Involvement of AMP-Activated Protein Kinase in Glucose Uptake Stimulated by the Globular Domain of Adiponectin in Primary Rat Adipocytes

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Adiponectin is an abundant adipocyte-derived plasma protein with anti-atherosclerotic and insulin-sensitizing properties that suppresses hepatic glucose production and enhances glucose uptake into skeletal muscle. To characterize the potential effects of adiponectin on glucose uptake into adipose cells, we incubated isolated epididymal rat adipocytes with the globular domain of recombinant adiponectin purified from an *E. coli* expression system. Globular adiponectin increased glucose uptake in adipocytes without stimulating tyrosine phosphorylation of the insulin receptor or insulin receptor substrate-1, and without enhancing phosphorylation of Akt on Ser-473. Globular adiponectin further enhanced insulin-stimulated glucose uptake at submaximal insulin concentrations and reversed the inhibitory effect of tumor necrosis factor-α on insulin-stimulated glucose uptake. Cellular treatment with globular adiponectin increased the Thr-172 phosphorylation and catalytic activity of AMP-activated protein kinase and enhanced the Ser-79 phosphorylation of acetyl CoA carboxylase, an enzyme downstream of AMP kinase in adipose cells. Inhibition of AMP kinase activation using two pharmacological inhibitors (adenine 9-β-D-arabinofuranoside and compound C) completely abrogated the increase in glucose uptake stimulated by globular adiponectin, indicating that AMP kinase is integrally involved in the adiponectin signal transduction pathway. Coupled with recent evidence that the effects of adiponectin are mediated via AMP kinase activation in liver and skeletal muscle, the findings reported here provide an important mechanistic link in the signaling effects of adiponectin in diverse metabolically responsive tissues. *Diabetes* 52:1355–1363, 2003

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ACC, acetyl CoA carboxylase; AICAR, 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside; AMP kinase, AMP-activated protein kinase; araA, adenine 9-β-D-arabinofuranoside; IRS-1, insulin receptor substrate-1; KRBH, Krebs-Ringer bicarbonate HEPES; PVDF, polyvinylidine fluoride; TNF-α, tumor necrosis factor-α.

Rather than being a simple depot of energy-rich triglyceride molecules, adipose tissue has recently been highlighted as an active secretory organ (1,2). Expansion of adipose tissue, in particular the visceral depot, leads to altered release into the bloodstream of a variety of factors that are associated with insulin resistance and increased cardiovascular risk in obese patients (3). Adiponectin is a relatively abundant adipocyte-derived circulating plasma protein whose levels have been shown to be dramatically reduced in association with obesity, insulin resistance, and cardiovascular disease (4–10). Accordingly, the physiological actions of adiponectin have been shown to include insulin-sensitizing, anti-hyperglycemic properties as well as anti-atherosclerotic vascular effects (11–14).

Adiponectin is highly conserved among mammalian species as a 244-amino acid polypeptide with a stretch of 22 collagen (Gly-X-Y) repeats and a globular domain with an overall homology similar to complement factor C1q (5,15). The full-length form of adiponectin forms high Mr oligomers in the bloodstream, and some studies have ascribed different signaling properties to various recombinant or processed forms of the protein (11,12,16–19). Adiponectin has been shown to enhance insulin sensitivity; improve plasma clearance of free fatty acids, glucose, and triglycerides; and suppress hepatic glucose production (11,12,16,20). These effects have been associated with increased cellular fat oxidation (12) and appear to involve the activation of AMP-activated protein kinase (AMP kinase) in liver and skeletal muscle (21,22). AMP kinase is a widely expressed serine kinase responsive to hypoxia and cellular stress that has been strongly implicated in a variety of pleiotropic cellular responses, including the insulin-independent activation of glucose transport in skeletal muscle (23).

The reduction in plasma adiponectin levels in visceral obesity is in sharp contrast to the increased secretion of cytokines such as tumor necrosis factor-α (TNF-α) in these pathological situations (24,25). TNF-α has been shown to have direct negative effects on insulin-signaling pathways in its target cells (26–29). In vascular cells, adiponectin can oppose various adverse effects of TNF-α; this observation has been especially well documented in animal models (6). Thus, a better understanding of the cellular mechanism of adiponectin's action may provide

insight into signaling abnormalities that occur in patients with obesity and type 2 diabetes as well as in vascular tissues.

In the present study, we explored whether the globular domain of adiponectin has effects on glucose uptake in isolated adipocytes. Our results indicate that globular adiponectin increases basal glucose uptake in adipocytes and enhances insulin-stimulated glucose uptake at submaximal insulin concentrations. Moreover, globular adiponectin reverses the inhibitory effect of TNF-α on insulin-stimulated glucose uptake. We also showed that AMP kinase is involved in the stimulation of glucose uptake by globular adiponectin in mature adipose cells, as inhibitors of AMP kinase activity abrogate the increase in glucose uptake elicited by globular adiponectin. These findings provide an important mechanistic link implicating AMP kinase in the signaling effects of adiponectin in diverse, metabolically responsive tissues.

RESEARCH DESIGN AND METHODS

Materials. General reagents were of the highest available grade and were obtained from Sigma Chemical (St. Louis, MO) or Fisher Scientific (Pittsburgh, PA). The pTrcHisA vector was obtained from Invitrogen (Carlsbad, CA). The nickel ion-agarose affinity column was from QIAGEN (Valencia, CA). The Acticlean Etox column was from Sterogene Bioseparations (Carlsbad, CA), and the limulus amebocyte lysate pyrogen plus detection kit was from BioWhittaker (Walkersville, MD). Collagenase, type I, was obtained from Worthington Biochemical (Lakewood, NJ). D-[^{14}C] glucose and 2-deoxy-D-[^{3}H]glucose were obtained from ICN (Costa Mesa, CA). [γ-^{32}P]ATP was obtained from Amersham Biosciences (Piscataway, NJ). All antibodies were polyclonal rabbit antisera, except as otherwise indicated. Anti−insulin receptor (β-subunit) and anti−insulin receptor substrate-1 (IRS-1) antibodies and anti-phosphotyrosine monoclonal antibodies (clone 4G10) were obtained from Upstate Biotechnology (Lake Placid, NY). Phospho-Akt Ser-473 antibody and pan-Akt isoform protein antibody were from Cell Signaling Technology (Beverly, MA). The phospho-AMP kinase Thr-172 and AMP kinase α1 + α2 isoform antibodies were from Cell Signaling Technology. The AMP kinase α2 isoform antibody, phospho-acetyl CoA carboxylase (ACC) Ser-79 antibody and SAMS peptide were from Upstate Biotechnology. Compound C (6-[4-(2-piperidin-1-yl-ethoxy)-phenyl)]-3-pyridin-4-yl-pyyrazolo[1,5-a] pyrimidine), a potent and selective small-molecule AMP kinase inhibitor (30), was generously provided by Merck Research Laboratories (Rahway, NJ). Adenine 9-β-D-arabinofuranoside (araA) was obtained from Sigma, and 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR) was obtained from Toronto Research Chemicals (North York, Ontario). Protein A agarose beads were obtained from Pierce (Rockford, IL). Secondary antibodies and reagents for enhanced chemiluminescence were obtained from Perkin-Elmer Life Sciences (Boston, MA).

