The Rab-GTPase-activating protein TBC1D1 regulates skeletal muscle glucose metabolism

Ferenc Szekeres,1* Alexandra Chadt,2* Robby Z. Tom,1 Atul S. Deshmukh,1 Alexander V. Chibalin,1 Marie Björnholm,1 Hadi Al-Hasani,2 and Juleen R. Zierath1,3

1Department of Molecular Medicine and Surgery, Karolinska Institutet, Stockholm, Sweden; 2German Institute of Human Nutrition, Potsdam-Rehbruecke, Department of Pharmacology, Nutritional, Germany; and 3Department of Physiology and Pharmacology, Karolinska Institutet, Stockholm, Sweden.

Submitted 28 November 2011; accepted in final form 6 June 2012.

Szekeres F, Chadt A, Tom RZ, Deshmukh AS, Chibalin AV, Björnholm M, Al-Hasani H, Zierath JR. The Rab-GTPase-activating protein TBC1D1 regulates skeletal muscle glucose metabolism. Am J Physiol Endocrinol Metab 303: E524–E533, 2012. First published June 12, 2012; doi:10.1152/ajpendo.00605.2011.—The Rab-GTPase-activating protein TBC1D1 has emerged as a novel candidate involved in metabolic regulation. Our aim was to determine whether TBC1D1 is involved in insulin as well as energy-sensing signals controlling skeletal muscle metabolism. TBC1D1-deficient congenic Nob1.10SJL mice, and wild-type littersmates were studied. Glucose and insulin tolerance, glucose utilization, hepatic glucose production, and tissue-specific insulin-mediated glucose uptake were determined. The effect of insulin, AICAR, or contraction on glucose transport was studied in isolated skeletal muscle. Glucose and insulin tolerance tests were normal in TBC1D1-deficient Nob1.10SJL mice, yet the 4-h-fasted insulin concentration was increased. Insulin-stimulated peripheral glucose utilization during a euglycemic hyperinsulinemic clamp was similar between genotypes, whereas the suppression of hepatic glucose production was increased in TBC1D1-deficient mice. In isolated extensor digitorum longus (EDL) but not soleus muscle, glucose transport in response to insulin, AICAR, or contraction was impaired by TBC1D1 deficiency. The reduction in glucose transport in EDL muscle from TBC1D1-deficient Nob1.10SJL mice may be explained partly by a 50% reduction in GLUT4 protein, since proximal signaling at the level of Akt, AMPK, and acetyl-CoA carboxylase (ACC) was unaltered. Paradoxically, in vivo insulin-stimulated 2-deoxyglucose uptake was increased in EDL and tibialis anterior muscle from TBC1D1-deficient mice. In conclusion, TBC1D1 plays a role in regulation of glucose metabolism in skeletal muscle. Moreover, functional TBC1D1 is required for AICAR- or contraction-induced metabolic responses, implicating a role in energy-sensing signals. Insulin resistance; glucose transport; glucose transporter 4; adenosine 5′-monophosphate-activated protein kinase.

TBC1D1 is a member of the TBC1D Rab-GAP family of proteins involved in the regulation of glucose transport (25). Silencing of Tbc1d1 in L6 muscle cells increases basal and insulin-stimulated GLUT4 translocation (12), whereas ectopic expression of TBC1D1 in 3T3-L1 adipocytes inhibits this response (4, 25). TBC1D1 is expressed at higher levels in skeletal muscle compared with adipose tissue (3, 4, 30). We have reported a mutation in the Tbc1d1 gene in Swiss Jim Lambert (SJL) mice that results in a truncated protein lacking the TBC Rab-GTPase-activating protein domain when expressed in a cell-free system (3). In vivo, a truncated form of the protein is not detected. Recombinant congenic mice lacking TBC1D1 are protected against diet-induced obesity, as shown by reduced body weight, decreased respiratory quotient, and increased basal lipid oxidation in isolated skeletal muscle (3). Moreover, a genetic variation in TBC1D1 (R125W) is associated with obesity predisposition in humans (20, 29). Overexpression of the obesity-associated TBC1D1 R125W mutant in mouse skeletal muscle decreases insulin-stimulated glucose uptake (1). Collectively, these studies provide evidence for a critical role of TBC1D1 in skeletal muscle glucose and lipid metabolism and the regulation of whole body energy homeostasis.

TBC1D1 is the closest relative of TBC1D4 [Akt substrate of 160 kDa (AS160)], and therefore, these proteins share several structural key features (25). TBC1D1 and TBC1D4 are 79% identical in their Rab-GAP domain (25) and are active toward similar Rabs (21, 25). Nevertheless, TBC1D1 and TBC1D4 appear to be differently regulated by dedicated upstream kinases in response to diverse stimuli, including insulin and muscle contraction/AMP-activated protein kinase (AMPK) activation (1, 24, 25, 31). Insulin treatment and AMPK activation promote specific phosphorylation patterns of TBC1D1 (24, 25) and TBC1D4 (27, 31, 33), which might determine the Rab-GAP activity within these molecules. Several lines of evidence suggest that insulin (16, 25, 27), AMPK activation (4, 8, 24, 32), and exercise/muscle contraction (2, 6, 24) promote the phosphorylation and inactivation of TBC1D4 and TBC1D1, leading to increased GAP loading of Rab proteins on GLUT4 vesicles, and increase GLUT4 translocation to the plasma membrane. Thus, TBC1D1 and TBC1D4 have emerged as novel candidates linking pathways by which insulin and energy status signals regulate glucose transport. Nevertheless, the precise role of TBC1D1 and TBC1D4 in the regulation of metabolic events in skeletal muscle is still incompletely understood.

