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Suppression of 5′-Nucleotidase Enzymes Promotes AMP-activated Protein Kinase (AMPK) Phosphorylation and Metabolism in Human and Mouse Skeletal Muscle*

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Sameer S. Kulkarni, Håkan K. R. Karlsson, Ferenc Szekeres, Alexander V. Chibalin, Anna Krook, and Juleen R. Zierath[1]

From the Departments of Molecular Medicine and Surgery and Physiology and Pharmacology, Karolinska Institutet, S-171 77 Stockholm, Sweden

Background: The 5′-nucleotidase (NT5) family of enzymes dephosphorylates non-cyclic nucleoside monophosphates to produce nucleosides and inorganic phosphates.
Results: NT5 silencing increases the intracellular availability of AMP/ATP and invokes AMP-activated protein kinase (AMPK) activation, glucose uptake, and lipid oxidation.
Conclusion: NT5C enzymes inhibit basal lipid oxidation and glucose transport in skeletal muscle.
Significance: Suppression of cytosolic NT5C expression or activity may bypass metabolic inflexibility in type 2 diabetes.

The 5′-nucleotidase (NT5) family of enzyme dephosphorylates non-cyclic nucleoside monophosphates to produce nucleosides and inorganic phosphates. We hypothesized that gene silencing of NT5 enzymes to increase the intracellular availability of AMP would increase AMP-activated protein kinase (AMPK) activity and metabolism. We determined the role of cytosolic NT5 in metabolic responses linked to the development of insulin resistance in obesity and type 2 diabetes. Using siRNA to silence NT5C2 expression in cultured human myotubes, we observed a 2-fold increase in the AMP/ATP ratio, a 2.4-fold increase in AMPK phosphorylation (Thr^{172}), and a 2.8-fold increase in acetyl-CoA carboxylase phosphorylation (Ser^{79}) ($p < 0.05$). siRNA silencing of NT5C2 expression increased palmitate oxidation by 2-fold in the absence and by 8-fold in the presence of 5-aminoimidazole-4-carboxamide 1-β-D-ribofuranoside. This was paralleled by an increase in glucose transport and a decrease in glucose oxidation, incorporation into glycogen, and lactate release from NT5C2-depleted myotubes. Gene silencing of NT5C1A by shRNA injection and electroporation in mouse tibialis anterior muscle reduced protein content (60%; $p < 0.05$) and increased phosphorylation of AMPK (60%; $p < 0.05$) and acetyl-CoA carboxylase (50%; $p < 0.05$) and glucose uptake (20%; $p < 0.05$). Endogenous expression of NT5C enzymes inhibited basal lipid oxidation and glucose transport in skeletal muscle. Reduction of 5′-nucleotidase expression or activity may promote metabolic flexibility in type 2 diabetes.

Type 2 diabetes is associated with metabolic inflexibility, as evidenced by impaired switching between lipid and glucose oxidation in response to feeding and insulin stimulation in skeletal muscle (1–3). Therapeutic strategies to enhance whole body lipid or glucose metabolism may improve insulin sensitivity and energy homeostasis in type 2 diabetic patients. AMP-activated protein kinase (AMPK)[2] is a metabolic stress-sensing protein kinase that has emerged as a potential drug target for diabetes because it plays a role in coordinating lipid and glucose metabolism and energy balance (4, 5). Metabolic stressors, such as glucose deprivation, hypoxia, and muscle contraction, increase the intracellular concentration of AMP relative to ATP and activate AMPK (4). Activated AMPK switches on catabolic pathways that generate ATP, such as lipid oxidation, glucose transport, and glucose oxidation, and switches off anabolic pathways that consume ATP, such as synthesis of glycogen, fatty acids, and protein (5–7). Although ADP is the direct product of ATP hydrolysis during metabolic stress, it is rapidly converted into AMP via adenylate kinase. This increases the intracellular AMP level, even though free cytosolic AMP is buffered by protein binding and deamination to IMP. Thus, the AMP/ATP ratio is critical for maintaining cellular energy homeostasis. Pharmacological strategies to modify AMP or ATP levels may boost metabolism in type 2 diabetes.

One challenge with pharmacologically targeting AMPK for the treatment of metabolic disease is the broad family of AMPK heterotrimers. AMPK has two catalytic α-subunits, two regulatory β-subunits, and three regulatory γ-subunits, thereby allowing for a total of 12 different heterotrimeric complexes. Overexpression of an activated AMPKγ3 mutation in skeletal muscle prevents the development of dietary-induced insulin resistance concomitant with enhanced lipid oxidation (8). Treatment of animals with the adenosine analog 5-aminoimidazole-4-carboxamide 1-β-D-ribofuranoside (AICAR), which

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[1] To whom correspondence should be addressed: Section for Integrative Physiology, Karolinska Institutet, von Eulers väg 4, 4th Floor, S-171 77 Stockholm, Sweden. E-mail: juleen.zierath@ki.se.