Animals. Male SD rats weighing 220–240 g were obtained from Charles River Laboratories (Wilmington, MA). The animal handling and euthanasia protocol was approved by the Institutional Animal Care and Use Oversight Committee of Thomas Jefferson University.

Preparation of the recombinant globular domain of adiponectin. The cDNA encoding the full-length adiponectin sequence (31) was obtained by PCR from human adipose tissue RNA, and the truncated globular domain (amino acids 108–244) was subcloned into the pTrcHisA bacterial expression vector. The recombinant adiponectin globular domain was expressed as an NH_2-terminal 6 × His-tagged fusion protein in *E. coli* DH5α, by induction with isopropyl β-thiogalactopyranoside. Bacterial cell lysates were prepared under native conditions, and the recombinant globular adiponectin was isolated over a nickel-ion agarose column according to the manufacturer's instructions (QIAGEN). After elution, the protein was applied to an Acticlean Etox column to remove potential endotoxin contamination, the absence of which was verified by limulus amebocyte lysate assay.

Isolation of rat adipocytes. Rat adipocytes were isolated as previously described (32). Briefly, excised rat epididymal adipose tissue was handled under sterile conditions and rinsed in warm Hank's balanced salt solution in polypropylene tubes (Falcon 2059). Visible blood vessels were removed, and the fat pads were minced into millimeter-size pieces. The adipose tissue was then digested in Krebs-Ringer bicarbonate HEPES (KRBH) buffer (120 mmol/l NaCl, 4 mmol/l KH_2PO_4, 1 mmol/l $MgSO_4$, 0.75 mmol/l $CaCl_2$, 10 mmol/l $NaHCO_3$, 30 mmol/l HEPES; pH 7.4) with 1 mg/ml collagenase type I, 1% (wt/vol) BSA, 2.5 mmol/l glucose, 100 μg/ml penicillin, 100 μg/ml streptomycin, and 1% (vol/vol) fungizone for 40–60 min in a 37°C water bath with gentle agitation. After being filtered sequentially through 500- and 250-μm nylon mesh, the adipocyte cell suspension was centrifuged at 800*g* at room temperature for 2 min. Cells were washed twice in warm KRBH buffer containing 1% BSA, 2.5 mmol/l glucose, and antibiotics.

Adipocyte treatments. For the glucose uptake assay, cells were washed two times in KRBH buffer (pH 7.4) with 1% BSA but without glucose; for the AMP kinase phosphorylation assay, cells were washed two times in KRBH buffer (pH 7.4) with 1% BSA containing 2.5 mmol/l glucose. Cells were then resuspended in the wash buffer at 20% cytocrit. Before assay or preparation of cell lysates, the adipose cells were treated at 37°C with the indicated concentrations of globular adiponectin for 2 h, AICAR for 20 min, insulin for 5 min, or TNF-α for 20 min. Where indicated, cell treatment with araA or compound C was initiated 20 min before the addition of globular adiponectin.

Measurement of D-[^{14}C]glucose and 2-deoxy-D-[^{3}H]glucose uptake. Glucose uptake was measured as previously described (32). Adipocyte suspensions (150–210 μl) were equilibrated in triplicate in polypropylene vials for 10 min at 37°C and uncapped with gentle agitation. KRBH buffer (50 μl) with or without insulin was then added and the incubation proceeded for 5–30 min at 37°C, as indicated. Next D-[^{14}C]glucose (1 μCi) or 2-deoxy-D-[^{3}H]glucose (0.5 μCi) was added to each vial for 5 or 2 min, respectively, and the transport reaction was terminated by the addition of cytochalasin B (10 μl of a 1.5 mmol/l solution). The adipocyte suspension was then pipetted into a 400-μl microfuge tube containing 100 μl dinonylphthalate oil, and immediately centrifuged for 2 min at 6,000*g* at room temperature. The tubes were cut through the oil layer, the tube fragments containing the packed cells were dropped into polyethylene vials containing 4 ml liquid scintillation fluid, and the cell-associated radioactivity was measured in a β-counter. The level of nonspecific radioactivity in the cell pellet was determined by adding cytochalasin B to a set of control samples before incubation with D-glucose or 2-deoxy-D-glucose. These background counts were subtracted from all the values, and after the cell concentration was determined, the glucose uptake was normalized by cell number, as described by Digirolamo et al. (33).

Immunoblotting for protein phosphotyrosine levels, phospho-Akt, phospho-AMP kinase, and phospho-ACC. After treatment of the adipocytes, the incubation buffer was removed. The cells were then washed twice in KRBH containing 2.5 mmol/l glucose and disrupted into 1 ml lysis buffer (50 mmol/l HEPES [pH 7.4], 150 mmol/l NaCl, 1% (vol/vol) Triton X-100, 5 mmol/l EDTA, 5 mmol/l EGTA, 20 mmol/l Na pyrophosphate, 20 mmol/l NaF, 1 mmol/l Na orthovanadate, 1 mmol/l β-glycerophosphate, 1 mmol/l phenylmethylsulfonyl fluoride, and proteinase inhibitor cocktail). The cell lysate was sonicated twice for 10 s each on ice and centrifuged at 2,000 rpm for 10 min and 12,000 rpm for 20 min at 4°C to gradually remove the fat layer. The lysate supernatant was then isolated by centrifugation at 100,000*g* for 1 h and the protein concentration was estimated using Bio-Rad protein dye reagent, as described by the manufacturer.