AMPK is an evolutionarily conserved cellular sensor of energy status. Upon activation, AMPK stimulates glucose uptake and lipid oxidation to produce energy while terminating other energy-consuming processes to restore energy balance (14, 18). AMPK is activated during muscle contraction (11, 13, 34), and this contributes partly to the regulation of glucose transport in response to exercise (22). AICAR (5-aminoimidazole-4-carboxamide-1-β-D-ribonucleoside), a pharmacological AMPK activator, increases glucose transport and lipid oxidation (19) in skeletal muscle. Although both muscle contraction and AICAR increase TBC1D1 phosphorylation (24), the requirement for TBC1D1 in energy-sensing signals controlling metabolic responses is unknown.

* These authors contributed equally to this work.
Given that TBC1D1 is an AMPK and Akt substrate (4, 7, 24, 25), we determined insulin- and AMPK-mediated metabolic responses in TBC1D1-deficient congenic B6.SJL-Nob1.10 (Nob1.10\textsuperscript{WT}) mice and wild-type (WT) littermates. We determined whether whole body and tissue-specific glucose homeostasis is dependent on TBC1D1. We hypothesized that signaling pathways emanating from AMPK involved in the regulation of glucose transport in skeletal muscle may require TBC1D1. Thus, we determined the direct role of TBC1D1 in isolated skeletal muscle on energy status signals controlling glucose metabolism. Our results provide evidence for a role of TBC1D1 in the regulation of AMPK-mediated responses controlling glucose metabolism in skeletal muscle.

**EXPERIMENTAL PROCEDURES**

**Experimental animals.** All experiments were approved by the Ethics Committee of the State Ministry of Agriculture, Nutrition, and Forestry (State of Brandenburg, Germany) and the Regional Animal Ethics Committee (Stockholm, Sweden). Mice were maintained on a 12:12-h light-dark cycle and had free access to standard chow and water. The recombinant congenic strain B6.SJL-Nob1.10 was generated by 12-fold backcross of SJL mice to the C57BL/6J strain, using marker selection for Nob1 QTTL and subsequent intercrosses, as described previously (3). Male 3- to 4-mo-old littermates homozygous for either the Nob1.10\textsuperscript{WT} (WT) or the Nob1.10\textsuperscript{H9262} allele were studied.

**Glucose tolerance test.** Mice (14 wk of age) were fasted for 4 h before the experiment. Each mouse received a single intraperitoneal injection of glucose (2 g/kg body wt). To minimize stress, mice were kept in an accustomed cage and had free access to water. Blood samples were obtained at 0, 15, 30, 60, and 120 min from the tail tip for glucose and insulin analysis. Blood glucose was measured using a glucose meter (OneTouch Ultra; Lifescan, Milpitas, CA). Plasma insulin levels were determined by an ELISA assay (insulin mouse ultrasensitive ELISA; Crystal Chem, Downers Grove, IL).

**Insulin tolerance test.** Nonfasted mice (14 wk old) were injected intraperitoneally with insulin (1 IU/kg body mass, Actrapid penfill; Novo Nordisk, Bagsværd, Denmark). Glucose concentration was determined during the insulin tolerance test using a glucose meter (Ascensia Elite; Bayer Health Care, Mishawaka, IN) in blood samples obtained from the tail tip at 0, 15, 30, and 60 min.

**Blood chemistry.** Blood from fed mice was collected for analysis of insulin, lepton, resistin, and plasminogen activator inhibitor-1 (PAI-1) using the Bio-Plex mice diabetes kit from Bio-Rad (Hercules, CA) according to the manufacturer’s instructions. Adiponectin was analyzed in serum from 4-h-fasted mice using an ELISA kit from Millipore (Billerica, MA).

**Euglycemic hyperinsulinemic clamp.** At least 4 days before the experiments, mice were anesthetized with isoflurane, and an indwelling catheter was inserted in the jugular vein and externalized through an incision in a skin flap behind the head. Before the surgery, the mice were subjected to MRI (EchoMRI; Echo Medical Systems, Houston, TX) to determine lean and fat mass. On the day of the clamp, mice were fasted for 5 h and placed in individual plastic containers for tail blood sampling. Glucose turnover rate was measured in the basal state and during euglycemic and hyperinsulinemic conditions, using a constant infusion of [3-\textsuperscript{3}H]glucose (2.5 μCi/ml) and a flow rate of 0.09 μCi/min as described (5). Basal glucose production and utilization was assessed for 65–75 min after the start of the tracer infusion. Blood samples (20 μl) were taken at 65, 70, and 75 min for the determination of the plasma [3-\textsuperscript{3}H]glucose and glucose concentration. Insulin concentration was measured in a sample taken at 75 min. At time 75 min, a euglycemic hyperinsulinemic clamp was started. A priming dose of insulin (12.5 mU/kg) was administered, followed by a constant infusion rate of 2.5 mU*kg\textsuperscript{-1}*min\textsuperscript{-1} (Actrapid; Novo Nordisk). The plasma glucose concentration was determined at 5-min intervals using an OneTouch Ultra glucose meter. Glucose (30%) was infused at a variable rate to maintain the plasma glucose concentration at the basal level. At steady state (~80 min after the start of the insulin infusion), blood samples were taken at 80, 85, and 90 min and processed as described for the baseline condition above to measure whole body glucose utilization and hepatic glucose production. Hepatic glucose production was determined by subtracting the average glucose infusion rate during the clamp condition from the whole body glucose uptake. Animals were euthanized by an overdose of pentobarbital sodium.