[2] The abbreviations used are: AMPK, AMP-activated protein kinase; AICAR, 5-aminoimidazole-4-carboxamide 1-β-D-ribofuranoside; ACC, acetyl-CoA carboxylase.

can be transported into the cell and converted to 5-aminoimidazole-4-carboxamide-1-β-D-ribofuronosil 5′-monophosphate (AICAR monophosphate) to mimic the effect of AMP on AMPK, improves glucose tolerance and insulin sensitivity in diabetic animal models (9–12). Thus, several lines of evidence suggest that activation of the AMPK pathway may improve glucose and lipid metabolism in type 2 diabetes. Theoretically targeting other components of this pathway may also improve glucose and energy homeostasis in type 2 diabetes. One strategy to increase the availability of intracellular AMP relative to ATP and to activate the AMPK pathway further is via altering the expression of AMP-metabolizing enzymes, such as 5′-nucleotidases.

5′-Nucleotidases (NT5), also known as nucleoside monophosphate phosphohydrolases, are a family of enzymes that dephosphorylate non-cyclic nucleoside monophosphates to nucleoside and inorganic phosphate (13). This family of enzymes is critical for the physiological control of energy balance, metabolic regulation, and cell replication (14). Of the seven identified human 5′-nucleotidases, five (NT5C1A, NT5C1B, NT5C2, NT5C3, and NT5C3L) are localized in the cytoplasm, one (eN) is bound to the extracellular part of the plasma membrane, and one is present in the mitochondrial matrix (mdN) (15, 16). 5′-Nucleotidases have broad substrate specificity and are involved in maintaining cellular nucleotide and nucleoside levels by catalyzing the hydrolysis of either AMP to adenosine and phosphate or IMP to inosine and phosphate (15). Tissue expression profiling studies have revealed that NT5C1A and NT5C2 are expressed in skeletal muscle (17–19). NT5C1A has a higher affinity for AMP, whereas NT5C2 has a higher affinity for IMP, and both are relevant for the intercellular turnover of AMP in skeletal muscle (20). Thus, we hypothesized that gene silencing of these enzymes to increase the intracellular availability of AMP relative to ATP may trigger the activation of AMPK to increase glucose uptake and lipid oxidation.

EXPERIMENTAL PROCEDURES

Animal Housing and Care—Experiments were approved by the Regional Animal Ethical Committee (Stockholm, Sweden). Mice were maintained on a 12-h light/12-h dark cycle and received standard rodent chow. Male C57BL/6 mice (12–16 weeks old) were purchased from Charles River and housed for at least 1 week before use.

Primary Human Skeletal Muscle Cells—Skeletal muscle biopsies (*rectus abdominis*) were obtained during scheduled abdominal surgery with informed consent from the donors. The subjects were 61 ± 5 years (body mass index of 26 kg/m^2), with no known metabolic disorder. Satellite cells were isolated from the biopsies by trypsin and collagenase digestion, grown to myoblasts, and differentiated to myotubes (21, 22). DMEM, Ham's F-10 medium, fetal bovine serum, penicillin, streptomycin, and Fungizone were obtained from Invitrogen. Unless specified otherwise, all reagents were purchased from Sigma. Radioactive reagents were purchased from Amersham Biosciences.

siRNA Transfections in Myotubes—Myotubes cultured in 6-well plates were transfected using Lipofectamine 2000 reagent (Invitrogen). Differentiation medium was changed to antibiotic-free growth medium on day 2 of the myotube differentiation protocol. On days 0 and 2, myotubes were transfected with individual siRNAs (1 μg/ml) using Lipofectamine 2000 in serum-free DMEM. Pools of four siRNAs directed against human NT5C2 or a scrambled sequence were used. siRNA oligonucleotides for NT5C2 was purchased from Dharmacon (Chicago, IL). Myotubes were washed with PBS, and 2 ml of DMEM containing 2% fetal bovine serum was then added to each well. On day 6 of the differentiation protocol, the myotubes were serum-starved overnight and incubated in the absence (basal) or presence of 1 mM AICAR (Toronto Research Chemicals Inc., Ontario, Canada) before each assay.

mRNA Expression Analysis—Myotubes were washed three times with RNase-free PBS and harvested directly for RNA extraction (RNeasy minikit, Qiagen). The total RNA concentration was measured and reverse-transcribed with random hexamers using the Superscript first strand synthesis system (Invitrogen). Reactions were performed in duplicate in a 96-well format using a Prism 7000 sequence detector and TaqMan-based technology (Applied Biosystems). TaqMan probes were from Applied Biosystems. The relative quantities of target transcripts were calculated after data normalization using the standard curve method.

Western Blot Analysis—Cell lysates (40 μg of protein) were resuspended in Laemmli buffer. Proteins were separated by SDS-PAGE, transferred to PVDF membranes (Millipore), blocked with 7.5% nonfat milk, washed with TBS/Tween (10 mM Tris-HCl, 100 mM NaCl, and 0.02% Tween 20), and incubated overnight at 4 °C with primary antibodies. Antibodies against total AMPK and acetyl-CoA carboxylase (ACC) protein and phospho-specific antibodies against AMPK (Thr^{172}) and ACC (Ser^{79}) were diluted 1:1000 (Cell Signaling Technology). A mouse monoclonal antibody against NT5C2 (diluted 1:1000) was from Abnova (Taipei City, Taiwan). A rabbit polyclonal antibody against NT5C1A (diluted 1:500) was from Agrisera AB (Umeå, Sweden). Membranes were washed with TBS/Tween and incubated with the appropriate horseradish peroxidase-conjugated secondary antibodies (diluted 1:25,000). Proteins were visualized by enhanced chemiluminescence and quantified by densitometry.