For evaluation of insulin receptor and IRS-1 phosphotyrosine levels, lysates from the treated adipocytes containing 200 μg protein were first incubated with 1.0 μg insulin receptor or IRS-1 antibody overnight at 4°C, then incubated with 40 μl protein A agarose beads for 2 h at 4°C. The agarose was pelleted by centrifugation at 2,000 rpm for 2 min and washed four times in cell lysis buffer. The agarose beads were mixed with 20 μl SDS protein sample buffer and boiled at 100°C for 5 min. After being centrifuged at 4,000 rpm for 2 min, the supernatant was resolved by SDS-PAGE (7.5% wt/vol) and transferred to polyvinylidine fluoride (PVDF) membrane. Primary blotting antibodies were used according to the manufacturer's instructions. Visualization of bound antibodies was performed by incubation with horseradish peroxidase−conjugated secondary antibodies and then enhanced chemiluminescence, exposure to X-ray film, and quantitation using the Image Station 440CF (Kodak, Rochester, NY). To normalize the blots for protein levels, after being immunoblotted with anti-phosphospecific antibodies, the blots were treated for 20 min at 37°C using a stripping buffer solution (Pierce), washed several times with Tris-buffered saline with Tween-20 (0.05% vol/vol), and reprobed using polyclonal antibodies to Akt, AMP kinase, or ACC.

Immunoprecipitation and AMP kinase enzyme assay. Cell lysates were prepared as described for immunoblotting. Aliquots containing 200 μg of lysate protein were incubated with 1.0 μg anti−AMP kinase α1 + α2 or anti−AMP kinase α2 polyclonal rabbit antibody overnight at 4°C, then incubated with 40 μl protein A agarose beads for 2 h at 4°C. The agarose was washed four times using AMP kinase reaction buffer (20 mmol/l HEPES-NaOH [pH 7.0], 0.4 mmol/l dithiothreitol with 300 μmol AMP) and then incubated with 20 μl AMP kinase reaction buffer, 10 μl SAMS substrate peptide (80 μmol/l final concentration), and 10 μCi [γ-^{32}P]ATP mixed with 75 mmol/l magnesium chloride and 500 μmol unlabeled ATP for 15 min at 30°C. Aliquots of 35 μl were spotted onto the center of a 2-cm square of Whatman P81 paper.

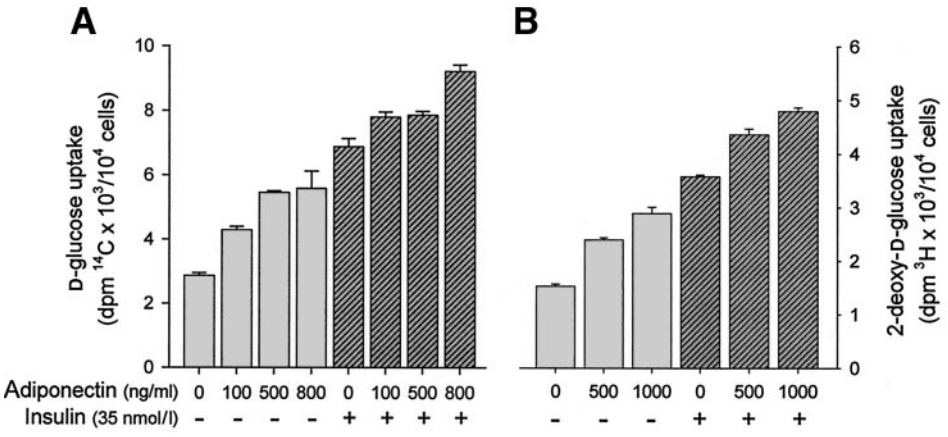

FIG. 1. Effect of adiponectin globular domain and insulin on uptake of D-glucose and 2-deoxy-D-glucose in rat adipocytes. Isolated rat adipocytes were treated with the recombinant globular domain of adiponectin at the concentration shown for 2 h with or without stimulation with insulin (35 nmol/l) for 5 min before measurement of uptake of D-[^{14}C]glucose (*A*) or 2-deoxy-D-[^{3}H]glucose (*B*), as described in RESEARCH DESIGN AND METHODS.

The P81 squares were washed three times with 0.75% (vol/vol) phosphoric acid and once with acetone and then transferred to a vial containing 5 ml of scintillation cocktail. The radioactivity was then counted in a β-counter. AMP kinase activity was expressed as the phosphorylation of SAMS peptide (dpm per microgram protein per minute).

Statistical analyses. Quantitative data are expressed as means ± SE from at least three replicate determinations. Student's *t* test was used for comparing two group means, and ANOVA was used for multiple comparisons. Differences were regarded as significant at $P < 0.05$.

RESULTS

Globular domain of adiponectin enhances glucose uptake into mature rat adipocytes. The effect of globular adiponectin on glucose uptake in mature rat adipose cells was evaluated using both D-[^{14}C]glucose and 2-deoxy-D-[^{3}H]glucose (Fig. 1). Treatment with the adiponectin globular domain alone for 2 h at concentrations of 100–800 ng/ml increased D-glucose uptake by 50–100% (all $P <$ 0.001 vs. control). Stimulation with insulin alone for 5 min at a submaximal concentration (35 nmol/l) increased D-glucose uptake by 2.4-fold ($P < 0.001$). D-glucose uptake was further increased by co-stimulation with insulin and globular adiponectin, by up to 34% compared to insulin alone with 800 ng/ml globular adiponectin ($P = 0.002$). Using the 2-deoxy-D-glucose assay (Fig. 1*B*), globular adiponectin alone at 500 and 1,000 ng/ml enhanced deoxyglucose uptake by 57 and 89%, respectively (both $P <$ 0.001). Insulin (35 nmol/l) stimulated the uptake of 2-deoxy-D-glucose by 2.3-fold ($P < 0.001$). Stimulation with insulin and globular adiponectin increased the uptake of deoxyglucose by up to 82%, compared to samples with no insulin stimulation ($P < 0.001$). These data established that globular adiponectin increased basal glucose uptake and significantly enhanced insulin-stimulated glucose uptake in a dosage-dependent manner.

