C1q Tumor Necrosis Factor α-related Protein Isoform 5 Is Increased in Mitochondrial DNA-depleted Myocytes and Activates AMP-activated Protein Kinase*

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Depletion of mtDNA in myocytes causes insulin resistance and alters nuclear gene expression that may be involved in rescuing processes against cellular stress. Here we show that the expression of C1q tumor necrosis factor α-related protein isoform 5 (C1QTNF5) is drastically increased following depletion of mtDNA in myocytes. C1QTNF5 is homologous to adiponectin in respect to domain structure, and its expression and secretion from myocytes correlated negatively with the cellular mtDNA content. Similar to adiponectin, C1QTNF5 induced the phosphorylation of AMP-activated protein kinase (AMPK), leading to increased cell surface recruitment of GLUT4 and increased glucose uptake. Treatment of cells with purified recombinant C1QTNF5 increased the phosphorylation of acetyl-CoA carboxylase and stimulated fatty acid oxidation. C1QTNF5-mediated phosphorylation of AMPK or acetyl-CoA carboxylase was unaffected by depletion of adiponectin receptors such as AdipoR1 or AdipoR2, which indicated that adiponectin receptors do not participate in C1QTNF5-induced activation of AMPK. Serum C1QTNF5 levels were significantly higher in obese/diabetic animals (OLETF rats, ob/ob mice, and db/db mice). These results highlight C1QTNF5 as a putative biomarker for mitochondrial dysfunction and a potent activator of AMPK.

Impaired mitochondrial function has been implicated in a number of human diseases, including diabetes and obesity (1). Previously, we demonstrated that the depletion of mtDNA in myocytes reduces the expression of insulin receptor substrate-1 (IRS-1),2 which results in insulin resistance and impaired glucose utilization (2). The signals from mitochondrial stress cause a variety of changes in nuclear gene expressions (3). Loss of mitochondrial membrane potential and ATP generation capacity as a result of mitochondrial stress activates some transcription factors that facilitate mitochondrial recovery from cellular stress (4). In this study, we performed annealing controlled primer (ACP)-based PCR to identify nuclear genes that were differentially expressed in response to changes in mtDNA content, and we identified a gene encoding C1q tumor necrosis factor α-related protein isoform 5 (C1QTNF5) that is drastically increased in mtDNA-depleted myocytes. C1QTNF5 belongs to the C1QTNFs family of proteins that are characterized by a specific domain structure, including an N-terminal signal peptide, a collagen repeat domain, and a C-terminal C1q-like globular domain (5). Nuclear DNA-encoded C1QTNF isoforms (C1QTNFs) are thought to be adiponectin paralogs in mammalian cells, because they contain similar modular organizational structure as adiponectin (6). The globular domain of C1QTNF5 is homologous (40%) in amino acid sequence to that of adiponectin (supplemental material 1), which suggests that the two proteins may have similar functions in cellular metabolism.

Adiponectin is an important adipokine, which participates in the regulation of energy metabolism (7). Unlike adiponectin, which is expressed exclusively in adipocytes, C1QTNFs are expressed in a wide variety of tissues and appear to have diverse functions (8). C1QTNF1, which is expressed by vascular smooth muscle cells, inhibits collagen-induced platelet aggregation (9) and activates Akt and MAPK (10). C1QTNF3 is expressed by chondrocytes, and recombinant C1QTNF3 stimulates cartilage development by activating extracellular signal-regulated kinase (ERK) and Akt signaling pathway (11, 12). Recently, it was reported that C1QTNF2 induces the phosphorylation of AMPK in C2C12 myocytes, resulting in increased glycogen accumulation and fatty acid oxidation (6). However, C1QTNF2 is not present in plasma, which indicates that other C1QTNFs act on muscle and liver cells to regulate metabolism.
In this study, we demonstrated that the expression and secretion of C1QTNF5 correlates negatively with mtDNA content in myocytes. Although the C1QTNF5 receptor has yet to be identified, C1QTNF5 exhibits similar biological activities to adiponectin, such as activating AMPK and augmenting glucose uptake and fatty acid oxidation. Serum C1QTNF5 levels were significantly higher in obese/diabetic animals as compared with normal animals.