5′-Nucleotidase Activity—5′-Nucleotidase activity was assayed as the release of [^{3}H]adenosine from [2-^{3}H]AMP or of [^{3}H] inosine from [8-^{3}H]IMP. An aliquot (20 μl) of homogenate was incubated for 15 min at 30 °C in a total volume of 50 μl containing 100 mM Tris-HCl, 2 mM $MgCl_2$, and 10 mM β-glycerophosphate/sodium salt with either [2-^{3}H]AMP or [8-^{3}H]IMP (200 μM AMP or IMP and 5 μCi of AMP or 1 mCi of IMP, respectively). Incubation was terminated by the addition of 10 μl of 150 mM $ZnSO_4$, followed by the addition of 10 μl of saturated $Ba(OH)_2$ to precipitate unhydrolyzed AMP. Samples were placed on ice for 10 min and subjected to centrifugation at 13,000 rpm for 15 min at 4 °C. The supernatant was collected for determination of radioactivity by scintillation counting (23).

Palmitate Oxidation—Lipid oxidation was assessed by exposing myotubes to [^{3}H]palmitic acid and measuring the tritiated water produced as described previously (24). Cells were washed once with PBS, exposed to 1 ml of DMEM (1 g of glu-

cose/liter) supplemented with 0.2% fatty acid-free BSA and 0.5 μCi of [9,10-^{3}H]palmitic acid, and incubated in the absence or presence of 1 mM AICAR for 4 h. To absorb non-metabolized palmitate, 0.2 ml of cell supernatant was mixed with 0.8 ml of charcoal slurry (0.1 g of charcoal powder in 1 ml of 0.02 M Tris-HCl at pH 7.5) and shaken for 30 min. Samples were subjected to centrifugation for 15 min at 13,000 rpm, 0.2 ml of supernatant with tritium-bound water was withdrawn, and radioactivity was measured by liquid scintillation counting.

Glucose Uptake—Myotubes were incubated in the absence or presence of insulin (120 nM) or AICAR (1 mM) for 1 h in glucose- and serum-free DMEM. Thereafter, myotubes were incubated in glucose-free DMEM containing 2-[1,2-^{3}H]deoxy-D-glucose (0.33 μCi/ml) and 10 μM unlabeled 2-deoxy-D-glucose (21, 22). Experiments were performed in triplicate, and results were normalized by protein concentration (BCA protein assay kit, Thermo Scientific, Rockford, IL).

Glucose Incorporation into Glycogen—Glycogen synthesis was determined by assessing the conversion of labeled glucose into glycogen (21, 22). Myotubes were incubated in DMEM containing 5 mM glucose and D-[U-^{14}C]glucose (1 μCi/ml; final specific activity of 0.18 μCi/μmol). Myotubes were incubated in the absence or presence of insulin (120 nM) for 90 min at 37 °C. Experiments were performed in triplicate.

Media Lactate Concentration—Myotubes were incubated overnight in the presence or absence of AICAR (1 mM) in serum-free DMEM. The medium (100 μl) was collected in duplicate, and lactate concentration was determined (25) using a commercially available kit (A-108, Biochemical Research Service Center, University of Buffalo, Buffalo, NY).

Glucose Oxidation—Myotubes were incubated for 4 h in 1 ml of serum-free DMEM supplemented with 0.1% fatty acid-free BSA, D-[U-^{14}C]glucose (1 μCi/ml; final specific activity of 0.18 μCi/μmol; Amersham Biosciences), 120 nM insulin, and 1 mM AICAR in the respective wells. Thereafter, 150 μl of SOLVABLE (aqueous-based tissue solubilizer, PerkinElmer Life Science) was dispensed into a center well positioned in each dish, and 150 μl of 35% perchloric acid was added to the medium. Myotubes were incubated for an additional 1 h, after which the center well was removed and subjected to scintillation analysis.

Gene Transfer by Electroporation in Intact Mouse Muscle—Adult C57BL/6 mice (20–25 g) were anesthetized with isoflurane. Hyaluronidase (30 μl of 1 unit/μl) was then injected in the tibialis anterior muscle through the skin, and mice were returned to separate cages for 2 h. Mice were anesthetized with isoflurane using a gas anesthesia machine (Harvard Scientific Instruments), and a pool of plasmids (30 μg) encoding either a scrambled sequence or shRNA against NT5C1A (SABiosciences, Frederick, MD) was injected in the tibialis anterior muscle of each leg through the skin. Electroporation was performed by delivering 220 V/cm in 8 pulses of 20 milliseconds using an ECM 830 electroporator (BTX Harvard Apparatus, Holliston, MA).

