The Rab-GTPase Activating Protein TBC1D1 Regulates Skeletal Muscle Glucose Metabolism

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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 B6.SJL-Nob1.10 ($Nob1.10^{SJL}$) and wild-type littermates 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 test was normal in TBC1D1-deficient $Nob1.10^{SJL}$ mice, yet the 4-hr 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 $Nob1.10^{SJL}$ mice may be partly explained 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.

Keywords: Insulin resistance, glucose transport, GLUT4, AMPK
INTRODUCTION

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), while ectopic expression of TBC1D1 in 3T3-L1 adipocytes inhibits this response (4, 25). TBC1D1 is expressed at higher levels in skeletal muscle compared to 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 towards 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/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,
promote the phosphorylation and inactivation of TBC1D4 and TBC1D1, leading to increased GTP 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 partly contributes 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. While muscle contraction and AICAR both increase TBC1D1 phosphorylation (24), the requirement for TBC1D1 in energy-sensing signals controlling metabolic responses is unknown.

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 (Nob1.10SJL) mice and wild-type littermates (WT). 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 Ethical Committee (Stockholm, Sweden). Mice were maintained on a 12 hr light/dark cycle and had free access to standard chow and water. The recombinant congenic strain B6.SJL-\textit{Nob1.10} was generated by twelve-fold backcross of SJL mice to the C57BL/6J strain using marker selection for \textit{Nob1} QTL and subsequent intercrosses as previously described (3). Male 3-4 months old littermates homozygous for either the \textit{Nob1.10}^{B6} (WT) or the \textit{Nob1.10}^{SJL} allele were studied.

*Glucose tolerance test*- Mice (14 weeks of age) were fasted for 4 hrs before the experiment. Each mouse received a single intraperitoneal injection of glucose (2 g/kg body weight). 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 ultra-sensitive ELISA; Crystal Chem, Downers Grove, IL).

*Insulin tolerance test*- Non-fasted mice (14-weeks old) were intraperitoneally injected 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, leptin, resistin and plasminogen activator inhibitor (PAI) -1 using the Bio-Plex mouse diabetes kit from Bio-Rad (Hercules, CA), according to the manufacturer instructions. Adiponectin was analyzed in serum from 4 hr 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 hrs 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 [3-3H]glucose (2.5 μCi bolus 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 [3-3H]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 x kg⁻¹ x min⁻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 basal 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 sodium pentobarbital.

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 hrs prior to the study. EDL and soleus muscles were removed from anesthetized mice (Avertin, 2,2,2-tribromo ethanol 99% and tertiary amyl alcohol, at 15–17 µl/g body weight i.p) and incubated for 30 min at 30°C in vials containing pre-oxygenated (95% O₂/5% CO₂) Krebs-Henseleit-buffer (KHB) containing 5 mM Hepes (pre-buffer) and supplemented with 15 mM mannitol and 5 mM glucose. Muscles were transferred to new vials containing fresh KHB pre-gassed as described above to which either AICAR (2 mM; Toronto Research Chemicals Inc., Toronto, 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 pre-buffer. 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 condition. Muscles were subjected to additional incubation, as described below for glucose transport.

Glucose transport- Skeletal muscle was transferred to new vials containing pre-oxygenized KHB supplemented with 20 mM mannitol and incubated for 10 min. Muscles were then transferred to new vials containing pre-oxygenized KHB supplemented with 1 mM 2-deoxy-[^1,2,3]H]glucose (2.5 µCi/ml) and 19 mM[^14]C]mannitol and incubated for 20 min. After the last incubation, muscles were washed in ice-cold Krebs-Henseleit buffer, blotted on filter paper, and quickly frozen with aluminum tongs pre-cooled in liquid nitrogen and stored at -80°C.
Glucose transport was determined as described (9). Results are expressed as nmol glucose x mg protein$^{-1}$ x 20 min$^{-1}$.