**Tissue collection.** To determine TBC1D1 and TBC1D4 protein level, mice were anesthetized (isoflurane), and skeletal muscle and adipose tissue ([Musculus tibialis anterior (TA), M. extensor digitorum longus (EDL), M. soleus, M. gastrocnemius, and brown and white adipose tissues]) were immediately dissected, freeze-clamped, and stored at −80°C.

**Muscle incubation.** Mice were fasted for 4 h prior to the study. EDL and soleus muscles were removed from anesthetized mice (Avertin, 99% 2,2,2-tribromo ethanol, and tertiary amyl alcohol at 15–17 μg body wt/μl) and incubated for 30 min at 30°C in vials containing preoxygenated (95% O\textsubscript{2}−5% CO\textsubscript{2}) Krebs-Henseleit buffer (KHB) containing 5 mM HEPES (prebuffer) and supplemented with 15 mM mannitol and 5 mM glucose. Muscles were transferred to new vials containing fresh pregressed KHB, as described above, to which either AICAR (2 mM; Toronto Research Chemicals, Toronto, ON, Canada), insulin (0.36 and 120 nM, Actrapid; Novo Nordisk), or insulin (120 nM) and AICAR (2 mM) were added. Muscles were subjected to additional incubation, as described below for glucose transport. The concentration of insulin and/or AICAR was maintained throughout all subsequent incubation steps. To assess the effects of contraction on glucose transport, isolated EDL and soleus muscles were incubated for 30 min in prebuffer. Thereafter, contraction was induced by electrical stimulation with 100 Hz (0.2-ms pulse duration, 20 V) at a rate of one 0.2-s contraction every 2 s for 10 min (26). Contralateral muscles were incubated for 10 min under resting conditions. Muscles were subjected to additional incubation, as described below for glucose transport.

**Glucose transport.** Skeletal muscle was transferred to new vials containing preoxygenized KHB supplemented with 20 mM mannitol and insulin (5 μg/mg muscle) for 10 min. Muscles were then transferred to new vials containing preoxygenized KHB supplemented with 1 mM 2-deoxy-[1,2,3\textsuperscript{3}H]glucose (2.5 μCi/ml) and 19 mM [1\textsuperscript{4}C]mannitol and incubated for 20 min. After the last incubation, muscles were washed in ice-cold KHB, blotted on filter paper, and quickly frozen with aluminum tongs precooled in liquid nitrogen and stored at −80°C. Glucose transport was determined as described (9). Results are expressed as nmol glucose-mg protein\textsuperscript{-1}•20 min\textsuperscript{-1}.

**Immunoblot analysis.** Tissues were homogenized in cold lysis buffer to extract total proteins [20 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 1 mM β-glycerophosphate, 2.5 mM sodium pyrophosphate, 1 mM NaF, and 1 mM Na\textsubscript{2}VO\textsubscript{4}, pH 7.4, with protease inhibitors (Roche Diagnostics, Mannheim, Germany)]. Homogenates were centrifuged at 10,000 g for 10 min at 4°C. Protein concentration was measured by the Bradford method. Total proteins (30–40 μg) were separated by SDS-PAGE on 10 or 4–10% separation gels and transferred to Hybond-P polyvinylidene difluoride membranes (GE Healthcare, Little Chalfont, UK). A polyclonal antiserum to the TGQPSAPGPRPMRKS-CONH\textsubscript{2} peptide against mouse TBC1D1 was raised in rabbits (Eurogentec, Seraing, Belgium). Affinity-purified antibodies to the peptide were used for immunoblot analysis of TBC1D1 at a dilution of 1:1,000 for 1 h in TBS with 5% (wt/vol) milk. Western blot analysis of AS160 (no. 07-741; Millipore), Akt (no. 9272; Cell Signaling Technology, Beverly, MA), phospho-Akt (Ser\textsuperscript{778}) (no. 9271; Cell Signaling Technology), phospho-AMPK (Thr\textsuperscript{172}) (no. 2531; Cell Signaling Technology), phospho-ACC (Ser\textsuperscript{79}) (no. 3661; Cell Signaling Technology), AMPK (no. 2532; Cell Signaling Technology), ACC (no. 4190; Cell

E525

Downloaded from http://ajpendo.physiology.org/ by 10.220.33.5 on June 24, 2017

AJP-Endocrinol Metab • doi:10.1152/ajpendo.00605.2011 • www.ajpendo.org

TBC1D1 REGULATES SKELETAL MUSCLE GLUCOSE METABOLISM
Signaling Technology), glucose transporter 4 (GLUT4) (no. CBL243; Millipore), insulin-responsive aminopeptidase (IRAP) (no. 3808; Cell Signaling Technology), vesicle-associated protein 2 (VAMP2) (no. PAI–766; Thermo Scientific, Rockford, IL), and myocyte enhancer factor 2C (MEF2C) (no. 9792; Cell Signaling Technology) was performed using commercially available antibodies. Hexokinase II antibody was a generous gift from Dr. Oluf Pedersen (Steno Memorial Hospital, Gentofte, Denmark). Membranes were subjected to immunoblot analysis with an antibody against glyceroldehyde phosphate dehydrogenase (GAPDH) (no. AM4300; Ambion, Grand Island, NY) to confirm equal protein loading. To visualize proteins, immunoblots were incubated with appropriate secondary antibodies, and specific protein bands were detected using ECL or ECL Plus Western Blot Reagents from GE Healthcare. Data are presented as arbitrary units (AU).