Measurement of Nucleotides—Differentiated skeletal muscle myotubes from 10-cm dishes were washed three times with ice-cold PBS and scraped in 300 μl of ice-cold 5% perchloric acid. Tibialis anterior muscle (~50 mg) was powdered in liquid nitrogen and then homogenized in 0.2 ml of 5% (by volume) perchloric acid. Samples were subjected to centrifugation at 14,000 rpm for 3 min at 4 °C to remove insoluble material. A solution containing an equal volume of 1,1-tri-*n*-octylamine and 1,1,2-trichlorotrifluoroethane (total volume of 220 μl) was added to the supernatant, and the sample was rigorously vortexed. After centrifugation, the upper aqueous layer was removed and extracted a second time with a further addition of 220 μl of the same organic solvent mixture as described above. An aliquot (20 μl) of the final aqueous phase was analyzed by capillary electrophoresis with on-column isotachophoretic pre-concentration using a leading buffer consisting of 50 mM sodium phosphate and 50 mM NaCl (pH 5.2) and a tailing buffer containing 100 mM MES/Tris (pH 5.2). To each buffer was added 0.2% hydroxyethylcellulose to decrease the electro-osmotic flow. Nucleotide peaks were detected by UV absorbance at 260 nm (reference of 400 nm) and integrated using System Gold software (Beckman). Nucleotide ratios were calculated from peak areas after correction for retention times (26).

Glucose Uptake in Intact Tibialis Anterior Muscle—Seven days after the electroporation procedure, mice were fasted for 4 h. Thereafter, a bolus of glucose (3 gm/kg) was administered by gavage, and [^{3}H]glucose (4.5 μl of 2-[^{3}H]deoxy-D-glucose/100 μl of saline/animal, 1 mCi/ml) was administered intraperitoneally. Mice were anesthetized 120 min after the glucose gavage, and the tibialis anterior muscle was dissected and directly frozen in liquid nitrogen for subsequent determination of [^{3}H]glucose accumulation. Frozen muscle samples were homogenized in ice-cold buffer (10% glycerol, 5 mM sodium pyrosulfate, 13.7 mM NaCl, 2.7 mM KCl, 1 mM $MgCl_2$, 20 mM Tris (pH 7.8), 1% Triton X-100, 10 mM NaF, 1 mM EDTA, 0.2 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 0.5 mM sodium vanadate, 1 mM benzamidine, and 1 μM microcystin) for 20 s using a motor-driven pestle. Homogenates were rotated end-over-end for 1 h at 4 °C and then subjected to centrifugation at 12,000 × *g* for 10 min at 4 °C. The supernatant (30 μl) was analyzed by liquid scintillation counting. A portion of the remaining supernatant was stored at −80 °C for immunoblot analysis.

Statistics—Differences between myotubes or skeletal muscle transfected with a scrambled sequence or siRNA/shRNA against specific NT5C enzymes were determined by Student's *t*-test. Results are presented as means ± S.E. Statistical significance was set at $p < 0.05$.

RESULTS

NT5C Silencing in Human Myotubes—NT5C1A mRNA was either low or undetectable, whereas NT5C2 mRNA was readily detectable in primary human myotubes. Given that NT5C2 was the only detectable enzyme of the two targets studied, gene silencing was directed against NT5C2. Gene silencing of NT5C2 reduced mRNA expression (Fig. 1*A*) and protein content (Fig. 1*B*) by 75 and 70%, respectively ($p < 0.05$), followed by a 3-fold increase in the ADP/ATP ratio and a 2-fold increase in the AMP/ATP ratio (Fig. 1, *D* and *E*). Growth and gross morphology as assessed by light microscopy of differentiated myotubes with depleted NT5C2 expression were similar to those in myotubes transfected with siRNA against a scrambled sequence (data not shown).

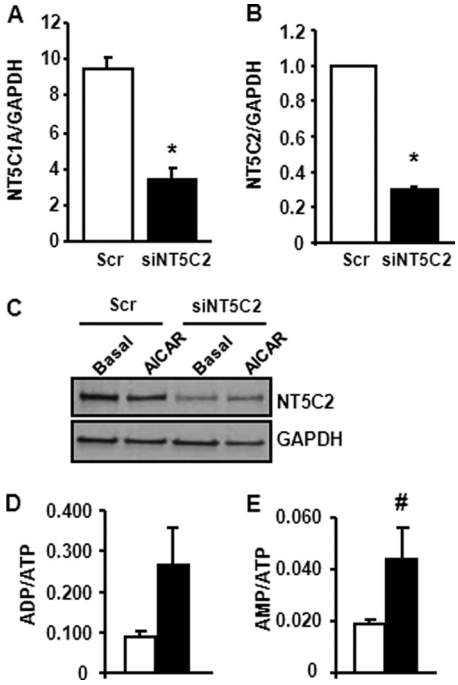

FIGURE 1. *A* and *B*, effect of siRNA-mediated silencing of NT5C2 on mRNA expression (*A*) and protein content (*B*) in primary human myotubes. *C*, representative immunoblot of NT5C2 protein content in myotubes transfected with siRNA against a scrambled sequence (*Scr*) or NT5C2 (*siNT5C2*) and incubated for 16 h in the absence (*Basal*) or presence of 1 mM AICAR. GAPDH protein was used as a loading control. *D* and *E*, ADP/ATP and AMP/ATP ratios, respectively. Results are means ± S.E. for n = five subjects. *, $p < 0.05$; #, $p = 0.08$ *versus* myotubes transfected with siRNA against a scrambled sequence for each condition.