**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-glycerol phosphate/2.5 mM sodium pyrophosphate/1 mM NaF/1 mM Na$_3$VO$_4$ pH 7.4 with protease inhibitors (Roche Diagnostics, Mannheim, Germany)). Homogenates were centrifuged at 10,000 x 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 (PVDF) membranes (GE Healthcare, Little Chalfont, UK). A polyclonal antiserum to the TGQPSAPGPRPMRKS-CONH$_2$ 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 hr in TBS with 5% (wt/vol) milk. Western blot analysis of AS160 (Millipore, #07-741), Akt (Cell Signaling Technology, Beverly, MA, #9272), phospho-Akt (Ser$^{473}$) (Cell Signaling, #9271), phospho-AMPK (Thr$^{172}$) (Cell Signaling, #2531), phospho-ACC (Ser$^{79}$) (Cell Signaling, #3661), AMPK (Cell Signaling, #2532), ACC (Cell Signaling, #4190), GLUT4 (Millipore, #CBL243), insulin-responsive aminopeptidase (IRAP) (Cell Signaling, #3808), vesicle-associated membrane protein 2 (VAMP2) (Thermo Scientific, Rockford, IL, #PA1-766) and myocyte enhancer factor 2C (MEF2C) (Cell Signaling, #9792) 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 glyceraldehyde phosphate dehydrogenase (GAPDH; Ambion, Grand Island, NY, #AM4300) 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 is 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 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 μl) was composed of TaqMan® Universal PCR Master Mix, NoAmpEraseUNG, 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. Beta-actin was utilized as a house keeping 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 GMBH, Freiburg, Germany) was administered as a bolus (3 μCi) ~60 min after the start of the clamp. Tail blood samples (20 μl) 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 mU x kg⁻¹ x min⁻¹) was infused during the 60 min clamp. Animals were euthanized at the end of the study by an overdose of sodium pentobarbital 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 pre-holed cork plates with OCT (Tissues-Tek, Sakura Finetek, NL). Forty-eight sections (12 µm) from soleus, EDL and TA per mouse were fixed in acetone (4°C; 5 min) and air-dried prior to staining. An alkaline phosphatase method with indoxyl-tetrazolium was used as described (36). Staining was carried out for 1 hr at 37-38°C, using incubation medium composed of nitroblue tetrazolium (Sigma, St. Louis, MO), 30 mg; 5-bromo 4-chloro 3-indolyl phosphate p-toluidine salt (Sigma), 6 mg in 30 ml of a buffer containing 6.9 mM MgSO₄ and 27.5 mM NaBO₂, adjusted to pH 9.2-9.4 with boric acid. A 5 min post-fixation in sucrose buffered formalin (4% formaldehyde, pH 7.3, 300 mOsmol) was performed after rinsing, and samples were then alcohol dehydrated and mounted. An Image-J program (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, MD, http://imagej.nih.gov/ij/, 1997-2011) was used for analysis. Background was subtracted, threshold was adjusted, and the image was converted to a binary form for watershed transformation. Individual fibers were manually counted.

Statistics- Data are reported as mean ± SEM. Differences were determined using a paired or unpaired t-test or ANOVA as appropriate. Bonferroni test was used for post hoc analysis. Significance was established at P<0.05.

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 (Figure 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 (Figure 1A). Expression of TBC1D1 and TBC1D4 in TA muscle from the TBC1D1-deficient (Nob1.10SJL) and wild-type (WT) mice have been previously reported (3). Here we report TBC1D1 protein is undetectable in EDL and soleus (Figure 1B and 1C) and gastrocnemius muscle (data not shown) from Nob1.10SJL mice, whereas TBC1D4 protein abundance is similar between the genotypes.

