TBC1D1 phosphorylation in human skeletal muscle. AMPK and Akt are established upstream kinases of TBC1D1 and AS160 in skeletal muscle (15, 31, 34). Both before and after the dietary intervention, 30 min of moderate-intensity exercise significantly increased activity of AMPKα2 (Fig. 2A), the predominant catalytic isoform expressed in human skeletal muscle, but had no effect on AMPKα1 activity (Fig. 2B). The 2-wk diet intervention had no effect on AMPK activity in the basal state or after exercise stimulation (Fig. 2, A and B). Consistent with these data, AMPK Thr172 phosphorylation (Fig. 2C) and phosphorylation of the AMPK substrate ACC (Fig. 2D) were increased with exercise, with no effect of the dietary intervention. Exercise did not lead to detectable increases in Akt Ser737 and Akt Thr591 phosphorylation before or after the dietary intervention (Supplemental Fig. S2). Thus, 30 min of moderate-intensity exercise in obese individuals increases AMPKα2 activity but has no effect on Akt or AMPKα1 activity. Skeletal muscle protein expression of AMPK (Fig. 2E), ACC (Fig. 2F), Akt, and the AMPK upstream kinase LKB1 (Fig. 2G) was not altered following the 2 wk on the low-carbohydrate diet.

Using phosphoproteomics and immunoblotting, we determined previously that Ser231, Ser660, and Ser700 on TBC1D1 are phosphorylated in mouse skeletal muscle after contraction and during stimulation with the AMPK activator 5-aminooimidazole-4-carboxamide-1-B-D-ribonanoside (AICAR) (31, 36). We also identified Thr590 as an Akt site that is phosphorylated with insulin stimulation (31, 36). Using site-specific antibodies that recognize TBC1D1 phosphorylation on these residues, we determined regulation of TBC1D1 phosphorylation in human skeletal muscle. Exercise significantly increased Ser231 and Ser660 phosphorylation, whereas there was only a slight tendency for exercise to increase TBC1D1 Ser700 phosphorylation (Fig. 3, A–C). The insulin-responsive TBC1D1
Thr591 site (Fig. 3D) was not affected by the acute bout of exercise, consistent with the absence of Akt phosphorylation. Diet intervention reduced the basal level of Ser660 phosphorylation but did not modify the response to exercise. There was no effect of the low-carbohydrate diet intervention on any of the other TBC1D1 phosphorylation sites under either the basal or exercise condition (Fig. 3, A, C, and D).

We recently identified AS160 Ser711 as an AMPK consensus sequence in mouse skeletal muscle (35). In the current study, 30 min of exercise robustly increased AS160 Ser711 phosphorylation in human skeletal muscle (Fig. 3E). Consistent with the absence of Akt phosphorylation, exercise did not increase AS160 phospho-Akt substrate (PAS) phosphorylation (Fig. 3F). There was no effect of the low-carbohydrate diet intervention on AS160 Ser711 or PAS phosphorylation (Fig. 3, E and F).

*Muscle glycogen and GLUT4 concentrations.* Muscle glycogen concentration is a major regulator of skeletal muscle glucose transport (10, 13). The 2-wk diet intervention, which was composed of low carbohydrate consumption, significantly reduced muscle glycogen concentrations (Fig. 4A). The single bout of acute exercise significantly decreased muscle glycogen concentrations both before and after the diet intervention (Fig. 4A). GLUT4 expression was not altered by the dietary intervention (Fig. 4B).

**DISCUSSION**

Understanding the mechanisms by which exercise and dietary interventions stimulate glucose transport could lead to novel treatments for metabolic diseases such as type 2 diabetes. Here, we studied a group of obese subjects at a high risk for type 2 diabetes (21) and examined the regulation of the AS160 and TBC1D1, Rab-GAP proteins known to be critical for glucose transport in skeletal muscle. We demonstrate that human muscle expresses multiple splice variants of TBC1D1 and that 30 min of moderate-intensity exercise in obese subjects increases site-specific TBC1D1 and AS160 phosphorylation. However, a diet intervention that resulted in significant weight loss and glycogen depletion did not alter basal or exercise-stimulated TBC1D1 and AS160 phosphorylation in muscle.

Most of our understanding of TBC1D1 phosphorylation comes from rodent models, where it has been shown that TBC1D1 is a substrate for multiple kinases (4, 22, 31, 36). In the current study, we found that exercise in human skeletal muscle robustly increased TBC1D1 Ser231 and Ser660 phosphorylation and that this increase mirrored the increase in AMPKα2 activity. Unlike Ser231 and Ser660, there was only a tendency to increase TBC1D1 Ser700 phosphorylation in response to 30 min of exercise. In mouse skeletal muscle, phosphorylation on the TBC1D1 Ser700 site during contractions is modest (36), whereas stimulation with the AMPK activator AICAR leads to potent Ser700 phosphorylation (36). The lower Ser700 phosphorylation compared with Ser231 and Ser660 in response to contraction and exercise could be due to numerous factors, including activation of specific phosphatases or a higher level of Ser700 phosphorylation in the basal state. In mouse skeletal muscle, mutation of multiple phosphorylation sites impairs contraction-stimulated glucose transport, but there is no effect of single site mutation (3, 36). This indicates that TBC1D1 is regulated by a combination of phosphorylation events rather than by phosphorylation on a single site. Given the pattern of TBC1D1 phosphorylation observed in the current study, it is likely...
that glucose transport in human skeletal muscle also entails phosphorylation on multiple TBC1D1 sites.