Quantitative real-time PCR. Total RNA was isolated from skeletal muscle and liver using a Trizol extraction (Invitrogen, Carlsbad, CA). The first-strand cDNA synthesis was prepared with 2.0 mg of total RNA, random hexamer primer, and Superscript III reverse transcriptase (Invitrogen). A quantitative real-time PCR analysis was performed with the Applied Biosystems 7500 FAST Real-time PCR System. The PCR mix (10 l) was composed of TaqMan Universal PCR Master Mix, NoAmp-EraseUNG, and cDNA amount corresponding to 10 ng RNA used for cDNA synthesis (each sample in a triplicate). Taqman assays from Applied Biosystems (Carlsbad, CA) were utilized to determine gene expression. b-Actin was utilized as a housekeeping gene.

Insulin-stimulated tissue-specific glucose uptake. Separate experiments were performed to assess tissue-specific insulin-stimulated glucose uptake, as described (5). Animals were studied essentially as described above for the euglycemic hyperinsulinemic clamp procedure; however, 2-deoxy-D-[1-14C]glucose (Amersham Bioscience Europe, Freiburg, Germany) was administered as a bolus (3 mg/kg + 30 min after the start of the clamp. Tail blood samples (20 l) were taken at 3, 6, 10, 15, 20, 30, 40, and 60 min after the 2-deoxy-D-[1-14C]glucose injection to measure blood 2-deoxy-D-[1-14C]glucose concentration (5). Insulin (10 mg/kg + 1 min) was infused during the 60-min clamp. Animals were euthanized after the study by an overdose of pentobarbital sodium, and tissues were removed. Tissue content of 2-deoxy-D-[1-14C]glucose and 2-deoxy-D-[1-14C]glucose 6-phosphate was determined as described (15). A portion of each tissue was digested for 60 min in 1 M NaOH at 60°C and then neutralized with 1 M HCl. 2-deoxy-D-[1-14C]glucose 6-phosphate and 2-deoxy-d-[1-14C]glucose were differentially precipitated by the use of a zinc hydroxide (0.3 M) precipitation or a perchloric acid solution (6%).

Capillarization. After dissection, muscles were rapidly frozen in liquid nitrogen and stored at −80°C until sectioning. Muscles were mounted on preholed cork plates with OCT (Tissues-Tek; Sakura Finetek). Forty-eight sections per mouse (12 mm) from soleus, EDL, and TA were fixed in acetone (4°C, 5 min) and air-dried prior to staining. An alkaline phosphatase method with indoxyl-tetrazolium and TA were fixed in acetone (4°C, 5 min) and air-dried prior to staining. An alkaline phosphatase method with indoxyl-tetrazolium and that TBC1D1 protein is undetectable in EDL (Fig 1A), whereas TBC1D4 protein abundance is notably lower in EDL, TA, and gastrocnemius muscles (Fig. 1A). Expression of TBC1D1 and TBC1D4 in TA muscle from the TBC1D1-deficient (Nob1.10SJL) and WT mice has been reported previously (3). Here, we report that TBC1D1 protein is undetectable in EDL (Fig 1B), soleus (Fig. 1C), and gastrocnemius tissue (data not shown) from Nob1.10SJL mice, whereas TBC1D4 protein abundance is similar between the genotypes.

RESULTS

TBC1D1 and TBC1D4 (AS160) protein abundance. Protein abundance of TBC1D1 and TBC1D4 was determined in white adipose tissue (WAT), brown adipose tissue (BAT), and different types of skeletal muscle (EDL, soleus, TA, and gastrocnemius) from C57BL/6J mice. TBC1D1 protein was readily detectable in all skeletal muscle types studied, with highest levels observed in TA and EDL muscle (Fig. 1A). Conversely, TBC1D1 protein was considerably lower in WAT or BAT. TBC1D4 protein was highly abundant in WAT, BAT, and soleus muscle, with notably lower levels in EDL, TA, and gastrocnemius muscles (Fig. 1A). Expression of TBC1D1 and TBC1D4 in TA muscle from the TBC1D1-deficient (Nob1.10SJL) and WT mice was reported previously (3). Here, we report that TBC1D1 protein is undetectable in EDL (Fig 1B), soleus (Fig. 1C), and gastrocnemius muscle (data not shown) from Nob1.10SJL mice, whereas TBC1D4 protein abundance is similar between the genotypes.

Fig. 1. TBC1D1 and TBC1D4 protein abundance. A: Western blot analysis of TBC1D1 and AS160 (TBC1D4) protein abundance in white adipose tissue (WAT), brown adipose tissue (BAT), and different skeletal muscle types from C57BL/6J mice. B and C: TBC1D1 and TBC1D4 abundance in extensor digitorum longus (EDL; B) and soleus (C) muscle from 16-wk-old male TBC1D1-deficient (Nob1.10SJL) and wild-type (WT) mice (n = 4). Note that the EDL muscle from the Nob1.10SJL mice is deficient for TBC1D1 protein and that TBC1D4 expression is unaltered between the genotypes. TA, tibialis anterior; gastroc, gastrocnemius.
Glucose homeostasis in TBC1D1-deficient mice. Glucose and insulin tolerance were determined in TBC1D1-deficient Nob1.10SJL mice and wild-type littermates. TBC1D1-deficient Nob1.10SJL mice displayed a normal glucose profile under the glucose and insulin tolerance tests (Fig. 2, A and C). An elevation in the fasting plasma insulin level was observed in the basal state in the TBC1D1-deficient mice (P < 0.05; Fig. 2B). However, the insulin concentration was similar in wild-type and TBC1D1-deficient mice 15 min after the glucose injection.