**Glucose homeostasis in TBC1D1-deficient mice** - Glucose and insulin tolerance was 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 (Figure 2A and 2C). An elevation in the fasting plasma insulin level was observed in the basal state in the TBC1D1-deficient mice (P<0.05, Figure 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 was similar between TBC1D1-deficient and wild-type littermates (Figure 2D). Conversely, hepatic insulin sensitivity was enhanced in TBC1D1-deficient mice (Figure 2D). The insulin infusion suppressed hepatic glucose production 69% in wild-type mice, while glucose production was suppressed 94% in TBC1D1-deficient mice (P<0.05, Figure 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 alpha (Acac), sterol regulatory
element binding factor 1 (Srebf1), glucokinase (Gck), pyruvate kinase (Pklr) and fatty acid synthase (Fasn) were unaltered between wild-type and TBC1D1-deficient mice (Table 2). PEPCK protein abundance was unaltered in liver from wild-type versus TBC1D1-deficient mice (*WT* 2.3 ± 0.1 versus *Nob1.10SJL* 2.1 ± 0.2 AU, *n*=8). Akt (Ser473) phosphorylation assessed in liver harvested after the euglycemic-hyperinsulinemic clamp was similar between wild-type versus TBC1D1-deficient mice (*WT* 3.5 ± 1.1 versus *Nob1.10SJL* 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, Figure 3A) and 1.6-fold in soleus muscle (*P*<0.05, Figure 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 approximately 48% reduction in basal (*P*=0.08) and insulin-stimulated (*P*<0.05) glucose transport in EDL muscle (Figure 3A). In contrast, rates of basal and insulin-stimulated glucose transport in soleus muscle were comparable between genotypes (Figure 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 was determined. GLUT4 mRNA in soleus (Figure 3C), EDL (Figure 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 versus *Nob1.10SJL* 12.2 ± 1.0 AU, *n*=4). Nevertheless, GLUT4 protein abundance was reduced 51% in EDL muscle from TBC1D1-deficient versus wild-type mice (*P*<0.01, Figure 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 (Figure 3D) and gastrocnemius (data not shown) muscle, GLUT4 levels were unaltered between the genotypes. Abundance of GLUT4-containing vesicle proteins, including IRAP in EDL muscle (WT 2.4 ± 0.4 versus Nob1.10SJL 2.6 ± 0.3 AU, n=7) and VAMP2 in TA muscle (WT 7.0 ± 0.9 versus Nob1.10SJL 7.6 ± 1.1 AU, n=7), were 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^{473}). Basal and insulin-stimulated (120 nM) Akt (Ser^{473}) phosphorylation, as well as Akt protein abundance, was unaltered in EDL muscle from TBC1D1-deficient versus wild-type mice (Figure 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 (Figure 4A and 4B). Glucose transport in EDL muscle from wild-type mice was increased 2.7-fold in response to contraction (P<0.05, Figure 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 to wild-type mice (P<0.001, Figure 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 (Figure 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, Figure 5A and 5B). Glucose transport in EDL muscle from wild-type mice was increased 2.4-fold in response to AICAR (P<0.05, Figure 5C). EDL muscle from TBC1D1-deficient mice was unresponsive to AICAR (Figure 5C). Moreover the rate of glucose transport in AICAR-treated EDL muscle was 60% lower in TBC1D1-deficient versus wild-type mice (P<0.001, Figure 5C). AICAR did not alter glucose transport in oxidative soleus muscle from either TBC1D1-deficient or wild-type mice (Figure 5D). AICAR exposure did not have an additive effect on insulin-stimulated glucose transport in isolated EDL muscle (Figure 5E) or soleus muscle (Figure 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 mU x kg$^{-1}$ x 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 (Figure 6). Furthermore, insulin-stimulated glucose uptake was also unaltered in white and brown adipose tissue 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 to 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 versus Nob1.10$^{SJL}$ 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 by alkaline phosphatase staining. As expected, the number of capillaries per area (mm$^2$) is higher in soleus compared to 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 (mm$^2$) were unaltered in soleus, EDL and TA muscle from TBC1D1-deficient versus wild-type mice (Table 4). Thus, alterations in capillarization is unlikely to account for the discrepancy between our findings of a normal rate of insulin-mediated whole body glucose utilization \textit{in vivo} versus a profound impairment in cellular glucose uptake in isolated EDL muscle \textit{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 \textit{Nob1.10SJL} mice are protected against the development of obesity, partly due to increased whole body lipid oxidation, as evidenced by a lower respiratory quotient and increased skeletal muscle lipid oxidation compared to wild-type mice (3). However, insulin-stimulated glucose transport
is impaired in skeletal muscle from high-fat-fed TBC1D1-deficient mice \textit{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 (\textit{Nob1.10SJL}) support 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 \textit{in vivo} and \textit{in vitro} conditions. These findings in adipose tissue and oxidative muscle may be
explained by the relatively low expression of TBC1D1, compared to 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 versus 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, level of insulin was increased or unaltered in the basal state, while leptin,
adiponectin and PAI-1 was 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 tibialis anterior 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 versus oxidative fibers such as the soleus (30). In
TBC1D1-deficient mice, GLUT4 protein level was reduced 50% in glycolytic EDL muscle
and unchanged in oxidative soleus muscle. However, GLUT4 (Slc2a4) mRNA levels in
glycolytic EDL muscle was 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, were 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 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 versus 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 enhanced insulin signaling does not improve in vivo glucose uptake in TBC1D1-deficient mice. These findings are consistent with our earlier studies showing 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 of 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<sup>+/−</sup> 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), as well as directly by purified AMPK (30). Muscle contraction increases the rate of glucose transport by an insulin-independent mechanism that partly involves AMPK, as well as AMPK-related kinases (10, 35). Similar to skeletal muscle-specific GLUT4<sup>+/−</sup> mice (37), contraction-induced glucose uptake is also impaired in EDL muscle from TBC1D1-deficient mice. AICAR-mediated glucose uptake was also impaired in EDL muscle from TBC1D1-deficient mice. We attribute the partial impairment in contraction- and AICAR-induced glucose transport in TBC1D1-deficient mice to both a reduction in GLUT4 protein abundance and impairments in TBC1D1 signaling towards glucose uptake. Since contraction- and AICAR-induced AMPK signaling to ACC was unaltered, our results are consistent with the notion that TBC1D1 is downstream of AMPK. Alternatively, a role for other contraction-induced kinases in the cross-talk between TBC1D1 and glucose uptake cannot be excluded.

