Adiponectin Sensitizes Insulin Signaling by Reducing p70 S6 Kinase-mediated Serine Phosphorylation of IRS-1

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Adiponectin functions as an insulin sensitizer, and yet the underlying molecular mechanism(s) remains largely unknown. We found that treating C2C12 myotubes with adiponectin or rapamycin enhanced the ability of insulin to stimulate IRS-1 tyrosine phosphorylation and Akt phosphorylation, concurrently with reduced p70 S6 kinase phosphorylation at Thr389 as well as IRS-1 phosphorylation at Ser302 and Ser636/639. Overexpression of dominant-negative AMP kinase (AMPK), but not dominant-negative p38 MAPK, reduced the insulin-sensitizing effect of adiponectin. Rapamycin, but not adiponectin, enhanced insulin-stimulated Akt phosphorylation in HeLa cells, which lack LKB1, and exogenous expression of LKB1 in HeLa cells rescued the insulin-sensitizing effect of adiponectin. Finally, overexpression of wild-type Rheb (Ras homology-enriched in brain) or the TSC2 mutant lacking the AMPK phosphorylation site (TSC2S1345A) inhibited the insulin-sensitizing effect of adiponectin in C2C12 cells. These results indicate that activation of the LKB1/AMPK/TSC1/2 pathway alleviates the p70 S6 kinase-mediated negative regulation of insulin signaling, providing a mechanism by which adiponectin increases insulin sensitivity in cells.

Adiponectin (Acrp30, AdipoQ, ApM1, and GBP28) is a collag-en-like adipokine that has anti-atherogenic, anti-diabetic, and insulin-sensitizing properties (1–3). Mice lacking adiponectin have severe hepatic insulin resistance (4), and administration of adiponectin to animal models of type 2 diabetes and insulin resistance significantly enhances insulin sensitivity (5). In humans, adiponectin levels are low in the plasma of obese and type 2 diabetes subjects (6–8). The combined data suggest that adiponectin plays an important role in sensitizing insulin action.

The molecular mechanism by which adiponectin acts as an insulin sensitizer remains largely unknown. Insulin initiates its action by binding to its membrane receptor, leading to tyrosine phosphorylation and activation of the insulin receptor (IR). A major pathway downstream of IR is the phosphatidylinositol 3-kinase (PI3K) signaling pathway, which mediates insulin-stimulated GLUT4 membrane translocation and glucose uptake. Recent studies have shown that the PI3K pathway is negatively regulated by the TOR complex 1 (TORC1)-induced signaling pathway, which is mediated by the S6K-dependent phosphorylation of IRS-1 at several sites including Ser302, Ser307, and Ser636/639 (9–12). The inhibitory effect of S6K is greatly enhanced in cells lacking the tuberous sclerosis complex (TSC1/2), suggesting that TSC1/2 is a negative regulator of the mTOR/S6K signaling pathway (13). Interestingly, TSC1/2 activity is stimulated by AMP kinase (AMPK) (14), a heterotrimeric serine/threonine protein kinase, which is activated by adiponectin (15). However, whether activation of AMPK by adiponectin leads to inhibition of S6K and whether this inhibition plays a role in the insulin sensitizing effect of adiponectin remain unknown.

In the present study, we have shown that adiponectin sensitizes insulin signaling in C2C12 myotubes. In addition, we found that treatment of cells with adiponectin inhibits S6K phosphorylation at Thr389, an indicator of reduced S6K activity. The insulin-sensitizing effect of adiponectin was suppressed in cells lacking TSC1/2 or LKB1, or overexpressing Rheb, a dominant-negative (DN) form of AMPK, or a mutant TSC2 lacking the AMPK phosphorylation site (TSC2S1345A). These results indicate that activation of the LKB1/AMPK/TSC pathway, which relieves the negative regulation of mTOR/S6K via inhibition of Rheb, may provide a molecular mechanism by which adiponectin increases insulin sensitivity in cells.

EXPERIMENTAL PROCEDURES

Reagents—Rapamycin was purchased from Sigma. Anti-IRS-1 and anti-IR antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Antibodies and phosphospecific antibodies for Akt, p70S6K, AMPK, p38 MAPK, and IRS-1 were purchased from Cell Signaling Technology (Beverly, MA). The phosphotyrosine antibody (4G10) was purchased from Upstate Biotechnology (Lake Placid, NY). His-tagged gAdiponec-tin was produced as described in our recent work (16).