**EXPERIMENTAL PROCEDURES**

**Materials**—Antibodies for AMPKα, phospho-AMPKα (Thr172), phospho-ACC (Ser79), Akt, and phospho-Akt (Ser473) were purchased from Cell Signaling Technology (Beverly, MA). Antibodies for adiponectin and its receptors (AdipoR1 and AdipoR2) were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-IRS-1 antibody was from Upstate Biotechnology, Inc. (Lake Placid, NY) and anti-phospho-IRS-1 antibody was from Dr. Pann-Gill Suh (Postech, Pohang, Korea). Oligonucleotide primers were from Bionics (Seoul, Korea). Unless otherwise indicated, all other antibodies and chemicals were from Sigma.

**Cell Culture and Transient Transfection**—The cell lines used in this study were L6 and L6 GLUT4myc rat skeletal myocytes (provided by Dr. Amira Klip, Hospital for Sick Children, Toronto, Canada) (13). Myocytes were cultured and differentiated as described previously (14). For mtDNA depletion, L6 GLUT4myc myocytes were incubated with EtBr (0.2 μg/ml) and uridine (50 μg/ml) for 3 weeks in α-minimum essential medium supplemented with 10% FBS. Under these experimental conditions, mtDNA was depleted to <10% of normal. The removal of EtBr from the medium normalized mtDNA content (>90% of normal) within 7 days. The control parental L6 GLUT4myc myocytes were maintained for the same time period in normal culture medium. The mtDNA content of L6 GLUT4myc myocytes cultured with or without EtBr was monitored routinely by amplifying genomic DNA as described previously (2). HEK293 and SK-Hep1 cells were cultured in Dulbecco’s modified Eagle’s medium with 10% FBS. Human C1QTNF5 full-length cDNA cloned in pcDNA3.1 (Invitrogen) was transfected into HEK293, L6 GLUT4myc, and SK-Hep1 cells using Lipofectamine 2000 (Invitrogen).

**ACP-based PCR and Cloning**—Differentially expressed genes in control, mtDNA-depleted, and -reverted myocytes were screened by the ACP-based PCR method (15) using a GeneFishing DEG kit (Seegene, Seoul, Korea). The PCR fragments were directly cloned into TOPO-TA vector (TOPO-TA, Invitrogen) and sequenced with ABI PRISM 3100 (Applied Biosystems, Foster City, CA). The identified genes were confirmed by RT-PCR using primer sets. The first strand cDNA was normalized with the β-actin gene and used as a template. Based on the conserved regions derived from alignment of the rat and human C1QTNF5 sequences, two primer sets were designed as follows: S-1 (forward-S1, 5'-GTT GCC CCC CCA CGA TCA GCC TTC-3'; reverse-S1, 5'-AGC GAA GAC TGG GGA GCT-3') and S-2 (forward-S2, 5'-GTC CCT CCG CGA TCC GCC TTC-3'; reverse-S2, 5'-AGC GAA GAC TGG GGA GCT-3'). The PCR amplification protocol with L6 GLUT4myc cDNA was an initial 3-min denaturation at 94°C, followed by 20–25 cycles of 94°C for 40 s, 60°C for 40 s, and 72°C for 40 s and a 5-min final extension at 72°C. Under these conditions, the S-1 and S-2 primer sets produced amplified DNA fragments of 301 and 754 bp, respectively. The products were purified with a QIAquick gel extraction kit (Qiagen, Alameda, CA) and sequenced. Quantitative Real Time RT-PCR (qRT-PCR)—Quantitative expression analysis of specific genes was carried out in a Rotor-Gene 2000 (Corbett Research, Mortlake, Australia) using SYBR-Green PCR master mix (Qiagen, Valencia, CA) and specific primers (supplemental material 2). After amplification, melting curve analysis and agarose-gel electrophoresis verified the authenticity of the PCR products. The qRT-PCR results were analyzed using Rotor-Gene analysis software version 6.0 (Corbett Research) as described previously (2).