Our results demonstrate that human skeletal muscles express three different splice variants of TBC1D1. The long form of TBC1D1 expresses all three AMPK consensus sites (Ser231, Ser660, Ser700), whereas the medium form contains the Ser231 and Ser700 sites, and the short form contains only Ser231. Therefore, the three splice variants may react differently to exercise. Our Western blot analysis indicates that the long form is the predominant splice variant expressed in the obese subjects recruited to this study. However, it is possible that alternative expression patterns may exist in other populations. Mutations in TBC1D1 are associated with an increased risk of obesity (19, 30), and in future studies it will be important to investigate...
whether variations in expression of the splice forms are associated with an increased risk of developing obesity and diabetes.

The exercise protocol used in this study did not activate Akt, indicating that the site-specific TBC1D1 and AS160 phosphorylation were not mediated through this kinase. Previous data show that only prolonged exercise for 60 min or more increases Akt and AS160 PAS phosphorylation in human skeletal muscle (7, 32). This is in agreement with the lack of a role of Akt in exercise-stimulated glucose transport because, whereas Akt plays a key role in the insulin signal to glucose transport, studies in Akt2-knockout mice and using the phosphatidylinositol 3-kinase inhibitor wortmannin clearly demonstrate that contraction-induced glucose transport is independent of Akt.

Fig. 3. TBC1D1 phosphorylation at sites Ser^{231}, Ser^{660}, Ser^{700}, and Thr^{590}, phosphorylation of AS160 at Ser^{711}, and phospho-Akt substrate (PAS) phosphorylation were measured by Western blot. Open bars are before exercise and closed bars after 30 min of exercise on a cycle ergometer at 70% of VO_{2 max}.A: exercise increased phosphorylation of TBC1D1 at Ser^{231}, and there was no diet effect. B: exercise increased the phosphorylation of TBC1D1 at Ser^{660}. There was a main effect of diet intervention on the phosphorylation level of Ser^{660}. C and D: TBC1D1 phosphorylation at Ser^{700} and Thr^{590} was not changed after exercise or diet intervention. E: AS160 phosphorylation at Ser^{711} increased after exercise, and this exercise response was not affected by diet intervention. F: both AS160 and TBC1D1 contain phosphorylation sites that can be expected to be detected using the PAS antibody, but we did not see changes in the PAS signal after exercise or diet intervention. †P ≤ 0.01; *P ≤ 0.05.
activation (13). We have shown previously that insulin regulates TBC1D1 phosphorylation on Thr\(^{590}\) through Akt2 in mouse skeletal muscle (36). In the current study, we did not obtain muscle biopsies during insulin stimulation, and therefore, we cannot determine whether increased Akt2 phosphorylation is associated with increased TBC1D1 Thr\(^{590}\) phosphorylation in human skeletal muscle.

The subjects investigated in the current study were obese individuals with a BMI >30 kg/m\(^2\). Several groups have found AMPK activity to be normal in insulin-resistant individuals after exercise (12, 20), but a recent report has found exercise-induced AMPK activity to be reduced in obese people and type 2 diabetic patients (29). Lean subjects were not the focus of this study, and therefore, we cannot conclude whether the activation of AMPK and phosphorylation of TBC1D1 and AS160 were immediate adaptations to exercise. However, this is clearly an important topic for future investigations.

In conclusion, human skeletal muscle expresses three splice variants of TBC1D1, and the long form of the protein, which contains multiple regulated phosphorylation sites, is the predominant form expressed. These phosphorylation sites are known to regulate contraction-stimulated glucose transport in mouse muscle, and two of these sites, Ser\(^{231}\) and Ser\(^{660}\), are phosphorylated in response to exercise in obese humans. In addition, we show that exercise regulates AS160 phosphorylation of Ser\(^{111}\) in obese individuals but that the expression of TBC1D1 and AS160 is independent of dietary intervention and muscle glycogen levels. Thus, the regulation of TBC1D1 in human skeletal muscle may resemble what has been observed in mouse muscle, suggesting an important role of TBC1D1 in modulating glucose transport in human skeletal muscle.

**GRANTS**

This work was supported by funding from National Institute of Arthritis and Musculoskeletal and Skin Diseases Grants R01-AR-42238 and R01-AR-45670 (L. J. Goodyear), the Graetz Challenge Grant from the Joslin Diabetes Center (L. J. Goodyear), the American Diabetes Association Mentor-based Award (L. J. Goodyear), and Diabetes and Endocrinology Research Center Grant
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P30-DK-36836 (Joslin Diabetes Center). Additional funds to support this work were provided by the Danish Agency for Science Technology and Innovation to N. Jessen (grant no. 271-07-0719).

DISCLOSURES

No conflicts of interest, financial or otherwise, are disclosed by the authors.

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