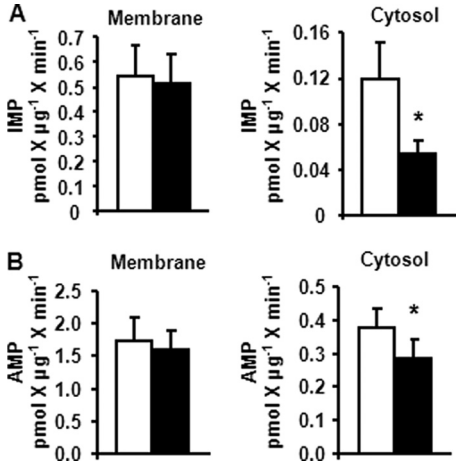

FIGURE 2. **Effect of siRNA-mediated silencing of NT5C2 on IMP-hydrolyzing (*A*) and AMP-hydrolyzing (*B*) activity.** Primary human myotubes were transfected with siRNA against a scrambled sequence (*white bars*) or NT5C2 (*black bars*). Membrane and cytosolic fractions were prepared. Results are means ± S.E. for n = six subjects. *, $p < 0.05$ *versus* myotubes transfected with siRNA against a scrambled sequence for each condition.

IMP and AMP Nucleotidase Activity—Myotubes were transfected with siRNA against a scrambled sequence or NT5C2. Cells were lysed, and cytosolic and total membrane fractions were prepared for measurement of IMP and AMP nucleotidase activity (Fig. 2). siRNA-mediated silencing of NT5C2 decreased IMP-hydrolyzing activity by 60% (Fig. 2*A*) and AMP-hydrolyzing activity by 25% (Fig. 2*B*) in the cytosolic fraction. Conversely, neither IMP- nor AMP-hydrolyzing activity in the membrane fraction was altered.

Effect of NT5C2 Silencing on AMPK and ACC Phosphorylation—Myotubes were incubated in the absence or presence of AICAR. NT5C2 silencing increased basal AMPK phosphorylation by 2.0-fold ($p < 0.05$) (Fig. 3*A*). AMPK phosphorylation was further increased ($p < 0.05$) in NT5C2-silenced myotubes upon AICAR exposure. NT5C2 silencing also increased basal ACC phosphorylation by 3.6-fold, with a further increase noted in the presence of AICAR (Fig. 3*B*).

Effect of NT5C2 Silencing on Palmitate Oxidation—Insulin suppressed (50%) and AICAR increased (8-fold) palmitate oxidation. NT5C2 silencing increased basal palmitate oxidation by 1.8-fold in primary human myotubes ($p < 0.05$) (Fig. 4). Although NT5C2 silencing did not modify palmitate oxidation under insulin-stimulated conditions, the response to AICAR was enhanced (1.5-fold; $p < 0.05$).

Effect of NT5C2 Silencing on Glucose Uptake and Metabolism—Insulin and AICAR increased glucose transport in primary myotubes (Fig. 5*A*). NT5C2 silencing increased glucose uptake under basal and insulin-stimulated conditions ($p < 0.05$). Conversely, AICAR-mediated glucose uptake was unaltered by NT5C2 silencing. Insulin, but not AICAR, increased glucose incorporation into glycogen in primary myotubes (Fig. 5*B*). NT5C2 silencing was without effect on basal, insulin-stimulated, or AICAR-stimulated glucose incorporation into glycogen. AICAR reduced lactate release from primary myotubes (Fig. 5*C*). NT5C2 silencing also reduced lactate release from primary myotubes by 22 and 40% under basal and AICAR-stimulated conditions ($p < 0.05$). Expression of lactate dehydrogenase was unaltered by NT5C2 silencing (data not shown). Insulin, but not AICAR, increased glucose oxidation in primary myotubes (Fig. 5*D*). Depletion of NT5C2 decreased glucose oxidation under basal and AICAR-stimulated conditions ($p < 0.05$) but not in the presence of insulin.

Effect of NT5C1 Silencing on AMPK and ACC Phosphorylation and Glucose Uptake in Intact Mouse Skeletal Muscle—Preliminary data suggested that NT5C1A mRNA expression is increased by 1.5-fold in vastus lateralis muscle from type 2 diabetic patients compared with people with normal glucose tolerance ($p < 0.05$). Thus, we determined the effect of NT5C1A silencing in adult mouse skeletal muscle. One week after electroporation, contralateral muscles transfected with either a scrambled sequence or shRNA against NT5C1A were removed, and the mRNA and protein content of NT5C1A was determined (Fig. 6*A*). Gene silencing reduced NT5C1A protein content by 60% ($p < 0.05$) (Fig. 6*B*) *versus* that in the sham-treated contralateral muscle. GAPDH protein was unaltered by gene silencing. The reduction in NT5C1A protein was accompanied by a 17% increase in the AMP/ATP ratio ($p = 0.05$) (Fig. 6*C*). Moreover, gene silencing of NT5C1A was associated with a 60% increase in AMPK phosphorylation ($p < 0.05$) (Fig. 6*D*) and a 50% increase in ACC phosphorylation ($p < 0.05$) (Fig. 6*E*), whereas AMPK and ACC protein content was unaltered. NT5C1A increased *in vivo* glucose uptake in intact tibialis anterior muscle by 20% ($p < 0.001$) (Fig. 6*F*).