Euglycemic hyperinsulinemic clamp. Whole body insulin sensitivity was assessed in conscious mice by a euglycemic hyperinsulinemic clamp. Wild-type and TBC1D1-deficient mice were studied under similar glucose and insulin concentrations (Table 1). Body weight and the body composition of the TBC1D1-deficient mice were not altered (Table 1). Basal and insulin-mediated whole body peripheral glucose utilization were similar between TBC1D1-deficient and wild-type littermates (Fig. 2D). Conversely, hepatic insulin sensitivity was enhanced in TBC1D1-deficient mice (Fig. 2D). The insulin infusion suppressed hepatic glucose production 69% in wild-type mice, whereas glucose production was suppressed 94% in TBC1D1-deficient mice (P < 0.05; Fig. 2D). TBC1D1 protein levels are undetectable in the livers of Nob1.10SJL mice and their wild-type littermates (3). Nevertheless, we quantified the expression of genes important for hepatic glucose and lipid metabolism in the liver. The mRNA expression of acetyl-CoA carboxylase-1, sterol regulatory element-binding factor 1, glucokinase, pyruvate kinase, and fatty acid synthase were unaltered between wild-type and TBC1D1-deficient mice (Table 2). Phosphoenolpyruvate carboxykinase protein abundance was unaltered in liver from wild-type vs. TBC1D1-deficient mice (WT 2.3 ± 0.1 vs. Nob1.10SJL 2.1 ± 0.2 AU; n = 8). Akt (Ser^73) phosphorylation was assessed in liver harvested after the euglycemic hyperinsulinemic clamp was found to be similar.

Table 1. Clamp characteristics

<table>
<thead>
<tr>
<th></th>
<th>Glucose Turnover Rate</th>
<th>Tissue-Specific Glucose Uptake</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>WT</td>
<td>Nob1.10SJL</td>
</tr>
<tr>
<td>Body weight, g</td>
<td>28.3 ± 0.4</td>
<td>27.2 ± 0.8</td>
</tr>
<tr>
<td>Lean mass, g</td>
<td>23.4 ± 0.5</td>
<td>22.5 ± 0.7</td>
</tr>
<tr>
<td>Fat mass, g</td>
<td>2.4 ± 0.2</td>
<td>2.6 ± 0.1</td>
</tr>
<tr>
<td>Basal</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plasma glucose, mmol/l</td>
<td>10.7 ± 0.4</td>
<td>9.9 ± 0.5</td>
</tr>
<tr>
<td>Plasma insulin, pmol/l</td>
<td>99.6 ± 4.1</td>
<td>121.3 ± 8.3*</td>
</tr>
<tr>
<td>Clamp</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plasma glucose, mmol/l</td>
<td>9.1 ± 0.4</td>
<td>8.7 ± 0.5</td>
</tr>
<tr>
<td>Plasma insulin, pmol/l</td>
<td>432.4 ± 16.4</td>
<td>487.5 ± 27.1</td>
</tr>
</tbody>
</table>

Results are means ± SE (n = 7–9). Data from 13- to 16-wk-old 4-h-fasted male wild-type (WT) and TBC1D1-deficient (Nob1.10SJL) mice are reported. *P < 0.05 vs. WT.
between wild-type vs. TBC1D1-deficient mice (WT 3.5 ± 1.1 vs. Nob1.10^{8/L} 3.9 ± 1.2 AU; n = 8).

Insulin signaling and glucose uptake in skeletal muscle. We determined insulin-stimulated glucose uptake in isolated EDL and soleus muscle from TBC1D1-deficient and wild-type littermates using a submaximal (0.36 nM) and a maximal (120 nM) concentration of insulin. Insulin (120 nM) increased glucose transport 2.5-fold in EDL muscle (P < 0.001; Fig. 3A) and 1.6-fold in soleus muscle (P < 0.05; Fig. 3B) from wild-type mice. Moreover, the insulin-stimulated fold changes in glucose transport in EDL and soleus muscle were similar between TBC1D1-deficient and wild-type mice. However, TBC1D1-deficient mice displayed an ∼48% reduction in basal (P = 0.08) and insulin-stimulated (P < 0.05) glucose transport in EDL muscle (Fig. 3A). In contrast, rates of basal and insulin-stimulated glucose transport in soleus muscle were comparable between genotypes (Fig. 3B). To determine the mechanism for the reduced basal and insulin-stimulated glucose transport in skeletal muscle from TBC1D1-deficient mice, GLUT4 mRNA expression (Slc2a4) and protein abundance were determined. GLUT4 mRNA in soleus (Fig. 3C), EDL (Fig. 3C), and TA (data not shown) muscle was unaltered between wild-type and TBC1D1-deficient mice. MEF2C protein abundance was also unaltered in TBC1D1-deficient EDL muscle (WT 12.9 ± 0.7 vs. Nob1.10^{8/L} 12.2 ± 1.0 AU; n = 4). Nevertheless, GLUT4 protein abundance was reduced 51% in EDL muscle from TBC1D1-deficient vs. wild-type mice (P < 0.01; Fig. 3D). This reduction is consistent with our earlier finding in TA muscle from TBC1D1-deficient mice raised on a high-fat diet (3). However, in soleus (Fig. 3D) and gastrocnemius muscle (data not shown), GLUT4 levels were unaltered between the genotypes. Abundance of GLUT4-containing vesicle proteins, including IRAP in EDL muscle (WT 2.4 ± 0.4 vs. Nob1.10^{8/L} 2.6 ± 0.3 AU; n = 7) and VAMP2 in TA muscle (WT 7.0 ± 0.9 vs. Nob1.10^{8/L} 7.6 ± 1.1 AU; n = 7), was unaltered in TBC1D1-deficient mice. GLUT1 and hexokinase II protein abundance was similar in EDL muscle from wild-type and TBC1D1-deficient mice (data not shown). To determine whether alterations in insulin signaling account for the reductions in glucose transport, we determined phosphorylation of Akt (Ser^{73}). Basal and insulin-stimulated (120 nM) Akt (Ser^{73}) phosphorylation, as well as Akt protein abundance, was unaltered in EDL muscle from TBC1D1-deficient vs. wild-type mice (Fig. 3E). The glucose transport defect in EDL muscle from TBC1D1-deficient mice does not appear to arise from impaired insulin signaling since the fold insulin-stimulated increase in glucose transport and Akt phosphorylation was similar to wild-type mice.