In conclusion, TBC1D1-deficient mice display alterations in glucose metabolism. TBC1D1 deficiency is associated with 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.
ACKNOWLEDGEMENTS

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FIGURE LEGENDS

Figure 1. TBC1D1 and TBC1D4 protein abundance. Western blot analysis of TBC1D1 and AS160 (TBC1D4) protein abundance (A) in white (WAT), brown adipose tissue (BAT) and different skeletal muscle types from C57BL/6J mice. (EDL: Extensor digitorum longus, TA: Tibialis anterior, gastroc: gastrocnemius). TBC1D1 and TBC1D4 abundance in EDL (B) and soleus (C) muscle from 16-week-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.

Figure 2. Glucose utilization and insulin sensitivity in TBC1D1-deficient mice. An intraperitoneal glucose tolerance test was performed in 4-hour fasted 14-week-old TBC1D1-deficient (Nob1.10SJL) (open circles/bars) and wild-type (WT) (filled circles/bars) mice (n=6-9). Plasma glucose (A) and insulin (B) concentrations under the glucose tolerance test are shown. The glucose profile under an insulin tolerance test was determined in fed TBC1D1-deficient (Nob1.10SJL) (open circles) and wild-type (WT) (filled circles) mice (C) (n=6-9). Basal and insulin-stimulated (Rd) peripheral glucose utilization and hepatic glucose production (Ra) (D) was determined during a euglycemic-hyperinsulinemic clamp in 4-hour fasted conscious wild-type WT (filled bars) and TBC1D1-deficient Nob1.10SJL (open bars) mice (n=7-8). Results are mean ± SEM, *P<0.05 versus wild-type WT mice.

Figure 3. Insulin-stimulated signaling and glucose transport in skeletal muscle. Basal and insulin-stimulated (0.36 and 120 nM) glucose transport in isolated EDL (A) and soleus (B) muscle from TBC1D1-deficient Nob1.10SJL (open bars) and wild-type WT (filled bars) mice (n=4-5). (C) mRNA expression of Slc2a4. (n=9-11) (D) GLUT4 protein abundance in TBC1D1-deficient Nob1.10SJL mice (open bars) and WT wild-type mice (filled bars). Upper panel shows representative immunoblot for GLUT4 protein. (n=8). (E) Basal and insulin-stimulated (120 nM) Akt (Ser473) phosphorylation in EDL muscle from 16-week-old
TBC1D1-deficient \textit{Nob1.10}^{SJL} (open bars) and wild-type \textit{WT} (filled bars) mice was determined by Western blot analysis. Representative immunoblot of total Akt protein and Akt (Ser\textsuperscript{473}) phosphorylation are shown above Akt (Ser\textsuperscript{473}) phosphorylation graphs (\(n=4\)). Data are mean ± SEM, *P<0.05, †P<0.01, #P<0.001 versus \textit{WT} wild-type mice.

\textbf{Figure 4. Contraction-stimulated signaling and glucose transport in skeletal muscle.}

Rest and contraction-mediated phosphorylation of AMPK (Thr\textsuperscript{172}) (A) and ACC (Ser\textsuperscript{79}) (B) was determined by Western blot analysis in EDL muscle. Representative immunoblot of AMPK and ACC protein expression and AMPK (Thr\textsuperscript{172}) and ACC (Ser\textsuperscript{79}) phosphorylation are shown above respective graphs (\(n=6\)). Glucose transport in isolated EDL (C) and soleus (D) muscle from TBC1D1-deficient \textit{Nob1.10}^{SJL} (open bars) and wild-type \textit{WT} (filled bars) mice (\(n=6\)). Data are mean ± SEM, #P<0.001 versus \textit{WT} wild-type mice.