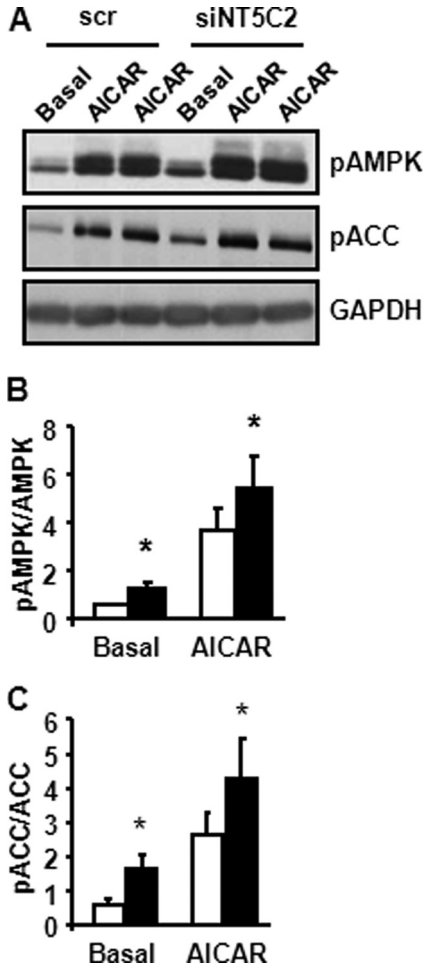

FIGURE 3. **Effect of siRNA-mediated silencing of NT5C2 on protein phosphorylation and total content of AMPK and ACC in primary human myotubes.** GAPDH protein content was determined and used as a loading control. Myotubes were transfected with siRNA against a scrambled sequence (*scr*; *white bars*) or NT5C2 (*siNT5C2*; *black bars*) and incubated for 16 h in the absence (*Basal*) or presence of 1 mM AICAR for protein phosphorylation analysis. Representative immunoblots are shown for AMPK and ACC (*A*). GAPDH protein content was determined and used as a loading control. Quantified data are shown for AMPK Thr^{172} (*B*) and ACC Ser^{79} (*C*). Results are means ± S.E. (arbitrary units) for protein phosphorylation/total protein content for $n = $ six subjects. *, $p < 0.05$ *versus* myotubes transfected with a scrambled sequence.

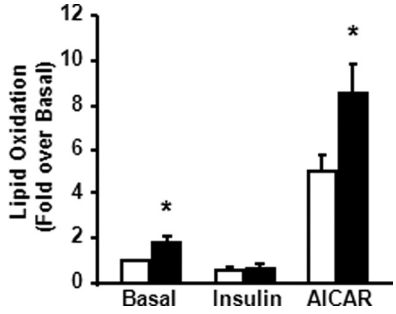

FIGURE 4. **Effect of siRNA-mediated silencing of NT5C2 on palmitate oxidation.** Primary human myotubes were transfected with siRNA against a scrambled sequence (*white bars*) or NT5C2 (*black bars*) and incubated for 4 h in the absence (*Basal*) or presence of either 120 nM insulin or 1 mM AICAR. Results are means ± S.E. for $n = $ six subjects. *, $p < 0.05$ myotubes transfected with a scrambled sequence for each condition.

DISCUSSION

Enzymes involved in cytosolic AMP catabolism, including the cytosolic 5′-nucleotidases NT5C1A and NT5C2, have been implicated in the balance of intracellular nucleotide pools (15). These enzymes are important for maintaining appropriate intracellular levels of AMP and ATP and therefore may indirectly modify AMPK activity. AMPK signaling is important for the regulation of cellular and whole body glucose and energy homeostasis (5–7). Because both glucose metabolism and lipid metabolism are impaired in type 2 diabetes patients, AMPK activation has been considered a potential target to reverse these metabolic abnormalities (4, 5, 27). AMPK switches on catabolic pathways that generate ATP while simultaneously switching off anabolic pathways that consume energy in the form of ATP (7). This study was designed to determine the role of the cytosolic 5′-nucleotidases in the metabolic responses linked to the development of insulin resistance in obesity and type 2 diabetes.