**Generation of C1QTNF5 Constructs and Production of Recombinant Proteins**—To produce the GST fusion of full-length rat C1QTNF5, cDNA was generated by PCR and cloned into the BamHI and Xhol sites of pGEX4T-1. The GST fusion globular domain of rat C1QTNF5 (Pro100–Ala243) was generated by PCR and cloned into the BamHI and Xhol sites of pGEX 4T-1. These recombinant GST fusion proteins were expressed in Escherichia coli BL21, harvested, and purified using glutathione-Sepharose™ Fast Flow according to the manufacturer’s instructions (Amersham Biosciences). pBAD/Thio-TOPO (Invitrogen) vector containing the thioredoxin-fused globular domain of human C1QTNF5 (5) was from Dr. A. F. Wright (Western General Hospital, Edinburgh, Scotland, UK). The recombinant protein was produced and purified as described (5). We also prepared the globular domain of human C1QTNF5 (with an additional Met) without a tagged sequence. The C1QTNF5 gene was amplified by PCR using a forward primer (5'-CAG TCT GAC ATA TGG TGC CTC CGC GAT CC-3') and a reverse primer (5'-AGA CTG GAA TTC CTA AGC AAA GAC TGG-3') and cloned into the NdeI and EcoRI sites of pREP A vector (Invitrogen). The globular domain of human C1QTNF5 was expressed in E. coli BL21 and purified with DEAE-Sepharose Fast Flow and Sephacryl S-200 (Amersham Biosciences). All recombinant proteins were subjected to 12% SDS-PAGE for purity confirmation. To remove endotoxin, the purified protein was loaded in Detoxi-Gel (Pierce) before storage at −70°C.

**Antibody Production and Specificity**—Antibody against human C1QTNF5 was raised in rabbits using the purified thioredoxin-fused recombinant globular domain of human C1QTNF5. Adequate titers were obtained after three boosters with the protein in Freund’s incomplete adjuvant. The IgG from the antiserum was purified by protein A-affinity chromatography, and the antibody specificity was confirmed by immunoblotting. The antibody was highly specific for the globular domain of human C1QTNF5, without any cross-reactivity against thioredoxin or adiponectin. The antibody also recognized the globular domains of rat and mouse C1QTNF5, whereas the globular domains of human, rat, and mouse C1QTNF5 were not detected by an adiponectin (Acrp30) polyclonal antibody (supplemental material 3).
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Phosphoprotein Analysis—Differentiated L6 GLUT4myc myocytes were serum-starved for 5 h and treated with AICAR (2 mM), insulin (100 nM), or various concentrations of recombinant proteins for the specified times. Cells were immediately washed three times with ice-cold phosphate-buffered saline and lysed in lysis buffer (1% Triton X-100, 1 mM Na3VO4, 1 mM Na4P2O7, 1 mM benzamidine, 0.2 mM phenylmethylsulfonyl fluoride, 10 μM E-64, 1 mM pepstatin A, 1 mM leupeptin, and phosphatase inhibitor mixture (Sigma) in ice-cold phosphate-buffered saline) by vortex mixing. After centrifugation (14,000 g, 1 min at 4 °C), the supernatant was assayed for protein levels and mixed with 2X Laemmli sample buffer containing 7.5% β-mercaptoethanol and heated for 5 min at 65 °C. Total protein (20 μg) was resolved by 6.5–10% SDS-PAGE and immunoblotted with the indicated phospho-specific antibodies.

siRNA-mediated Knockdown of AdipoR1 and AdipoR2 Expression—All RNA interference experiments were carried out with SMARTpool siRNAs from Dharmacon, according to the manufacturer’s instructions. After 4 h of transfection, the medium was replaced with α-minimum essential medium containing 10% FBS, and the cells were harvested for 48 h. Isolation of RNA, cDNA synthesis, primers, and qRT-PCR condition for AdipoR1 and AdipoR2 were conducted as described (16).

Measurement of 2-Deoxyglucose (2-DG) Uptake and GLUT4 Translocation—Myocytes were serum-starved for 5 h and washed twice with HEPES-buffered saline solution (140 mM NaCl, 20 mM HEPES/Na, 2.5 mM MgSO4, 1 mM CaCl2, and 5 mM KCl, pH 7.4). 2-DG uptake and the movement of Myc-tagged GLUT4 to the cell surface were measured as described (2).

Fatty Acid Oxidation—The fatty acid oxidation rate was measured as 14CO2 generation from [14C]palmitate (PerkinElmer Life Sciences), as described previously (17).

Animals—All animal experiments were carried out in accordance with the principles of laboratory animal care (NIH publication 85-23) and with the ethics committee approval from Dongguk University. Eight-week-old male OLETF rats and nondiabetic control male LETO rats were provided by Otsuka Pharmaceutical Co. (Tokushima, Japan). C57BL/6 lean, db/db, and ob/ob mice were purchased from SLC (Japan). Animals were conditioned in a controlled environment (23 ± 1 °C, 12-h light/dark cycle) and placed individually in cages with standard rodent chow and tap water. Food intake and body weight were monitored daily. The animals were sacrificed at 39 weeks (rat) and 12 weeks of age (mice) when they were obese and/or diabetic. The animals were fasted for 16 h prior to all experimental analyses. Blood was withdrawn from tail vein or retro-orbital plexus.