Plasmids—cDNAs encoding full-length LKB1 or Akt were cloned into the mammalian expression vector pcDNA3.1/ Myc-Hist (+) (Invitrogen). Plasmids encoding Wt-TSC2 and MutTSC2 (S1345A) tagged with hemagglutinin constructs were

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2 The abbreviations used are: IR, insulin receptor; AMPK, AMP kinase; DN, dominant negative; IRS, IR substrate; mTOR, mammalian target of rapamycin; Rheb, Ras homology enriched in brain; S6K, p70 S6 kinase; TORC, TOR complex; TSC, tuberous sclerosis complex; PI3K, phosphatidylinositol 3-kinase; MAPK, mitogen-activated protein kinase; DMEM, Dulbecco’s modified Eagle’s medium.
described previously (14). Plasmids encoding wild-type AMPK and DN-AMPK (K45R), wild-type and DN-p38 MAPK, and wild-type and DN-Rheb (D60K) were generous gifts from Drs. Michael Quon, Jiahuai Han, and Jie Chen, respectively.

**Cell Lines, Transfection, and Western Blot**—C2C12 cells were maintained in DMEM (ATCC) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. Differentiation of C2C12 cells to myotubes was done by growing cells in low-serum medium (98% DMEM, 2% (v/v) horse serum, 4.0 mM glutamine, 25 mM Hepes, pH 7.4). TSC1−/−, TSC2−/−, and wild-type control mouse embryonic fibroblasts were generous gifts of Dr. David Kwiatkowski (17). These cells were maintained in DMEM with 10% fetal calf serum. HeLa cells were maintained in DMEM with 10% fetal calf serum, 1% penicillin/streptomycin. All cell lines were grown at 37 °C with 5% CO₂.

Transfections were performed using Lipofectamine or Lipofectamine 2000 according to the manufacturer’s protocol (Invitrogen). Cells were normally serum-starved for 4–6 h before treatment. Cells were lysed with lysis buffer (50 mM Hepes, pH 7.6, 150 mM NaCl, 1% Triton X-100, 10 mM NaF, 20 mM sodium pyrophosphate, 20 mM β-glycerol phosphate, 1 mM sodium orthovanadate, 10 μg/ml leupeptin, 10 μg/ml aprotinin, and 1 mM phenylmethanesulfonyl fluoride). After centrifugation, the cell lysates were denatured, separated by SDS-PAGE, and analyzed by Western blot using specific antibodies as indicated in the figure legends. Western blot results are typically representative of at least three independent experiments with similar results.

**Quantification and Statistics**—Quantification of the relative increase in insulin-stimulated protein phosphorylation was performed by analyzing Western blots using the Scion Image software and was normalized with the amount of protein expression in each experiment. Values presented are mean values ± S.E. Statistical significance was determined by analysis of variance (ANOVA).

**RESULTS AND DISCUSSION**

To elucidate the molecular mechanisms underlying the insulin sensitizing effect of adiponectin, we treated C2C12 myotubes with insulin in the absence or presence of the globular form of adiponectin (gAd), which has been shown to bind to the adiponectin receptor-1 (AdipoR1) with a higher affinity than the full-length adiponectin (15). As expected, treatment of cells with insulin led to increased IRS-1 and Akt phosphorylation (Fig. 1, A, lane 2, and B, lane 3). Insulin-stimulated IRS-1 and Akt phosphorylation (Fig. 1, A, lane 2, and B, lane 3). Insulin-stimulated IRS-1 and Akt phosphorylation (Fig. 1, A, lane 2, and B, lane 3).
Akt phosphorylation was markedly increased in cells pretreated with adiponectin (Fig. 1A, lanes 3–5) or the S6K inhibitor rapamycin (Fig. 1B, lanes 4–6). A similar increase in insulin-stimulated IRS-1 and Akt phosphorylation was also observed in mouse hepatocytes pretreated with full-length adiponectin (data not shown). Interestingly, the adiponectin or rapamycin-enhanced insulin signaling correlated with a reduction of S6K phosphorylation at Thr389, which also correlated with S6K activity (Fig. 1A and B, fifth panel from top). Because activation of S6K has been shown to negatively regulate insulin-stimulated IRS-1 tyrosine phosphorylation and downstream signaling (12, 13), these results suggest that adiponectin may increase insulin sensitivity by alleviating the inhibitory effect of S6K. Consistent with this idea, suppression of S6K1 levels has been shown to significantly improve insulin sensitivity (18). In addition, we found that pretreatment of cells with rapamycin blunted the insulin sensitizing effect of adiponectin (Fig. 1C). On the other hand, treatment of C2C12 myotubes with adiponectin had no significant effect on insulin-stimulated IR tyrosine phosphorylation (Fig. 1D), suggesting that the insulin-sensitizing effect of adiponectin directly targets IRS-1 rather than the IR.