To evaluate the time course of globular adiponectin–stimulated glucose uptake, rat adipocytes were incubated with 1,000 ng/ml globular adiponectin for up to 3 h. The uptake of 2-deoxy-D-glucose was significantly increased by fivefold after treatment with globular adiponectin for 30 min, and the increase was sustained for at least an additional 2 h (Fig. 2).

Adiponectin globular domain reverses the inhibition of basal or insulin-stimulated glucose uptake by TNF-α in rat adipocytes. To determine whether globular adiponectin could reverse the inhibition of adipocyte glucose transport mediated by TNF-α, adipose cells were incubated with 500 ng/ml globular adiponectin for 1.5 h after treatment with or without an inhibitory concentration of 20 ng/ml TNF-α for 20 min (34), followed by 35 nmol/l insulin for the last 5 min of the cell incubation before measurement of 2-deoxy-D-glucose uptake (Fig. 3). TNF-α attenuated the stimulation of glucose uptake by globular adiponectin, insulin, and the two agents together by 45.0, 21.0, and 16.7%, respectively ($P < 0.01$). Compared to samples treated with TNF-α alone or TNF-α with insulin, stimulation by globular adiponectin enhanced glucose uptake by 89 and 31%, respectively (both $P < 0.01$), effectively reversing the suppression of glucose uptake by TNF-α (Fig. 3).

Adiponectin globular domain does not affect insulin-stimulated tyrosine phosphorylation of the insulin receptor or IRS-1 or serine phosphorylation of Akt in mature rat adipocytes. To evaluate the signaling pathways used by globular adiponectin in the adipose cells, we first tested whether globular adiponectin enhanced the level of tyrosine phosphorylation of the insulin receptor or IRS-1. Cellular stimulation under conditions identical to those used to enhance glucose transport noted above significantly increased the tyrosine phosphorylation of the

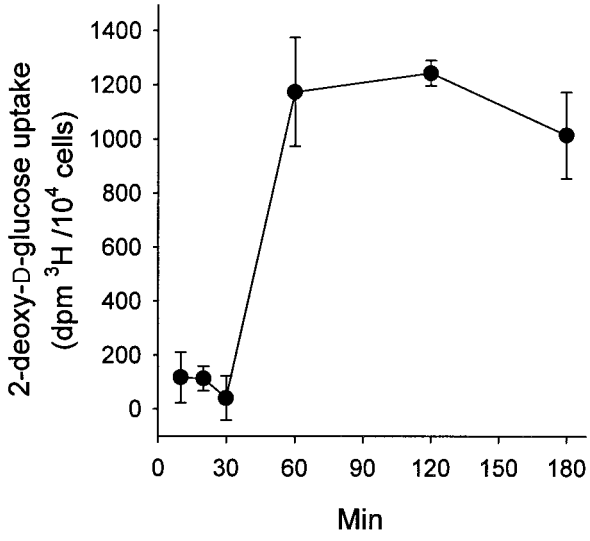

FIG. 2. Time course of adiponectin globular domain stimulation of 2-deoxy-D-glucose uptake in rat adipocytes. Isolated rat adipocytes were treated with the globular domain of adiponectin (1,000 ng/ml) for the indicated time period before measurement of uptake of 2-deoxy-D-[^{3}H]glucose, as described in RESEARCH DESIGN AND METHODS.

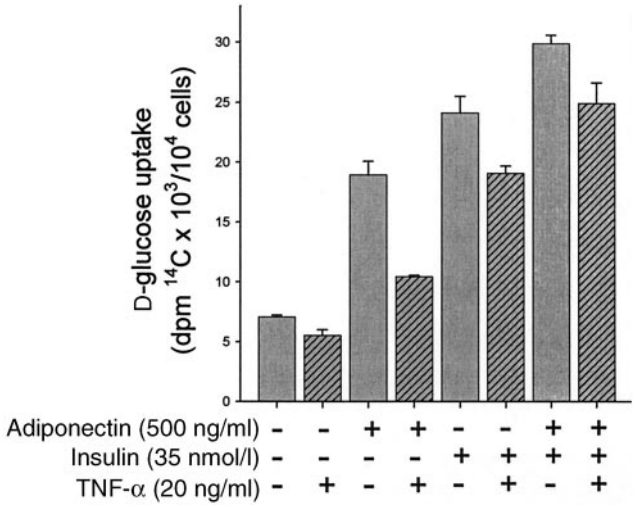

FIG. 3. Effect of adiponectin globular domain and insulin on suppression of glucose transport by TNF-α in rat adipocytes. Isolated adipose cells were treated where indicated with the globular domain of adiponectin (500 ng/ml) for 2 h, TNF-α (20 ng/ml) for 20 min, and/or insulin (35 nmol/l) for 5 min before measurement of the uptake of 2-deoxy-D-[^{3}H]glucose, as described in RESEARCH DESIGN AND METHODS.

insulin receptor and IRS-1 in immunoprecipitated cell lysates (Fig. 4). In contrast, globular adiponectin treatment of the adipocytes had no effect on insulin receptor and IRS-1 tyrosine phosphorylation. Similarly, the downstream activation of Akt was significantly enhanced by insulin under these conditions, whereas globular adiponectin had no effect on Akt Ser-473 phosphorylation (Fig. 4).

Adiponectin globular domain stimulates phosphorylation and activity of AMP-activated protein kinase in rat adipocytes. To evaluate the potential role of AMP kinase in the cellular effects of globular adiponectin in rat adipocytes, lysates from cells incubated with globular adiponectin were blotted with phospho-AMP kinase (Thr-172) antibody (Fig. 5*A*). As quantitated in Fig. 5*B*, over the dosage range of 250–1,000 ng/ml, globular adiponectin increased the phosphorylation of AMP kinase up to twofold.