\textbf{Figure 5. AICAR-stimulated signaling and glucose transport in skeletal muscle.}

Phosphorylation of AMPK (Thr\textsuperscript{172}) (A) and ACC (Ser\textsuperscript{79}) (B) was determined in basal and AICAR-stimulated EDL muscle from 16-week old TBC1D1-deficient \textit{Nob1.10}^{SJL} (open bars) and wild-type \textit{WT} (filled bars) mice. 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 (D) muscle from TBC1D1-deficient \textit{Nob1.10}^{SJL} (open bars) and \textit{WT} wild-type (filled bars) mice (\(n=6-7\)). The combined effect of insulin- and AICAR-stimulated glucose transport (Ins+AIC) was determined in EDL (E) and soleus (F) muscle from TBC1D1-deficient \textit{Nob1.10}^{SJL} (open bars) and \textit{WT} wild-type (filled bars) mice (\(n=4-7\)). Data are mean ± SEM, *P<0.05, †P<0.01 versus \textit{WT} wild-type mice.

\textbf{Figure 6. Tissue-specific glucose uptake in conscious mice.} Insulin-stimulated tissue-specific glucose uptake was determined during a euglycemic-hyperinsulinemic clamp in 4-hour fasted wild-type \textit{WT} (filled bars) and TBC1D1-deficient \textit{Nob1.10}^{SJL} (open bars) mice (\(n=7\)). Results are mean ± SEM, *P<0.05, †P<0.01 versus wild-type mice.
Figure 1

A

C57BL6J

WT Nob1.10SJL

TBC1D1

TBC1D4

GAPDH

B

WT Nob1.10SJL

TBC1D1

TBC1D4

GAPDH

C

WT Nob1.10SJL

TBC1D1

TBC1D4

GAPDH
Figure 3

A

Glucose uptake (nmol x mg⁻¹ x 20 min⁻¹)

B

Glucose uptake (nmol x mg⁻¹ x 20 min⁻¹)

C

Slc2a4 mRNA (Fold over WT)

D

GLUT4 protein (AU)

E

pAkt (Ser⁴⁷³) (AU)

Insulin (nmol/l)

EDL  Soleus

Insulin (nmol/l)

EDL  Soleus

WT  Nob

Insulin (nmol/l)

WT  Nob

EDL

pAkt (Ser⁴⁷³) (AU)

Insulin (nmol/l)

WT  Nob

Insulin (nmol/l)

WT  Nob

Insulin (nmol/l)
**Figure 4**

(A) WT vs Nob1.10SJL

- **Contraction**
  - Rest: -
  - Contraction: +

- **Glucose uptake**
  - (nmol x mg⁻¹ x 20 min⁻¹)

- **EDL**
  - Rest: 1
  - Contraction: 5

- **Soleus**
  - Rest: 3
  - Contraction: 4

(B) WT vs Nob1.10SJL

- **Contraction**
  - Rest: -
  - Contraction: +

- **pACC (Ser⁷⁹)**
  - Rest: 1
  - Contraction: 5

- **pAMPK (Thr¹⁷²)**
  - Rest: 0.5
  - Contraction: 2.5

- **AMPK**
  - Rest: 1.5
  - Contraction: 3

(C) EDL vs Soleus

- **Glucose uptake**
  - (nmol x mg⁻¹ x 20 min⁻¹)

- **EDL**
  - Rest: 2
  - Contraction: 6
  - #

- **Soleus**
  - Rest: 3
  - Contraction: 4

(D) EDL vs Soleus

- **Glucose uptake**
  - (nmol x mg⁻¹ x 20 min⁻¹)

- **EDL**
  - Rest: 1
  - Contraction: 4

- **Soleus**
  - Rest: 3
  - Contraction: 5
Figure 5

A

WT Nob1.10SJL
AICAR - + - +

B

WT Nob1.10SJL
AICAR - + - +

pAMPK (Thr172)
AMPK
pACC (Ser79)
ACC

C

EDL
Glucose uptake (nmol x mg⁻¹ x 20 min⁻¹)

D

Soleus
Glucose uptake (nmol x mg⁻¹ x 20 min⁻¹)