Adiponectin activates two major pathways in muscle cells, the AMPK and the p38 MAPK signaling pathways (15). To determine whether any or both of these signaling pathways are involved in mediating the insulin-sensitizing effect of adiponectin, we overexpressed DN-AMPK (19) or DN-p38 MAPK (20) in C2C12 cells. We found that overexpression of DN-AMPK, but not DN-p38 MAPK, significantly reduced the insulin-sensitizing effect of adiponectin on Akt phosphorylation (Fig. 2, A and B), which coincided with an increase in both basal and insulin-stimulated S6K phosphorylation and a decrease in insulin-stimulated tyrosine phosphorylation of IRS-1 (Fig. 2C). These results suggest that adiponectin may sensitize insulin signaling by inhibiting S6K through an AMPK-dependent signaling pathway.

One of the AMPK upstream kinases is LKB1, which phosphorylates AMPK on Thr172 in the activation loop (21). To determine whether adiponectin-stimulated AMPK activation is mediated by LKB1, we examined the effect of adiponectin on AMPK phosphorylation in HeLa cells, which express the adiponectin receptor AdipoR1 (data not shown) but not LKB1 (22). We found that treatment of HeLa cells with the globular form of adiponectin led to a small but notable increase in insulin-stimulated IRS-1 tyrosine phosphorylation (Fig. 3A, lane 4 versus 2) and Akt phosphorylation at Thr308 (Fig. 3B, lane 4 versus 2). Interestingly, the same anti-Akt antibody, which detected only a single band in C2C12 cells (Fig. 1), recognized two protein bands in the HeLa cell lysates (Fig. 3B, lower panel), which is consistent with the findings of others (23). The slower migrating band corresponds to the phospho-Akt band as shown in the top panel (Fig. 3B), but the identity of the faster migrating band is currently unknown. The insulin-sensitizing effect of adiponectin was greatly enhanced in HeLa cells transiently expressing LKB1 (Fig. 3, A and B, lane 6 versus 4). Together, these results suggest that adiponectin may sensitize insulin signaling via pathways dependent and independent of LKB1, which is consistent with the findings that AMPK is activated by both LKB1 and the Ca$^{2+}$-dependent kinase CaMKKβ (24).

Unlike adiponectin, rapamycin enhanced insulin-induced Akt and IRS-1 phosphorylation in HeLa cells in a dose-dependent manner (Fig. 3D), which suggested that rapamycin increased insulin sensitivity via a mTOR/S6K-dependent but LKB1-independent mechanism.
AMPK phosphorylates and activates TSC2 in response to cellular energy starvation (14). Activated TSC2 together with TSC1 negatively regulates the TOR complex 1 (TORC1) upstream activator, termed Rheb, leading to inhibition of mTOR/S6K (21). Consistent with results from others (25), insulin-stimulated Akt phosphorylation was greatly inhibited in both TSC1/+/H11002 and TSC2/+/H11002 mouse embryonic fibroblasts (data not shown). Overexpression of Wt-TSC2 in C2C12 cells moderately increased insulin-stimulated Akt phosphorylation at Thr308 (Fig. 4, top panel, lane 2 versus 5). In contrast, overexpression of a mutant TSC2 in which the AMPK phosphorylation site Ser1345 was replaced with alanine (14), markedly inhibited Akt phosphorylation induced by insulin or adiponectin plus insulin (Fig. 4, lanes 7–9). These results provide strong evidence for the involvement of the AMPK/TSC signaling pathway in the insulin-sensitizing effect of adiponectin.

Activated TSC2 together with TSC1 serves as a GTPase-activating protein, which subsequently inhibits the activity of the Rheb, leading to inhibition of mTOR/S6K (21). The effect of Rheb on TORC1 is specific, and Rheb does not activate TORC2 (27), which phosphorylates Akt at Ser473 (28), the so-called PDK2 or hydrophobic motif site (29). Overexpression of DN-Rheb inhibits not only the basal but also the nutrient- or serum-induced activity of p70S6K (30). In contrast, overexpression of wild-type Rheb leads to activation of mTOR and S6K (26). Consistent with the observation that activation of S6K negatively regulates insulin action (9–12), overexpression of DN-Rheb in C2C12 cells led to increased Akt phosphorylation in response to insulin and/or insulin plus adiponectin.
treatment, whereas overexpression of wild-type Rheb significantly inhibits the stimulatory effect of insulin and adiponectin (Fig. 5). These data suggest an involvement of Rheb in the cross-talk between adiponectin and insulin signaling pathways.