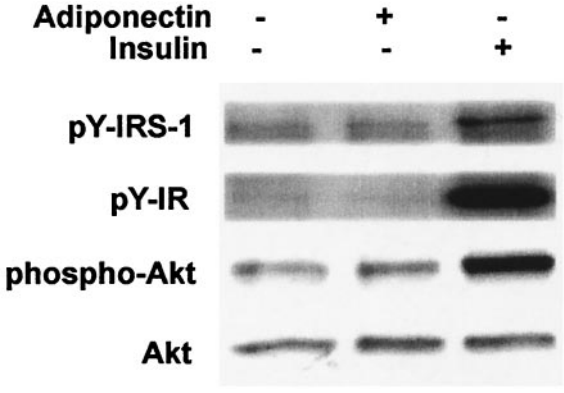

FIG. 4. Effect of adiponectin globular domain or insulin on protein-tyrosine phosphorylation of the insulin receptor and IRS-1 and Ser-473 phosphorylation of Akt in rat adipocytes. Isolated adipose cells were treated where indicated with the adiponectin globular domain (500 ng/ml) for 2 h or insulin (100 nmol/l) for 5 min. Cell lysates were subjected to immunoprecipitation with anti–insulin receptor β-subunit antibody or anti-IRS-1 antibody, and the immunoadsorbed samples were subjected to SDS-PAGE, transferred to PVDF membranes, and immunoblotted with monoclonal anti-phosphotyrosine antibodies (4G10), as described in RESEARCH DESIGN AND METHODS. Samples of the same cell lysates were also directly electrophoresed in SDS gels and subjected to immunoblotting with anti-phospho-Akt (Ser-473) antibodies as shown, after which the membranes were stripped and re-probed to normalize the blotted samples with antibody to Akt protein.

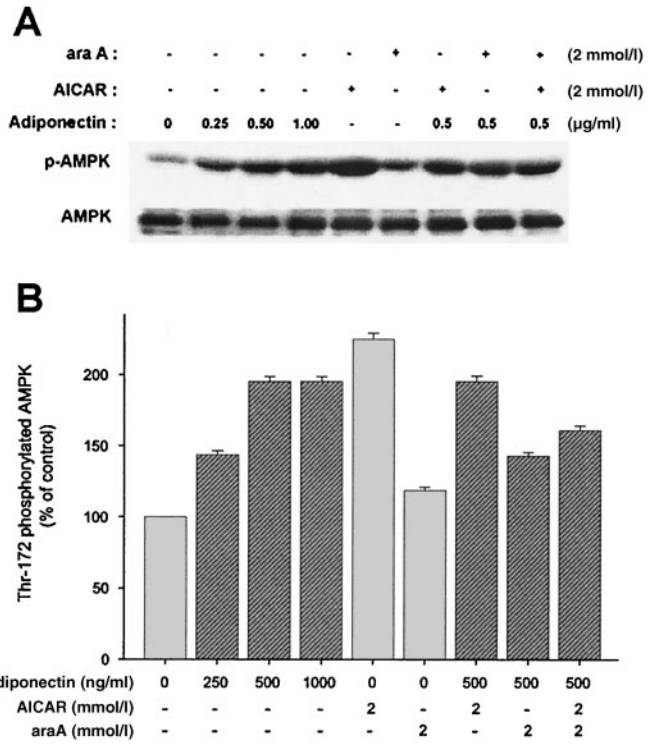

FIG. 5. Effect of adiponectin globular domain on the phosphorylation (Thr-172) of AMP kinase in rat adipocytes. After treatment of isolated adipose cells with the indicated concentration of the adiponectin globular domain for 2 h, cell lysates were directly electrophoresed in SDS-gels and subjected to immunoblotting with anti-phospho-AMP kinase (Thr-172) antibodies (indicated by p-AMPK). Where shown, some samples were also treated with the indicated concentration of araA starting at 20 min before the addition of globular adiponectin or AICAR during the final 30 min of incubation before cell lysis and immunoblotting. The membranes were also stripped and re-probed to normalize the blotted samples with antibody to the level of AMP α-subunit protein, as described in RESEARCH DESIGN AND METHODS. *A*: representative immunoblot; *B*: quantitated, normalized immunoblot data from several experiments as shown in *A*.

The time course of globular adiponectin–induced AMP kinase phosphorylation was also evaluated (Fig. 6). Using 1,000 ng/ml globular adiponectin, the phosphorylation of AMP kinase was significantly increased starting at 30 min of incubation. A saturating level of activation was reached at an increase of ~1.8-fold by 2–3 h. The overall time course of increased AMP kinase phosphorylation was significantly delayed compared to insulin activation of the insulin signaling pathway (Fig. 4) and, interestingly, preceded the activation of glucose uptake by globular adiponectin, which did not appear until after 30 min of stimulation (Fig. 2).

In control experiments, cells treated with the AMP kinase activator AICAR alone increased AMP kinase phosphorylation more than twofold compared to controls (Fig. 5*B*). Using samples of cell lysates immunoprecipitated with the AMP kinase α2 subunit antibody, globular adiponectin (250–1,000 ng/ml) was also shown to significantly increase the activity of AMP kinase by up to 47% ($P < 0.001$) (Fig. 7*A*). Similarly, the immunoprecipitates of

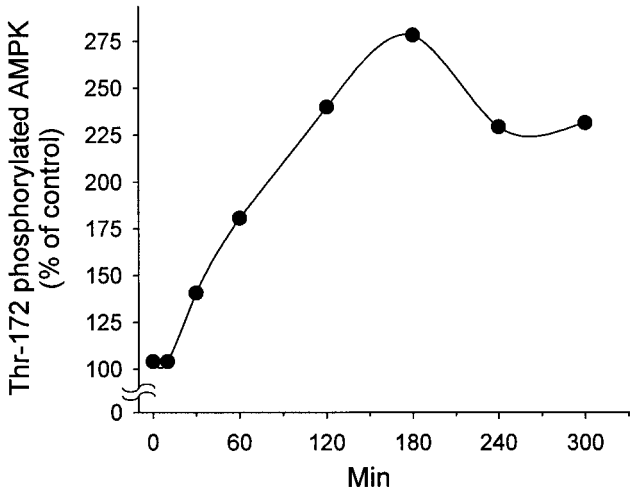

FIG. 6. Time course of adiponectin globular domain stimulation of the phosphorylation (Thr-172) of AMP kinase in rat adipocytes. After treatment of isolated adipose cells with 1,000 ng/ml of the adiponectin globular domain for the indicated period of time, cell lysates were directly electrophoresed in SDS-gels and subjected to immunoblotting with anti-phospho-AMP kinase (Thr-172) antibodies. The membranes were stripped and re-probed to normalize the blotted samples with antibody to the level of AMP α-subunit protein, as described in the legend to Fig. 5.