E

EDL

F

Soleus

Glucose uptake (nmol x mg⁻¹ x 20 min⁻¹)
Figure 6

2-deoxyglucose uptake (ng glucose x mg tissue\(^{-1}\) x min\(^{-1}\))
Table 1 Clamp characteristics

<table>
<thead>
<tr>
<th></th>
<th>Glucose turnover rate</th>
<th>Tissue-specific glucose uptake</th>
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<tbody>
<tr>
<td></td>
<td>WT</td>
<td>Nob1.10^SJL</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>
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<tr>
<td>Fat mass (g)</td>
<td>2.4 ± 0.2</td>
<td>2.6 ± 0.1</td>
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**Basal**

<table>
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<th>Plasma glucose (mmol/l)</th>
<th>Plasma insulin (pmol/l)</th>
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<tbody>
<tr>
<td>Body weight (g)</td>
<td>10.7 ± 0.4</td>
<td>99.6 ± 4.1</td>
</tr>
<tr>
<td>Lean mass (g)</td>
<td>9.9 ± 0.5</td>
<td>121.3 ± 8.3^&lt;sup&gt;+&lt;/sup&gt;</td>
</tr>
<tr>
<td>Fat mass (g)</td>
<td>10.0 ± 0.9</td>
<td>2714.9 ± 281.8</td>
</tr>
<tr>
<td></td>
<td>9.7 ± 0.5</td>
<td>103.8 ± 17.3</td>
</tr>
</tbody>
</table>

**Clamp**

<table>
<thead>
<tr>
<th></th>
<th>Plasma glucose (mmol/l)</th>
<th>Plasma insulin (pmol/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (g)</td>
<td>9.1 ± 0.4</td>
<td>432.4 ± 16.4</td>
</tr>
<tr>
<td>Lean mass (g)</td>
<td>8.7 ± 0.5</td>
<td>487.5 ± 27.1</td>
</tr>
<tr>
<td>Fat mass (g)</td>
<td>9.5 ± 0.7</td>
<td>2714.9 ± 281.8</td>
</tr>
<tr>
<td></td>
<td>9.1 ± 0.6</td>
<td>3191.0 ± 282.7</td>
</tr>
</tbody>
</table>

Data from 13-16 week old 4-hour fasted male wild-type (WT) and TBC1D1-deficient (Nob1.10^SJL) mice is reported. Results are mean ± SEM. (n=7-9). *P<0.05 versus WT.
Gene expression in liver from 16 week old wild-type (WT) and TBC1D1-deficient (Nob1.10SJL) mice is reported. Data is expressed as fold over WT. Mean ± SEM for n=14 mice per group.

<table>
<thead>
<tr>
<th>Gene</th>
<th>WT</th>
<th>Nob1.10SJL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acac</td>
<td>1.00 ± 0.08</td>
<td>0.92 ± 0.06</td>
</tr>
<tr>
<td>Srebfl</td>
<td>1.00 ± 0.10</td>
<td>0.79 ± 0.08</td>
</tr>
<tr>
<td>Gck</td>
<td>1.00 ± 0.12</td>
<td>0.76 ± 0.08</td>
</tr>
<tr>
<td>Pklr</td>
<td>1.00 ± 0.11</td>
<td>0.84 ± 0.10</td>
</tr>
<tr>
<td>Fasn</td>
<td>1.00 ± 0.14</td>
<td>0.76 ± 0.09</td>
</tr>
</tbody>
</table>
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>

Data from fed (insulin, leptin, resistin and PAI-1) and 4-hour fasted (adiponectin) male wild-type (WT) and TBC1D1-deficient (Nob1.10SJL) mice is reported. Results are mean ± SEM. (n=7-12). #P<0.001 versus WT.
Table 4 Histological analysis of skeletal muscle capillarization

<table>
<thead>
<tr>
<th></th>
<th>Capillaries/fiber</th>
<th>Capillaries/mm²</th>
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<tbody>
<tr>
<td></td>
<td>WT</td>
<td>Nob1.10&lt;sup&gt;SJL&lt;/sup&gt;</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>

Capillarization of soleus, EDL (extensor digitorum longus) and TA (tibialis anterior) muscle from 12-15 week old male wild-type (WT) and TBC1D1-deficient (Nob1.10<sup>SJL</sup>) mice is reported. Data are mean ± SEM for n=4-7 mice.