Contraction-mediated signaling and glucose uptake in isolated skeletal muscle. The effects of in vitro muscle contraction on AMPK signaling and glucose transport were investigated in isolated EDL and soleus muscle. To determine whether energy-sensing signals are intact in TBC1D1-deficient mice, phosphorylation of AMPK and ACC was measured. Contraction increased phosphorylation of AMPK (Thr^{172}) and ACC (Ser^{79}) (P < 0.05) in EDL muscle independent of genotype (Fig. 4, A and B). Glucose transport in EDL muscle from wild-type mice was increased 2.7-fold in response to contraction (P < 0.05; Fig. 4C). In TBC1D1-deficient mice, contraction increased glucose transport 2.0-fold (P < 0.05) in EDL muscle, but the overall rate of glucose transport was reduced 44% compared with wild-type mice (P < 0.001; Fig. 4C). We limited our studies of energy-sensing pathways to EDL muscle, since contraction did not alter glucose transport in oxidative soleus muscle from either TBC1D1-deficient or wild-type mice (Fig. 4D).

AICAR-mediated signaling and glucose transport in isolated skeletal muscle. We next determined the effects of AICAR on AMPK signaling and glucose transport in isolated skeletal muscle. AICAR increased phosphorylation of AMPK (Thr^{172}) and ACC (Ser^{79}) similarly in EDL muscle from wild-type and TBC1D1-deficient mice (P < 0.05; Fig. 5, A and B). Glucose transport in EDL muscle from wild-type mice was increased 2.4-fold in response to AICAR (P < 0.05; Fig. 5C). EDL muscle from TBC1D1-deficient mice was unresponsive to AICAR (Fig. 5C). Moreover, the rate of glucose transport in AICAR-treated EDL muscle was 60% lower in TBC1D1-deficient vs. wild-type mice (P < 0.001; Fig. 5C). AICAR did not alter glucose transport in oxidative soleus muscle from either TBC1D1-deficient or wild-type mice (Fig. 5D). AICAR exposure did not have an additive effect on insulin-stimulated glucose transport in isolated EDL muscle (Fig. 5E) or soleus muscle (Fig. 5F) from TBC1D1-deficient mice.

Tissue-specific insulin-stimulated in vivo glucose uptake. To further explore differences in glucose handling between wild-type and TBC1D1-deficient mice, we determined tissue-specific insulin-stimulated glucose uptake by a euglycemic hyperinsulinemic clamp. Conscious mice were infused with 10 μU·kg^{-1}·min^{-1} insulin. Glucose and insulin concentrations were similar between wild-type and TBC1D1-deficient mice (Table 1). Insulin-stimulated glucose uptake in soleus, gastrocnemius, diaphragm, and heart muscle was similar between TBC1D1-deficient mice and wild-type mice (Fig. 6). Furthermore, insulin-stimulated glucose uptake was also unaltered in WAT and BAT from TBC1D1-deficient mice. However, insulin-stimulated glucose uptake was increased in glycolytic TA (70%, P < 0.01) and EDL (38%, P < 0.05) muscle from TBC1D1-deficient mice compared with wild-type mice. Phosphorylation of Akt at Ser^{473} in TA muscle harvested after the euglycemic hyperinsulinemic clamp was unaltered between the genotypes (WT 7.1 ± 1.8 vs. Nob1.10^{8/L} 5.8 ± 1.7 AU; n = 7).

Blood chemistry. Blood was collected from male wild-type and TBC1D1-deficient mice. Serum insulin, leptin, PAI-1, and adiponectin concentrations were unchanged in male TBC1D1-deficient mice (Table 3). Serum resistin levels were increased in TBC1D1-deficient mice (P < 0.001; Table 3).

Capillarization. We determined capillarization of soleus, EDL, and TA muscle from wild-type and TBC1D1-deficient mice.
mice by alkaline phosphatase staining. As expected, the number of capillaries per area (mm²) was higher in soleus compared with EDL and TA muscle (Table 4). The number of capillaries per muscle fiber was similar between wild-type and TBC1D1-deficient mice (Table 4). Furthermore, capillaries per area (mm²) were unaltered in soleus, EDL, and TA muscle from TBC1D1-deficient vs. wild-type mice (Table 4). Thus, alterations in capillarization are unlikely to account for the discrepancy between our findings of a normal rate of insulin-mediated whole body glucose utilization in vivo vs. a profound impairment in cellular glucose uptake in isolated EDL muscle in vitro in TBC1D1-deficient mice.

DISCUSSION

TBC1D1 is a paralog of TBC1D4, and it is recognized as a critical regulator of glucose transport in skeletal muscle (1, 12, 23). Clinical genetic studies provide evidence that TBC1D1 is
also a candidate for severe obesity in humans, since the R125W coding variant confirms risk for familial obesity (20, 29). In skeletal muscle, expression of the obesity-associated TBC1D1 R125W mutant decreases insulin- but not contraction-induced glucose transport (1). Thus, mutant forms of TBC1D1 may contribute to the development of obesity by modulating skeletal muscle insulin sensitivity. High-fat-fed TBC1D1-deficient Nob1.10SJL mice are protected against the development of obesity, due partly to increased whole body lipid oxidation, as evidenced by a lower respiratory quotient and increased skeletal muscle lipid oxidation compared with wild-type mice (3). However, insulin-stimulated glucose transport is impaired in skeletal muscle from high-fat-fed TBC1D1-deficient mice in vitro (3). Here, we report that even on a low-fat diet, TBC1D1-deficient mice display impaired insulin-stimulated glucose transport in glycolytic EDL muscle, which is accompanied by a 50% reduction in protein abundance of GLUT4.