Statistical Analysis—Values are expressed as the means ± S.E. Where applicable, the significance of difference was analyzed using the Student’s t test for unpaired data.
RESULTS

Depletion of mtDNA Increases the Expression and Secretion of C1QTNF5

mtDNA-depleted myocytes were generated by exposing L6 GLUT4myc myocytes to a low dose of EtBr (0.2 μg/ml) (2). As shown in Fig. 1 (A and B), cytochrome oxidase subunits I (COX-I) and II (COX-II), both encoded only in mtDNA, were hardly amplified from the genomic DNA of the cells treated with EtBr for 3 weeks. In contrast, nuclear DNA-encoded genes such as COX-IV and β-actin (Fig. 1A) were detected at similar levels in both control and EtBr-treated cells, indicating that prolonged treatment with EtBr depleted cellular mtDNA content without altering the nuclear DNA replication. In addition, the removal of EtBr from medium successfully normalized mtDNA content within 7 days (Fig. 1, A and B). Because the changes in mtDNA content have been proposed to initiate a stress signal that leads to alterations in nuclear gene expression (3), we analyzed differentially expressed genes in mtDNA-depleted and -reverted L6 GLUT4myc myocytes using ACP-based PCR. The levels of a number of amplicons were increased or decreased in mtDNA-depleted myocytes (data not shown). We cloned and sequenced over 30 of these genes, and we identified one, a 432-bp amplicon that was drastically increased (~6-fold) in mtDNA-depleted myocytes, which exhibited high sequence homology to rat C1QTNF5 (GenBank™ BC089992). Using RT-PCR, we verified that C1QTNF5 was expressed in L6 GLUT4myc myocytes and that expression was increased in mtDNA-depleted myocytes and normalized in reverted myocytes (Fig. 1C). We also investigated whether other C1QTNF isoforms were differentially expressed in mtDNA-depleted and -reverted myocytes by semi-quantitative RT-PCR (Fig. 1D) and qRT-PCR (Fig. 1E). Although C1QTNF1–5 were expressed in myocytes, only the expression of C1QTNF5 was significantly increased (~8-fold) in mtDNA-depleted myocytes (Fig. 1, D and E). When mtDNA content was restored to near-control levels, the expression of C1QTNF5 also returned to control levels. These results indicated that the selective increase of C1QTNF5 correlates with impaired mtDNA replication and transcription in myocytes.

All C1QTNF isoforms possess a putative secretion signal peptide in their N terminus, and transient transfection studies in COS-7 cells have shown that C1QTNF1, -2, and -7 are secreted (6). To determine whether C1QTNF5 was secreted from cells, we transfected HEK293 cells, L6 GLUT4myc myocytes, and SK-Hep1 hepatocytes with an expression vector encoding full-length human C1QTNF5, and we then analyzed protein secretion by immunoblot analysis. C1QTNF5 distributed predominantly to the culture medium rather than cell lysate, which indicated that ectopically expressed C1QTNF5 is secreted from mammalian cells (Fig. 2A). Quantitative analysis of C1QTNF5 secretion using proportional amounts of cell lysate and culture medium revealed that, on a daily basis, transfected cells spontaneously secreted as much as 85–90% of the C1QTNF5 they produced. Because the transcription of C1QTNF5 was increased in mtDNA-depleted myocytes (Fig. 1, D and E), we examined whether the secretion of C1QTNF5 was also increased in mtDNA-depleted myocytes (Fig. 2B). We expected, the secretion of C1QTNF5 was increased about 4-fold in mtDNA-depleted myocytes as compared with control cells. When mtDNA content was restored to near-control levels, the expression and secretion of C1QTNF5 also returned to control levels. Taken together, these findings clearly indicated that C1QTNF5 is a secreted protein and that its expression is negatively correlated with the depletion of cellular mtDNA and/or mitochondrial dysfunction in myocytes.