AMP kinase α1 + α2 showed increased activity by up to 24% (Fig. 7*B*).

araA, a metabolic precursor of araATP, is an intracellular competitive inhibitor of AMP kinase and blocks AMP kinase–related glucose uptake in skeletal muscle (35,36). Treatment of the cells with araA had no effect on basal AMP kinase (Thr-172) phosphorylation or enzyme activity in α2 or α1 + α2 isoform immunoprecipitates (Figs. 5 and 7). However, araA significantly decreased AMP kinase phosphorylation stimulated by globular adiponectin treatment as well as by globular adiponectin and AICAR (Fig. 5). Importantly, araA fully suppressed the enzyme activity of AMP kinase α2 and α1 + α2 isoforms stimulated by globular adiponectin (Fig. 7). Less of an effect was noted on araA suppression of α2 isoform activity in immunoprecipitates from cells also treated with AICAR (Fig. 7*A*).

Adiponectin globular domain enhances the phosphorylation of acetyl CoA carboxylase. ACC is the rate-controlling enzyme downstream of AMP kinase for fatty acid synthesis and generation of intracellular malonyl-CoA, which regulates fatty acid oxidation (37). Consistent with the upstream activation of AMP kinase discussed above, treatment of adipocytes with globular adiponectin (250–1,000 ng/ml) also enhanced the phosphorylation (Ser-79) of ACC by up to 86% compared to the basal level ($P < 0.001$) (Fig. 8). AICAR treatment also increased ACC phosphorylation by more than twofold, whereas araA decreased the basal phosphorylation of ACC by 20%. Consistent with the inhibitory effect of araA on AMP kinase activation by globular adiponectin treatment, araA also dramatically suppressed the level of ACC phosphorylation stimulated by globular adiponectin.

Inhibition of AMP kinase activation blocks glucose uptake stimulated by the adiponectin globular domain but not by insulin. Two inhibitors of AMP kinase were used to characterize the role of this enzyme in globular adiponectin–stimulated glucose uptake: araA

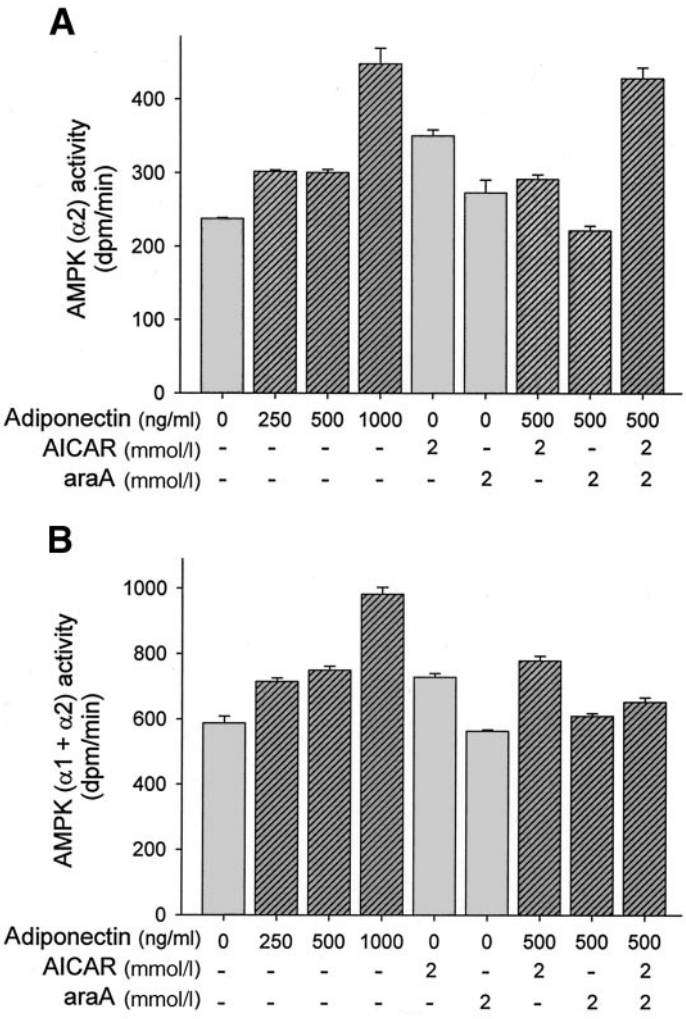

FIG. 7. Adiponectin globular domain stimulation of AMP kinase enzyme activity in immunoprecipitates from rat adipocytes. After treatment of isolated adipose cells as described in the legend to Fig. 5, cell lysates were prepared and samples were subjected to immunoprecipitation with antibodies to the AMP kinase α2 subunit (*A*) or with antibodies reacting with both α 1 + α2 AMP kinase subunit isoforms (*B*). AMP kinase enzyme activity was then measured in the washed immunoprecipitates as described in RESEARCH DESIGN AND METHODS.

and compound C. Adipocytes were preincubated with or without 2 mmol/l araA or 10 μmol/l compound C for 20 min after treatment with 500 ng/ml adiponectin globular domain for 2 h; then uptake of 2-deoxy-D-[^{3}H]glucose was measured (Fig. 9). Where indicated, cells were also stimulated with or without insulin (35 nmol/l) for 5 min before assay of glucose uptake. Adiponectin globular domain alone, insulin alone, and globular adiponectin plus insulin enhanced the uptake of 2-deoxyglucose by 2.1-, 2.3-, and 2.6-fold, respectively ($P < 0.001$). Treatment of cells with araA or compound C had no effect on the level of basal or insulin-stimulated glucose uptake. However, both araA and compound C completely suppressed the level of glucose uptake stimulated by globular adiponectin.