The mechanism by which mutations in the TBC1D1 gene cause obesity is unknown. Our earlier finding of reduced body weight and protection against diet-induced obesity in homozygous carriers of the TBC1D1-deficient SJL allele (Nob1.10SJL) supports a role for TBC1D1 in energy homeostasis (3). TBC1D1 appears to play a metabolic role in skeletal muscle rather than adipose tissue since mRNA profiling studies indicate TBC1D1 is highly expressed in skeletal muscle and to a lower extent in brown and white adipose tissue (3). TBC1D1 is linked to the regulation of glucose transport in cell cultures (12, 23) and isolated skeletal muscle (1).

Insulin-induced TBC1D1 phosphorylation is involved in the regulation of cellular glucose uptake in 3T3-L1 adipocytes (25) and C2C12 myotubes (23). Our results are consistent with these earlier studies since insulin-stimulated glucose transport was impaired in isolated EDL muscle from TBC1D1-deficient mice. However, at the whole body level, glucose handling in response to an intraperitoneal administration of glucose or insulin was normal in TBC1D1-deficient mice. Moreover, peripheral glucose utilization during a euglycemic hyperinsulinemic clamp was unaltered between wild-type and TBC1D1-deficient mice. Interestingly, hepatic insulin sensitivity was enhanced in TBC1D1-deficient mice, indicating tissue-specific responses. We further investigated the tissue-specific role of TBC1D1 in glucose uptake in conscious mice. Adipose tissue-specific glucose uptake was similar between wild-type and TBC1D1-deficient mice. Moreover, insulin-stimulated glucose uptake in oxidative muscle was unaltered between wild-type and TBC1D1-deficient mice under in vivo and in vitro conditions. These findings in adipose tissue and oxidative muscle may be explained by the relatively low expression of TBC1D1 compared with glycolytic muscle. In glycolytic muscle, insulin-stimulated glucose uptake in TBC1D1-deficient mice was enhanced in vivo but profoundly impaired in vitro. The mechanism for the differential effects of TBC1D1 deficiency under in vivo vs. in vitro conditions remains unclear. One possibility is that a circulating factor may act on skeletal muscle in vivo and override the effect of the reduced GLUT4 protein on glucose uptake in the TBC1D1-deficient mice. In TBC1D1-deficient mice, the level of insulin was increased or unaltered in the basal state, whereas leptin, adiponectin, and PAI-1 were unaltered. However, resistin was increased in TBC1D1-deficient mice, despite a normal level of body fat. Thus, we were unable to correlate changes in circulating factors with insulin
sensitivity in this model. Another possibility is that an alteration in the supply of nutrients or hormones directly to skeletal muscle in TBC1D1-deficient mice may contribute to the paradoxical difference between in vivo and in vitro glucose uptake. However, capillarization of the soleus, EDL, and TA muscle was similar in wild-type and TBC1D1-deficient mice, indicating that microvascular changes do not alter peripheral glucose uptake in this model.

In mice, TBC1D1 is expressed at high levels in skeletal muscle composed predominantly of glycolytic fibers, such as the EDL, vs. oxidative fibers, such as the soleus (30). In TBC1D1-deficient mice, GLUT4 protein level was reduced 50% in glycolytic EDL muscle and was unchanged in oxidative soleus muscle. However, GLUT4 (Slc2a4) mRNA levels in glycolytic EDL muscle were similar between TBC1D1-deficient and wild-type mice, indicating a potential role for TBC1D1 in posttranscriptional modifications of GLUT4. Since TBC1D1 is a negative regulator of GLUT4 translocation (12, 23), deletion of TBC1D1 may promote an excessive rate of GLUT4 exocytosis and may consequently reduce GLUT4 protein as a compensatory mechanism. However, abundance of the GLUT4-containing vesicle proteins IRAP and VAMP2 was unaltered in skeletal muscle from TBC1D1-deficient mice, implicating an effect specific for GLUT4. Although TBC1D1 is linked to insulin-stimulated glucose uptake in L6 muscle cells (12) and 3T3-L1 adipocytes (25), overexpression of wild-type TBC1D1 in mouse skeletal muscle does not alter insulin-stimulated glucose uptake (1). Nevertheless, the reduction in insulin-stimulated glucose transport in glycolytic EDL muscle from TBC1D1-deficient mice may be attributed to the reduction in GLUT4 protein since in oxidative soleus muscle insulin-stimulated glucose transport, as well as GLUT4 protein, was similar between the genotypes. We also found that insulin-signaling defects are unlikely to account for the reduction in glucose transport in glycolytic EDL muscle from TBC1D1-deficient vs. wild-type mice since Akt phosphorylation was unaltered. Insulin-stimulated Akt phosphorylation was also unaltered in TA muscle harvested after the euglycemic hyperinsulinemic clamp, suggesting that enhanced insulin signaling does not improve in vivo glucose uptake.