To further elucidate the mechanism by which adiponectin enhances insulin sensitivity, we examined IRS-1 serine phosphorylation by Western blot using phosphospecific antibodies. Both adiponectin (Fig. 6A) and rapamycin (Fig. 6B) inhibited insulin-stimulated Ser302 phosphorylation in C2C12 myotubes. These results are consistent with previous findings that phosphorylation of Ser302 negatively regulates IRS-1 function (12, 13), although controversial results exist on the roles of phosphorylation at this site (31, 32). In addition to Ser302, the insulin-induced Ser636/639 phosphorylation of IRS-1 was greatly inhibited by adiponectin or rapamycin (Fig. 6A and B, respectively). This finding is interesting because phosphorylation of Ser636/639 of IRS-1 by mTOR/S6K has recently been shown to interrupt the interaction between IRS-1 and PI3K, leading to reduced PI3K activity and its mediated downstream events (33). IRS-1 hyperphosphorylated at this site has also been observed in the skeletal muscle of type 2 diabetes subjects (34). Interestingly, adiponectin had little effect on insulin-stimulated IRS-1 phosphorylation at Ser302 (Fig. 6A). A major c-Jun N-terminal kinase (JNK)-mediated phosphorylation site (35). This finding suggests that inhibition of S6K, not JNK, plays a major role in the insulin sensitizing effect of adiponectin. This result is consistent with a recent finding that activation of AMPK reduces IRS-1 serine phosphorylation and potentiates insulin action in C2C12 cells (36).

Based on these findings, we describe a model of the cross-talk between the adiponectin and insulin signaling pathways (Fig. 7). In this model, adiponectin binds to its membrane receptors, AdipoR1 and AdipoR2, leading to recruitment of adaptor proteins such as APPL1 and activation of downstream kinases such as mTOR, S6K, PDK1, and Akt. AMPK activation further potentiates insulin signaling by reducing S6K activity and enhancing IRS tyrosine phosphorylation and insulin signaling.

FIGURE 5. **The role of Rheb in the insulin-sensitizing effect of adiponectin.** A, C2C12 cells were transfected with a plasmid encoding Myc-tagged Akt together with either a control plasmid or a plasmid encoding FLAG-tagged DN or wild-type (Wt) Rheb. At 18 h post transfection, cells were treated with 1.5 µg/ml globular adiponectin (gAd) for 20 min and then with 50 nM insulin for 5 min. Akt phosphorylation and protein expression were determined by Western blot using specific antibodies as indicated. B, quantification of Akt phosphorylation. The relative Akt phosphorylation levels were determined by densitometry and normalized with protein levels. *p < 0.05. PBS, phosphate-buffered saline; Ins, insulin.

FIGURE 6. **The effects of adiponectin and rapamycin on insulin-stimulated IRS-1 serine phosphorylation.** A, differentiated C2C12 myotubes were serum-starved and then treated with globular adiponectin (gAd) at different concentrations for 15 min followed by 10 nM insulin for 5 min. Cell lysates were analyzed by Western blot using specific antibodies as indicated. B, serum-starved C2C12 myotubes were treated with rapamycin (Rap) at different concentrations for 1 h followed by 10 nM insulin for 5 min. IRS-1 serine phosphorylation and protein expression were determined by Western blot using specific antibodies as indicated. Data are representative of at least three independent experiments with similar results.

FIGURE 7. **A model of the cross-talk between the adiponectin and insulin signaling pathways.** Adiponectin binds to the adiponectin receptor on the cell membrane, activating AMPK via LKB1-dependent and -independent pathways. Activation of the AMPK/TSC1/2 signaling pathway reduces mTOR/S6K-mediated serine phosphorylation of IRS proteins leading to enhanced IRS tyrosine phosphorylation and insulin signaling. P3K, phosphatidylinositol 3-kinase; APPL, adaptor protein containing pleckstrin homology domain, phosphotyrosine domain, and leucine zipper motif.
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as AMPK (16). The activated AMPK is able to phosphorylate TSC2 on Ser1108 and activate it. Activated TSC2 together with TSC1 serves as a GTPase-activating protein, which inhibits the activity of the Rheb. Inhibition of Rheb reduces mTOR/S6K-mediated serine phosphorylation of IRS, leading to enhanced Akt phosphorylation. This model provides a molecular mechanism by which adiponectin functions as an insulin sensitizer in cells.

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