Functional Significance of C1QTNF5

Rat C1QTNF5 Increases the Phosphorylation of AMPK and ACC but Not IRS-1 and Akt in Myocytes—The identification of C1QTNF5 and its negative correlation to cellular mtDNA content in myocytes implicate C1QTNF5 in muscle metabolism. We examined the effect of C1QTNF5 on AMPK activation, because AMPK plays an important role in the energy homeostasis through the regulation of glucose and fatty acid utilization (18). L6 GLUT4myc myocytes were treated with purified recombinant GST fusion proteins of full-length (GST-rC5), or globular domain (Pro100–Ala243, GST-rgC5) of rat C1QTNF5, and then the activation of AMPK and insulin signaling molecules such as IRS-1 and Akt was monitored by immunoblot analysis. As a positive control, we treated myocytes with a cell-permeable AMP analog, AICAR, which stimulates AMPK activity and glucose transport independently of phosphatidyl-
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![Diagram](image)

**FIGURE 3. Phosphorylation of AMPK and ACC in rat C1QTNF5-treated myocytes.** L6 GLUT4myc myocytes were serum-starved for 5 h and treated with AICAR or the indicated recombinant proteins for 30 min. Myocytes were lysed, subjected to SDS-PAGE, and immunoblotted with the indicated antibodies. A, myocytes were treated with GST (2 μg/ml), AICAR (2 μM), or GST fusions of full-length rat C1QTNF5 (GST-rfC5, 2 μg/ml) or its globular domain (GST-rgC5, 2 μg/ml) during the last 30 min of starvation. Immunoblot analysis was carried out using antibodies specific for AMPK, ACC, phospho-AMPK (Thr172, pAMPK), or phospho-ACC (Ser79, pACC). B, immunoblot intensities for pAMPK/AMPK (open columns) and pACC/ACC (filled columns) were quantified by densitometry and expressed in relative ratio. The intensity of the GST-treated control (Con) was set to 1. Values are expressed as means ± S.E. from five independent experiments. C, myocytes were treated with GST (2 μg/ml), insulin (100 nM), AICAR (2 μM), or 0.2, 1, or 5 μg/ml GST-rfC5 during the last 30 min of starvation. Immunoblot analysis was carried out using antibodies specific for AMPK, ACC, phospho-AMPK, IRS-1, Akt, or phospho-Akt (pAkt). The immunoblots are representative of five independent experiments. D, immunoblot intensities for pAMPK/AMPK (open columns) and pACC/ACC (filled columns) were quantified by densitometry and expressed in relative ratio. The intensity of the GST-treated control was set to 1 (data not shown). Values are expressed as means ± S.E. of five independent experiments. *, p < 0.05; **, p < 0.01; ***, p < 0.001 versus control.

Although GST appeared to be biologically inactive in our system, fusion of a GST moiety to a protein can alter the structure and function of that protein. To control for these variables, we generated GST-free recombinant human C1QTNF5 globular domain (hgC5) and evaluated its ability to induce the phosphorylation of AMPK and ACC in myocytes. Human and rat C1QTNF5 are 95% identical in amino acid sequence, and within the globular domain, they are 97% identical. Treatment of myocytes with hgC5 increased the phosphorylation of AMPK and ACC in a dose-dependent manner but had no effect on Akt phosphorylation (Fig. 4A). AMPK phosphorylation was rapidly induced by hgC5 (2 μg/ml), peaked at 15 min of treatment, and persisted for up to 60 min (Fig. 4B). The phosphorylation of ACC peaked at 30 min, and was maintained for up to 60 min.

In skeletal muscle, treatment with AICAR results in the AMPK-dependent phosphorylation of p38 MAPK, which in turn stimulates GLUT4 translocation to the plasma membrane (PM) (20). Similar to AICAR, hgC5 induced the phosphorylation of p38 MAPK within 15 min of treatment (Fig. 4B). The hgC5-induced phosphorylation of AMPK, ACC, and p38 MAPK was significantly inhibited by pretreatment of cells with araA, an inhibitor of AMPK (Fig. 4, C and D). These results suggested that hgC5 stimulates the phosphorylation of ACC and p38 MAPK through the activation of AMPK, and that ACC and p38 MAPK are downstream targets of AMPK in C1QTNF5-treated cells.