DISCUSSION

Over the past few years, our understanding of adiponectin as a potential key regulator of glucose and fat homeostasis as well as of vascular function in states of obesity and insulin resistance has grown dramatically (38,39). More

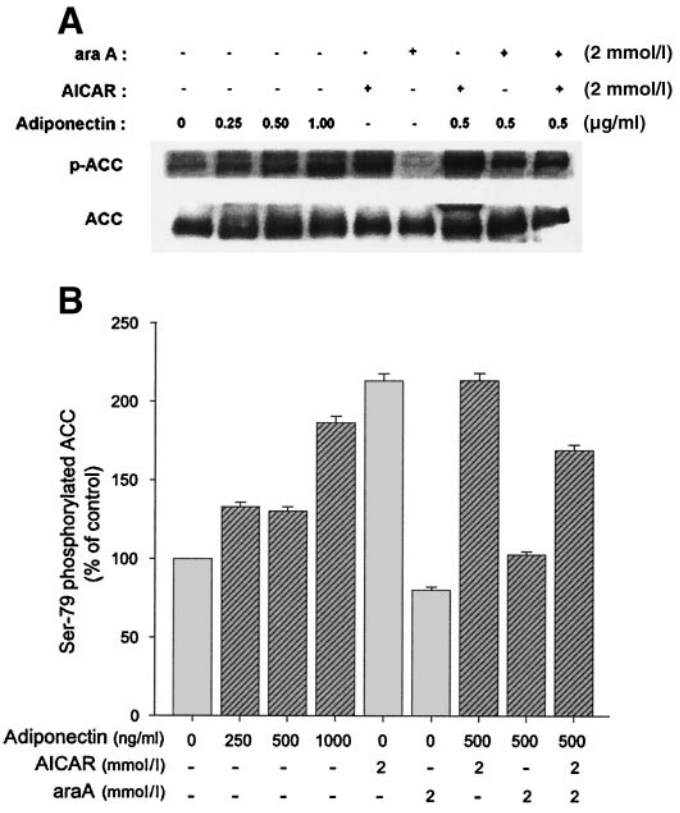

FIG. 8. Effect of adiponectin globular domain on the phosphorylation (Ser-79) of ACC in rat adipocytes. After treatment of isolated adipose cells with the indicated concentration of the adiponectin globular domain for 2 h, cell lysates were directly electrophoresed in SDS-gels and subjected to immunoblotting with anti-phospho-ACC (Ser-79) antibodies (indicated by p-ACC). Where shown, some samples were also treated with araA or AICAR, as described in the legend to Fig. 5. The membranes were also stripped and re-probed to normalize the blotted samples with antibody to the level of ACC enzyme protein, as described in RESEARCH DESIGN AND METHODS. *A*: representative immunoblot; *B*: quantitated, normalized immunoblot data pooled from data obtained as shown in *A*.

recently, insight has been gained into some of the properties of this protein in cellular signal transduction mechanisms, particularly in skeletal muscle and liver tissue; however, previous reports have not examined adiponectin action in adipose cells. In the present work, we demonstrated that the adiponectin globular domain alone has significant effects on glucose uptake in adipose tissue and that globular adiponectin also enhances insulin-stimulated glucose uptake and opposes TNF-α–inhibited glucose uptake in isolated adipocytes. Interestingly, the mechanism of globular adiponectin action in these cells does not involve changes in the tyrosine phosphorylation of key regulatory proteins in the insulin action pathway, but does involve activation of AMP kinase, providing a link between adiponectin signaling in skeletal muscle and liver with adipose tissue.

In contrast to our data in adipose tissue, adiponectin has recently been shown to enhance insulin-stimulated receptor tyrosine phosphorylation in skeletal muscle in animals and human subjects. Adiponectin is positively associated with insulin sensitivity and receptor tyrosine phosphorylation in skeletal muscle in human subjects (40). Administration of the adiponectin globular domain to lipoatrophic mice deficient in circulating adiponectin also potentiates insulin-stimulated tyrosine phosphorylation of the insulin receptor and IRS-1 and enhanced Akt activation in skeletal muscle (16). These findings suggest that the mechanism of some of the regulatory effects of adiponectin on cellular signaling may be different between adipose and skeletal muscle tissue. Furthermore, because our data were obtained by directly treating isolated adipocytes with the globular domain of adiponectin in vitro, it is possible that some of the effects observed with adiponectin on skeletal muscle in vivo were indirect.

In isolated adipose cells, globular adiponectin increases the threonine phosphorylation of AMP kinase, activates AMP kinase enzyme activity, and enhances the phosphorylation of ACC, a metabolic enzyme known to be downstream of AMP kinase (37). Moreover, we found that globular adiponectin–induced glucose uptake can be blocked by inhibitors of AMP kinase, indicating that AMP kinase activation mediates the effects of globular adiponectin on glucose uptake. This is in contrast to insulin-stimulated glucose uptake, which is not affected by the inhibitors of AMP kinase. AMP kinase is a highly conserved heterotrimeric signaling kinase responsive to hyp-

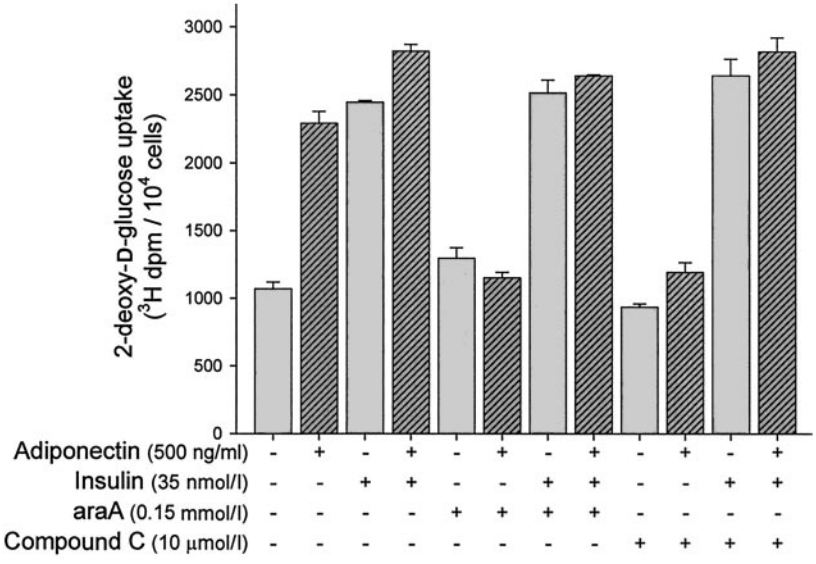

FIG. 9. Effect of AMP kinase inhibition on the stimulation of 2-deoxyglucose uptake by the adiponectin globular domain and insulin in rat adipocytes. After treatment of isolated rat adipocytes with globular adiponectin (500 ng/ml for 2 h) with or without insulin (35 nmol/l for the final 5 min of incubation) and also with araA and compound C, where indicated, uptake of 2-deoxy-D-[^{3}H]glucose was measured, as described in RESEARCH DESIGN AND METHODS.