**Fig. 5.** 5-Aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR)-stimulated signaling and glucose transport in skeletal muscle. Phosphorylation of AMPK (Thr172; A) and ACC (Ser79; B) was determined in basal and AICAR-stimulated EDL muscle from 16-wk-old Nob1.10[+/-] (open bars) and WT mice (filled bars). Representative immunoblots showing AMPK and ACC phosphorylation and protein expression are shown above respective graphs (n = 8). Glucose transport in isolated EDL (C) and soleus muscle (D) from Nob1.10[+/-] (open bars) and WT mice (filled bars) (n = 6–7). The combined effect of insulin- and AICAR-stimulated glucose transport (Ins + AIC) was determined in EDL (E) and soleus muscle (F) from Nob1.10[+/-] (open bars) and WT mice (filled bars) (n = 4–7). Data are means ± SE. *P < 0.05 and #P < 0.001 vs. WT mice.
uptake in TBC1D1-deficient mice. These findings are consistent with our earlier studies showing that insulin-stimulated glucose transport is reduced in high-fat-fed TBC1D1-deficient mice (3).

Our results provide evidence that TBC1D1-deficient mice are a partial phenocopy of heterozygous GLUT4-knockout mice (28). GLUT4 heterozygous mice (GLUT4+/−) display the full spectrum of the metabolic phenotype associated with insulin resistance and type 2 diabetes (28). Targeted disruption of GLUT4 in skeletal muscle reduces basal and insulin-stimulated glucose uptake and leads to whole body glucose intolerance (37). Muscle-specific heterozygous deletion of GLUT4 leads to a partial reduction in insulin-stimulated glucose transport in EDL and soleus muscle (37). Here, we report that TBC1D1-deficient mice have reduced insulin-stimulated glucose transport in EDL muscle, whereas insulin-stimulated glucose transport in soleus muscle was unaltered. Skeletal muscle-specific GLUT4+/− mice have a 40–50% reduction in GLUT4 protein in all muscle groups (37). Conversely, in TBC1D1-deficient mice, GLUT4 protein was reduced 50% only in glycolytic muscle. Skeletal muscle-specific GLUT4+/− mice are insulin intolerant (37), whereas TBC1D1-deficient mice have normal glucose and insulin tolerance, suggesting differences between these experimental models that may possibly be related to the fiber type-specific reductions in GLUT4 protein.

AMPK is an important mediator of contraction-induced glucose transport (10, 35). AMPK activation increases glucose uptake (19), concomitant with GLUT4 appearance at the plasma membrane (17). TBC1D1 is involved in the energy-sensing pathways involving AMPK that are linked to glucose uptake (4, 7, 24, 30). TBC1D1 is also phosphorylated by the pharmacological AMPK activator AICAR (24, 30). Our results provide evidence that TBC1D1-deficient mice display alterations in glucose metabolism. TBC1D1 deficiency is associated with a reduction in GLUT4 protein and in vitro glucose uptake in glycolytic but not oxidative skeletal muscle. Conversely, whole body glucose homeostasis is normal, or in glycolytic muscle even enhanced, in TBC1D1-deficient mice. Thus, functional TBC1D1 is required for insulin and energy-sensing signals controlling glucose metabolism in skeletal muscle.

ACKNOWLEDGMENTS

Present address of A. Chadt and H. Al-Hasani: German Diabetes Center, Düsseldorf, Germany.

GRANTS

This work was supported by grants from the European Research Council Ideas Program (ERC-2008-AdG23285 to J. R. Zierath), the Swedish Research Council, the Swedish Diabetes Association, the Novo Nordisk Foundation, the Foundation for Scientific Studies of Diabetology, the Strategic Research Foundation, the Wallenberg Foundation, the Strategic Research Program in Diabetes at Karolinska Institutet, the Commission of the European Communities EU FP6 (Contract Nos. LSHM-CT-2004-05272 EXGENESIS and LSHM-CT-2004-512013 EU-GENE2), and the Deutsche Forschungsgemeinschaft (GRK1208).

DISCLOSURES

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

AUTHOR CONTRIBUTIONS


Table 3. Blood chemistry in WT and Nob1.10SJL mice

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>Nob1.10SJL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Insulin pmol/l</td>
<td>158.4 ± 25.8</td>
<td>153.2 ± 56.8</td>
</tr>
<tr>
<td>Leptin ng/ml</td>
<td>1.0 ± 0.1</td>
<td>1.2 ± 0.1</td>
</tr>
<tr>
<td>Resistin ng/ml</td>
<td>194.5 ± 20.5</td>
<td>319.3 ± 16.3#</td>
</tr>
<tr>
<td>PAI-1, ng/ml</td>
<td>1.1 ± 0.1</td>
<td>0.9 ± 0.1</td>
</tr>
<tr>
<td>Adiponectin µg/ml</td>
<td>6.0 ± 0.8</td>
<td>6.9 ± 1.1</td>
</tr>
</tbody>
</table>

Results are means ± SE; n = 7–12. PAI-1, plasminogen activator inhibitor-1. Data from fed (insulin, leptin, resistin, and PAI-1) and 4-h-fasted (adiponectin) male WT and Nob1.10SJL mice are reported. #P < 0.001 vs. WT.

Table 4. Histological analysis of skeletal muscle capillarization

<table>
<thead>
<tr>
<th></th>
<th>Capillaries/Fiber</th>
<th>Capillaries/mm²</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>Nob1.10SJL</td>
<td>WT</td>
</tr>
<tr>
<td>Soleus</td>
<td>2.6 ± 0.0</td>
<td>2.5 ± 0.1</td>
</tr>
<tr>
<td>EDL</td>
<td>1.7 ± 0.1</td>
<td>1.7 ± 0.1</td>
</tr>
<tr>
<td>TA</td>
<td>3.1 ± 0.2</td>
<td>2.8 ± 0.2</td>
</tr>
</tbody>
</table>

Data are means ± SE; n = 4–7 mice. EDL, extensor digitorum longus; TA, tibialis anterior. Capillarization of soleus, EDL, and TA muscle from 12- to 15-wk-old male WT and Nob1.10SJL mice is reported.
REFERENCES