To evaluate the effect of full-length human C1QTNF5 on AMPK, we transfected myocytes with an expression vector for full-length human C1QTNF5 (hC5). As shown in Fig. 5A, the majority of ectopic hC5 was secreted into the medium. The overexpression of hC5 drastically increased the phosphorylation of AMPK, ACC, and p38 MAPK but had no effect on the

inositol 3-kinase activity (19). As shown in Fig. 3, AICAR induced phosphorylation of AMPK as well as its downstream target ACC, whereas GST alone did not. Interestingly, treatment of myocytes with GST-rfC5 or GST-rgC5 (2 μg/ml) increased the phosphorylation of AMPK and ACC without altering AMPK and ACC expression levels, similar to AICAR. Because of limited success in generating purified recombinant full-length C1QTNF5, and the fact that the isolated globular domain of C1QTNF5 was more potent than the full-length protein (Fig. 3, A and B), we examined the dose-dependent effect of C1QTNF5 on AMPK phosphorylation using GST-rgC5 (Fig. 3, C and D). As little as 0.2 μg/ml (~25 nM) recombinant GST-rgC5 induced a significant increase in the phosphorylation of AMPK, resulting in phosphorylation of ACC. In contrast, GST-rgC5 had no effect on the phosphorylation of IRS-1 and Akt, whereas insulin had a dramatic effect on the phosphorylation of both proteins (Fig. 3C). These results clearly showed that C1QTNF5 is a potent and selective activator of AMPK and has no effect on IRS-1 and Akt in the insulin signaling pathway in myocytes.

**Human C1QTNF5 Also Activates AMPK in Myocytes—** Although GST appeared to be biologically inactive in our system, fusion of a GST moiety to a protein can alter the structure and function of that protein. To control for these variables, we generated GST-free recombinant human C1QTNF5 globular domain (hgC5) and evaluated its ability to induce the phosphorylation of AMPK and ACC in myocytes. Human and rat C1QTNF5 are 95% identical in amino acid sequence, and within the globular domain, they are 97% identical. Treatment of myocytes with hgC5 increased the phosphorylation of AMPK and ACC in a dose-dependent manner but had no effect on Akt phosphorylation (Fig. 4A). AMPK phosphorylation was rapidly induced by hgC5 (2 μg/ml), peaked at 15 min of treatment, and persisted for up to 60 min (Fig. 4B). The phosphorylation of ACC peaked at 30 min, and was maintained for up to 60 min.

In skeletal muscle, treatment with AICAR results in the AMPK-dependent phosphorylation of p38 MAPK, which in turn stimulates GLUT4 translocation to the plasma membrane (PM) (20). Similar to
phosphorylation of IRS-1 or Akt (Fig. 5B). Thus, both the full-length and globular domain of human C1QTNF5 induced the phosphorylation of AMPK, ACC, and p38 MAPK independently of the insulin signaling pathway.

**C1QTNF5 Increases Glucose Uptake and Fatty Acid Oxidation in Myocytes**—Because the phosphorylation of AMPK by the cellular treatment of AICAR or adiponectin increases glucose uptake and fatty acid oxidation in skeletal muscle (18, 21), we next examined whether C1QTNF5 increased glucose uptake and fatty acid oxidation in L6 GLUT4myc myocytes. Both the globular domains of GST-rgC5 and hgC5 significantly increased 2-DG uptake to a similar extent as insulin and AICAR (Fig. 6A). Similar to its effect on AMPK phosphorylation, GST-rgC5 was more potent than full-length C1QTNF5 (GST-rfC5) in inducing glucose uptake. The effect of C1QTNF5 on glucose uptake was primarily due to the activation of AMPK, because C1QTNF5-mediated AMPK phosphorylation was independent of IRS-1 and Akt phosphorylation (Fig. 3C and Fig. 5B). We also analyzed whether C1QTNF5 affected the recruitment of GLUT4, a glucose transporter specific to muscle and adipose tissue, from intracellular storage pools to the PM. As shown in Fig. 6B, the globular domain of C1QTNF5, similar to AICAR, significantly increased the cell surface localization of GLUT4myc by 50% over basal. There was no additive effect of treatment with GST-rgC5 and AICAR. In addition, pretreatment with araA strongly inhibited GST-rgC5- and AICAR-stimulated GLUT4 translocation. These results suggested that the globular domain of C1QTNF5 stimulates the recruitment of GLUT4 to the PM, thereby increasing glucose uptake in myocytes.

The activation of AMPK in muscle increases the phosphorylation of ACC, leading to decreased fatty acid synthesis and a concomitant increase in β-oxidation of fatty acids (22). Treatment of myocytes with the globular domain of human C1QTNF5 significantly increased fatty acid oxidation in myocytes, as measured by palmitate oxidation (Fig. 6C). Because C1QTNF5-induced ACC phosphorylation (Fig. 4C and D) and palmitate oxidation (Fig. 6C) were abolished by pretreatment of araA, C1QTNF5-induced fatty acid oxidation was mainly due to the activation of AMPK.