oxia, exercise, and cellular stress that has been strongly implicated in a variety of cellular responses, including suppression of gluconeogenesis in the liver, promotion of glucose uptake in skeletal muscle, inhibition of fatty acid and sterol synthesis, increases in fatty acid oxidation, and inhibition of lipolysis (23,37,41–43). The α_2 isoform of the catalytic subunit is predominant in liver, skeletal muscle, and endothelial cells (44,45); however, both subunits are reportedly expressed in 3T3-L1 adipocytes (46,47).

Only a few previous studies have focused on the potential role of AMP kinase in glucose uptake in adipose cells. The use of the AMP kinase activator AICAR in attempts to delineate a role of AMP kinase in glucose transport activation in adipocyte models, has generated inconclusive results. AICAR increases AMP kinase activity and enhances glucose uptake in muscle tissue (36,48). Although treatment of differentiated 3T3-L1 adipocytes with AICAR reportedly enhanced glucose transport by up to twofold, overexpression of a dominant negative AMP kinase α2 construct abolished AMP kinase activation without affecting glucose transport, raising questions about whether AMP kinase is directly involved in AICAR-stimulated glucose transport in this adipocyte model (47,49). In the primary adipocytes, we also found that AICAR significantly increased AMP kinase phosphorylation at Thr-172 (Fig. 5), but did not increase AMP kinase enzyme activity (Fig. 7) or glucose uptake (data not shown) to a similar degree. As an intermediate in de novo purine biosynthesis, AICAR may exert additional cellular effects and may not be specific for AMP kinase activation (50). This may potentially explain why AICAR has little effect on adipocyte glucose uptake. Also, globular adiponectin may exert effects through additional pathways that may influence the ultimate stimulation of glucose uptake by globular adiponectin in addition to its enhancement of AMP kinase activity.

Studies in other cellular systems (including undifferentiated 3T3-L1 cells, C2C12 myoblasts, and clone 9 cells) have also implicated AMP kinase in GLUT1-mediated glucose transport, including actual activation of GLUT1 transporters preexisting in the plasma membrane (51,52). The mechanism(s) by which AMP kinase enhances cellular glucose transport has been best studied in skeletal muscle, where it has been linked to changes in GLUT4 transcription and translocation (41,48,53). Clearly, further work in this area may help to distinguish AMP kinase signaling mechanisms involving glucose uptake in muscle and adipose tissue.

The potent effect of araA and compound C in blocking globular adiponectin–stimulated glucose uptake strongly suggests a role for AMP kinase in the signaling pathway for insulin-independent glucose uptake stimulated by globular adiponectin in adipose cells. Our data demonstrating that araA decreases globular adiponectin–stimulated AMP kinase activation and ACC phosphorylation also provide further evidence in support of previous work showing that araA serves as an effective inhibitor of AMP kinase (35,36). The use of these inhibitors also helps to differentiate the mechanism of the globular adiponectin effect from that of insulin-stimulated glucose uptake, which was not affected by the AMP kinase inhibitors. Recently, AMP kinase has also been implicated in the action of adiponectin in skeletal muscle and liver tissue. Yamauchi et al. (22) first reported that AMP kinase is activated in mouse skeletal muscle by treatment in vivo with either the globular domain or full-length adiponectin, and that AMP kinase activation mediates the stimulation of glucose utilization and fatty acid oxidation by adiponectin. Interestingly, in this work, only full-length adiponectin was active in the liver to stimulate AMP kinase and metabolic signaling (22). Tomas et al. (21) have also reported that treatment of fast-twitch skeletal muscle with the globular domain of adiponectin increased AMP kinase activity twofold within 30 min, which was also associated with increased serine phosphorylation of ACC and a 1.5-fold increase in 2-deoxyglucose uptake. In contrast, the full-length hexameric form of adiponectin did not activate AMP kinase or ACC in fast-twitch muscle, and the globular adiponectin domain did not affect AMP kinase or glucose transport in slow-twitch muscle. Clearly, additional work will be necessary to further characterize whether the various molecular forms of adiponectin differ in their effects on AMP kinase activation or downstream signaling in these major tissues responsible for metabolic regulation.

The action of globular adiponectin in adipocytes also reverses the inhibitory effect of TNF-α on insulin-stimulated glucose uptake. The adverse effect of TNF-α has been previously shown to involve early events in insulin signaling involving insulin receptor autophosphorylation and IRS-1 tyrosine phosphorylation in fat tissue (54,55). Some of the beneficial effects of adiponectin on vascular cells appear to be mediated by inhibition of the deleterious actions of TNF-α, which can initiate an inflammatory cascade in obesity and insulin-resistant states (56). In cultured myocytes, TNF-α decreased and adiponectin enhanced FATP-1 mRNA, IRS-1–associated phosphatidylinositol 3-kinase activity, and glucose uptake (57). Without blocking TNF-α binding, adiponectin inhibits TNF-α–induced expression of adhesion molecules on cultured endothelial cells and TNF-α–induced endothelial adhesion of monocytic cells (6), an action likely mediated by adiponectin suppression of TNF-α–induced Iκ-B phosphorylation and nuclear factor-κB activation (58). Thus, adiponectin may play an important salutary role, potentially using shared signaling pathways in metabolic as well as in vascular cell types.

The finding that glucose uptake is enhanced by globular adiponectin in adipose cells is consistent with the observations that human subjects with type 2 diabetes have low circulating concentrations of adiponectin (9) and Pima Indians with high circulating concentrations of adiponectin are significantly less likely to develop hyperglycemia (59). The identification of enhanced glucose uptake in response to adiponectin in a mature primary cell type, and the elucidation of the role of the AMP kinase pathway in this process, provides a novel system for further detailed analysis of the adiponectin signal transduction cascade.

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