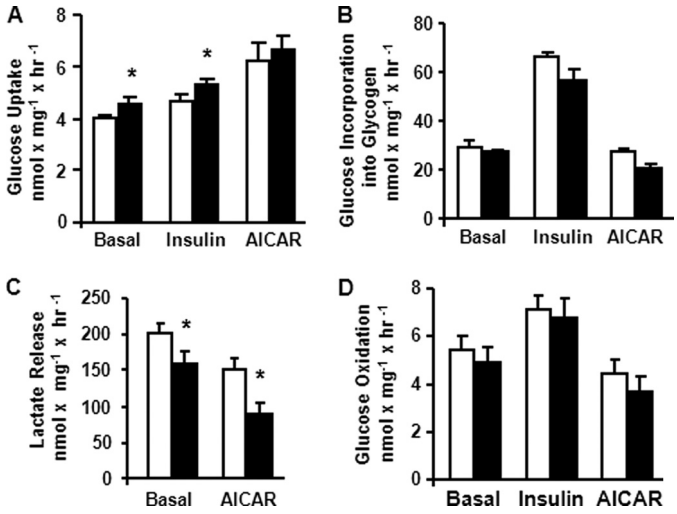

FIGURE 5. **Effect of siRNA-mediated silencing of NT5C2 on glucose uptake and metabolism.** Primary human myotubes were transfected with siRNA against a scrambled sequence (*white bars*) or NT5C2 (*black bars*) and incubated in the absence (*Basal*) or presence of 120 nM insulin or 1 mM AICAR for measurement of glucose uptake (*A*), glucose incorporation into glycogen (*B*), lactate release from the myotubes (*C*), and glucose oxidation (*D*). Results are means ± S.E. for $n = $ seven subjects. *, $p < 0.05$ myotubes transfected with a scrambled sequence.

We confirmed the role of cytosolic 5′-nucleotidases in the regulation of glucose and lipid metabolism in skeletal muscle using siRNA. Because NT5C1A is expressed at low levels in cultured human myotubes, we silenced NT5C2, an IMP-preferring enzyme that has overlapping specificity for AMP (18, 19). We hypothesized that siRNA against NT5C2 may increase the availability of intracellular AMP and IMP, which would alter the AMP/ATP ratio and increase AMPK activity. siRNA-mediated gene silencing markedly reduced NT5C2 mRNA and protein expression. Although an increase in cellular IMP cannot directly contribute to AMPK activation, IMP is metabolized and converted into adenylosuccinate by adenylosuccinate synthetase and is then converted into AMP by adenylosuccinate lyase (28), which may subsequently increase the AMP/ATP ratio and increase AMPK activity. Eukaryotic cells have an active adenylate kinase, which interconverts ATP, ADP, and AMP and maintains this reaction close to equilibrium (29). At equilibrium, the ADP/ATP ratio will vary as the square root of the AMP/ATP ratio. Our experimentally deter-

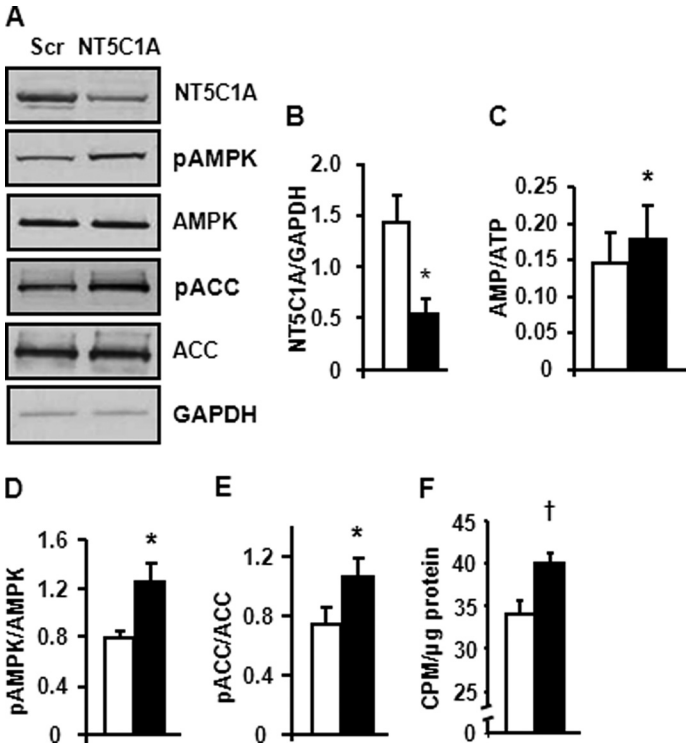

FIGURE 6. **shRNA-mediated gene silencing of NT5C1A in mouse tibialis anterior muscle.** Shown is the NT5C1A protein content, as well as protein phosphorylation and total content of AMPK and ACC after transfection with a pool of plasmids encoding a scrambled sequence (*Scr*; *open bars*) or shRNA against NT5C1A (*black bars*). *A*, representative immunoblots for phosphorylated (*p*) and total AMPK and ACC content. *B*, *D*, and *E*, quantified data for the immunoblot analysis for NT5C1A protein content (*B*) and phosphorylation of AMPK Thr^{172} (*D*) and ACC Ser^{79} (*E*). *C*, AMP/ATP ratio. *F*, *in vivo* glucose uptake. Results are means ± S.E. for n = seven mice. *, $p < 0.05$; †, $p < 0.001$ *versus* the scrambled sequence.

mined AMP/ATP and ADP/ATP ratios (Fig. 1, *D* and *E*) are close to the theoretically predicted values. Interestingly, like AMP, ADP can also bind to the AMPK γ-subunit, invoking AMP-induced phosphorylation of AMPK (30). NT5C2 silencing decreased NT5 activity in the cytoplasmic fraction concomitant with an increase in the AMP/ATP and ADP/ATP ratios. NT5C2 silencing increased AMPK (Thr^{172}) and ACC (Ser^{79}) phosphorylation in primary human myotubes. This response was increased in an additive manner by AICAR, a compound that mimics the effects of AMP on AMPK without affecting ATP or ADP levels (31, 32).