**Adiponectin Receptors Are Not Involved in the C1QTNF5-induced AMPK Activation**

To investigate whether the adiponectin receptors AdipoR1 and AdipoR2 were involved in C1QTNF5-mediated AMPK activation, we examined the effect of C1QTNF5 on myocytes transfected with synthetic siRNAs that targeted AdipoR1 (siR1, Fig. 7A) and AdipoR2 (siR2, Fig. 7B). qRT-PCR (data not shown) and immunoblot analysis confirmed that the expres-
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A

Lysate

hC5

Medium

hC5

AICAR

- - - +

pcDNA3.1

2 - - 2

pcDNA3.1-hC5

0.2 2 - -

µg/ml

B

AMPK

pAMPK

ACC

pACC

p38

pp38

IRS-1

pIRS-1

Akt

pAkt

AICAR

- - - +

pcDNA3.1

2 - - 2

pcDNA3.1-hC5

0.2 2 - -

µg/ml

FIGURE 5. Phosphorylation of AMPK, ACC, and p38 MAPK in C1QTNF5-overexpressed myocytes. Myocytes were transfected with empty pcDNA3.1 vector (2 µg/ml) or pcDNA3.1 containing full-length human C1QTNF5 (pcDNA3.1-hC5; 0.2 or 2 µg/ml) and incubated for 48 h. The media (20 µl) and cell lysates (40 µg) were analyzed for expression of C1QTNF5 (A) or phosphorylated AMPK, ACC, p38 MAPK, IRS-1, and Akt using corresponding antibodies (B). Some pcDNA3.1-transfected myocytes were treated with AICAR (2 mM) during the last 30 min of incubation. The immunoblots are representative of four independent experiments.

Serum C1QTNF5 Levels Are Higher in Diabetic Animal Models

To determine the clinical significance and implication of C1QTNF5, we analyzed serum C1QTNF5 levels in several animal models of diabetes, such as OLETF rats, ob/ob mice, and db/db mice. The OLETF rat is a well established animal model of insulin resistance and obesity-associated type 2 diabetes (23). OLETF rats exhibit impaired glucose tolerance accompanied by a marked increase in plasma insulin after 24 weeks of age.

FIGURE 6. Effect of C1QTNF5 on glucose uptake, GLUT4 translocation, and fatty acid oxidation in myocytes. Myocytes were serum-starved for 5 h and treated with a vehicle (Control), insulin (100 nM), AICAR (2 mM), or recombinant C1QTNF5 for 30 min. A, myocytes were treated with vehicle, insulin, AICAR, GST-fused full-length rat C1QTNF5 (GST-rfC5; 2 µg/ml), the GST-fused globular domain of rat C1QTNF5 (GST-rgC5; 2 µg/ml), or the globular domain of human C1QTNF5 (hgC5; 1 µg/ml). Intracellular radioactivity corresponding to [14C]2-DG uptake was expressed relative to that in the untreated control cells, which was set to 1. Values are the means ± S.E. of three independent experiments. *, p < 0.05; **, p < 0.01; ***, p < 0.001 versus control.

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sion of AdipoR1 or AdipoR2 was significantly reduced in myo-
cytes transfected with siR1 (Fig. 7A) or siR2 (Fig. 7B) but not
with a nontargeting scrambled siRNA. Knockdown of AdipoR1
or AdipoR2 by specific siRNAs had no effect on the C1QTNF5-
induced phosphorylation of AMPK and ACC (Fig. 7, A and B),
which clearly indicated that AdipoR1 and AdipoR2 are not
involved in the C1QTNF5-mediated activation of AMPK.
These results also suggested that there is a distinct C1QTNF5
receptor in myocytes, the identity and characterization of
which warrants further investigation.

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To determine the clinical significance and implication of
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OLETF rats exhibit impaired glucose tolerance accompanied
by a marked increase in plasma insulin after 24 weeks of age.
OLETF rats at 39 weeks of age developed hyperglycemia and impaired oral glucose tolerance as compared with control LETO rats (supplemental material 4). mtDNA content was significantly reduced in skeletal muscle of OLETF rats (Fig. 8A), and serum C1QTNF5 levels were significantly higher in OLETF rats as compared with LETO rats (Fig. 8B). Thus, there appeared to be a negative correlation between serum C1QTNF5 levels and mtDNA content in skeletal muscle in OLETF rats. To verify these results in other animal models of obesity/diabetes, we analyzed serum C1QTNF5 levels in db/db and ob/ob mice at 14 weeks of age (Fig. 8C). In both of these well characterized mouse models of obesity-associated diabetes, there were significantly higher levels of serum C1QTNF5 than in lean mice. These results suggested that serum C1QTNF5 levels may be correlated with impaired glucose tolerance, insulin resistance, and hyperglycemia in vivo.

DISCUSSION

C1QTNF5 is a 243-amino acid soluble protein containing structural features that are common to all C1QTNF family members as follows: an N-terminal signal peptide, a collagen repeat domain, and a C-terminal C1q-like globular domain (6). In this study, we investigated the expression pattern and biological function of C1QTNF5 in muscle metabolism. Above all, we revealed that C1QTNF5, and the globular domain of C1QTNF5 in particular, activates AMPK in myocytes. AMPK is a key enzyme in the regulation of energy homeostasis (18). It is activated primarily by an increase in the cellular ratio of AMP to ATP (18, 21). Exposure of skeletal muscle to AICAR, an AMPK agonist, stimulates an acute increase in glucose uptake (24), whereas overexpression of an AMPK mutant completely abrogates the effect of AICAR (25). AMPK increases glucose uptake by stimulating the recruitment of GLUT4 to the PM and inducing GLUT4 expression (24, 26). In this study, we demonstrated for the first time that treatment of myocytes with recombinant C1QTNF5 rapidly induces AMPK phosphorylation and increases glucose uptake through the stimulation of GLUT4 translocation to the PM.

C1QTNF5 also stimulated ACC phosphorylation and fatty acid oxidation through the phosphorylation of AMPK in myocytes. AMPK plays an important role in fat metabolism in skeletal muscle. The activation of AMPK induces the phosphorylation of ACC and a consequent reduction of malonyl-CoA synthesis, thereby stimulating fatty acid oxidation (24, 27). During the progression of type 2 diabetes, high levels of free fatty acids interfere with insulin-stimulated GLUT4 translocation and are closely associated with the development of insulin resistance (28). The observation that C1QTNF5 is involved in glucose uptake and fatty acid oxidation increases its therapeutic potential as a metabolic regulator and insulin sensitizer. Of note, C1QTNF5 also induced the phosphorylation of p38 MAPK through the activation of AMPK. In skeletal muscle, AICAR increases the phosphorylation of p38 MAPK, which in turn stimulates GLUT4 translocation to the PM (20). Activation of p38 MAPK results in the phosphorylation of peroxisome proliferator-activated receptor α, and it induces the association of
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peroxisome proliferator-activated receptor α with its coactivator, PGC-1α, resulting in the activation of fatty acid oxidation (20). It has been suggested that p38 MAPK and AMPK, by activating both glucose uptake and fatty acid oxidation, are key components through which adiponectin increases insulin sensitivity in skeletal muscle cells (29). Our results indicate that C1QTNF5 activates glucose uptake and fatty acid oxidation through the phosphorylation of p38 MAPK and AMPK, similar to AICAR and adiponectin.

One of the most interesting findings of this study is that adiponectin receptors were not involved in C1QTNF5-induced AMPK activation in myocytes. There are two different adiponectin receptors, AdipoR1 and AdipoR2 (29). AdipoR1 is expressed primarily in skeletal muscle and exhibits high binding affinity for the globular domain of adiponectin, whereas AdipoR2 is mainly expressed in liver and has a higher affinity for full-length adiponectin. In agreement with a previous report (30), AdipoR1 and AdipoR2 were expressed in L6 GLUT4myc myocytes, and AdipoR1 was the preferred receptor for adiponectin in myocytes. Interestingly, we did not observe any changes in the C1QTNF5-induced phosphorylation of AMPK and ACC in cells in which the expression of either AdipoR1 or AdipoR2 was significantly reduced by siRNAs. These results strongly suggest that the activation of AMPK by C1QTNF5 is independent of AdipoR1 and AdipoR2. It is noteworthy that the isolated globular domain of C1QTNF5 was a much more potent activator of AMPK than the full-length protein, which has well-characterized adiponectin-like activity that involves AdipoR1 in muscle (29). It is possible that the as-yet unidentified C1QTNF5 receptor in myocytes has a domain structure that is similar to AdipoR1. The identification of the putative C1QTNF5 receptor and other C1QTNF5-binding proteins is an interesting challenge for future studies.

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