NT5C2 depletion increased palmitate oxidation in primary human myotubes, and this effect was further increased in the presence of AICAR. AMPK activation by AICAR phosphorylates and inhibits ACC at Ser^{79}, leading to a decrease in malonyl-CoA, which releases the inhibitory loop on CPT1 and stimulates β-oxidation of long chain acyl-CoAs in the mitochondrial matrix (33–35). NT5C2 depletion increased glucose uptake, whereas glucose incorporation into glycogen was unaltered. Interestingly, lactate release from the cultured myotubes was reduced in NT5C2-depleted myotubes. Whereas acute AICAR exposure increases lactate production in isolated epitrochlearis muscle exposed to high glucose levels (36), AMPK activation in neonatal hearts increases lactate oxidation (37). The reduced lactate release in NT5C2-depleted myotubes may be consistent with a shift from non-oxidative to oxidative glucose metabolism. Nevertheless, the predicted increase in glucose oxidation was not observed upon NT5C2 depletion. This contrasts with our previous observation of a reciprocal relationship between lactate production and glucose oxidation in malonyl-CoA decarboxylase-depleted myotubes (25). The concomitant decrease in glucose oxidation and lactate release upon NT5C2 depletion may be attributed to an inhibitory action from an increase in ATP production on phosphofructokinase and pyruvate kinase, two key regulatory enzymes of the glycolytic pathway, via β-oxidation of lipids. Consequently, a partial metabolic block of glycolysis could limit the flux from phosphoenolpyruvate to pyruvate. Alternatively, the decrease in glucose oxidation and lactate release in the NT5C2-depleted myotubes may be attributed to a reduction in intracellular pyruvate levels; however, this remains to be addressed experimentally. Glucose may also be diverted toward the pentose pathway to provide substrates for RNA synthesis and cofactors for *de novo* lipogenesis (38).

In vivo electrotransfer to overexpress DNA from endogenous (39, 40) or mutant (41, 42) proteins in skeletal muscle has been used to address the role of specific genes in signal transduction and metabolism. Our expression profiling revealed that NT5C1A was abundantly expressed in mouse extensor digitorum longus muscle and human vastus lateralis muscle (data not shown). To determine the role of this isoform in AMPK signaling and glucose metabolism, we took an alternative approach by applying *in vivo* electrotransfer of shRNA to reduce NT5C1A protein in intact skeletal muscle. Similar to our results for NT5C2 siRNA in human myotubes, gene silencing of NT5C1A increased AMPK and ACC phosphorylation with a concomitant increase in glucose uptake, providing further support for a metabolic role of cytosolic NT5 isoforms. The protein content

of AMPK and ACC was unaltered, indicating that the NT5C1A-mediated changes in signal transduction directly increase glucose uptake.

AMPD1 (AMP deaminase 1) is another critical enzyme involved in the intracellular availability of AMP. AMPD1 plays a major role in regulating cellular AMP levels by converting AMP to IMP and competes with cytosolic 5′-nucleotidases for available AMP (43). Defects in the *AMPD1* gene decrease enzyme activity and increase AMP accumulation in skeletal muscle. Variations in the *AMPD1* gene are associated with alterations in the metabolic clearance rate of insulin (44). The common C34T polymorphism in the *AMPD1* gene is associated with lower prevalence of type 2 diabetes, reduced frequency of obesity, and lower systolic blood pressure in people with coronary artery disease without heart failure (45), possibly through increased AMPK activity. Conversely, genetic reduction of AMP in adenylate kinase 1-deficient mice reduces contraction-induced AMPK phosphorylation (46) and glucose transport (47) in skeletal muscle. Alterations in the adenine nucleotide levels by these approaches may have beneficial effects on glucose and energy homeostasis.

We have highlighted a novel role for NT5 in maintaining the intracellular energy status via AMPK. Gene silencing of NT5C2, the predominant cytosolic 5′-nucleotidase in human myotubes, increased AMPK and ACC phosphorylation and promoted lipid oxidation and glucose transport. Silencing of NT5C1A, the predominant form in mouse glycolytic muscle, increased AMPK and ACC phosphorylation with a concomitant increase in glucose uptake, providing further support for a metabolic role of cytosolic NT5 isoforms. Endogenous cytosolic NT5 isoforms may restrain basal lipid oxidation and glucose transport in resting skeletal muscle. The balance between cytosolic 5′-nucleotidases and AMPK activity in type 2 diabetes and obesity may play an important role in the regulation of insulin action and lipid metabolism in skeletal muscle. Suppression of cytosolic 5′-nucleotidase expression or activity may be one potential avenue to bypass metabolic inflexibility in type 2 diabetes.

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