Full-Length Adiponectin Attenuates Insulin Signaling and Inhibits Insulin-Stimulated Amino Acid Transport in Human Primary Trophoblast Cells

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OBJECTIVE—Maternal adiponectin levels are reduced and placental nutrient transporters are upregulated in obesity and gestational diabetes mellitus; however, the effects of adiponectin on placental function are unknown. We hypothesized that adiponectin regulates placental amino acid transport.

RESEARCH DESIGN AND METHODS—Human primary trophoblast cells were cultured and incubated with globular adiponectin (gAd) or full-length adiponectin (fAd) alone or in combination with insulin. System A and L amino acid transport and SNAT1, SNAT2, and SNAT4 isoform expression was measured. The activity of the AMP-activated protein kinase (AMPK), phosphatidylinositol 3 kinase–AKT, and peroxisome proliferator–activated receptor-α (PPARα) signaling pathways was determined.

RESULTS—In the absence of insulin, gAd stimulated AMPK Thr172 phosphorylation, SNAT2 protein expression, and system A activity. This effect appeared to be mediated by interleukin-6 release and signal transducer and activator of transcription 3 (STAT3) signaling because gAd failed to stimulate system A in cells in which STAT3 had been silenced using small interfering RNA. fAd alone had no effect on system A activity or SNAT expression. Insulin increased AKT and insulin receptor substrate 1 (IRS-1) phosphorylation, system A activity, and SNAT2 expression. When combined with insulin, gAd did not affect system A activity or SNAT expression. In contrast, fAd abolished insulin-stimulated AKT Thr308 and IRS-1 Tyr612 phosphorylation, system A activity, and SNAT2 expression. Furthermore, fAd increased PPARα expression and PPARα (Ser21) phosphorylation.

CONCLUSIONS—In contrast to the insulin-sensitizing actions of adiponectin in liver and muscle reported in the literature, fAd attenuates insulin signaling in primary human trophoblast cells. As a result, fAd inhibits insulin-stimulated amino acid transport, which may have important implications for placental nutrient transport and fetal growth in pregnancy complications associated with altered maternal adiponectin levels. Diabetes 59: 1161–1170, 2010

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states such as obesity and type 2 diabetes (17,18). The mechanisms underlying the increase in insulin sensitivity in response to adiponectin remains to be fully established and may include a multitude of pathways (19–21). For example, in skeletal muscle, adiponectin stimulates glucose transport by increased GLUT4 translocation (22), activates insulin signaling, and upregulates molecules involved in fatty acid transport, fatty acid oxidation, and energy dissipation, resulting in decreased triglyceride content (20). In the liver, adiponectin increases β-oxidation and decreases gluconeogenesis (12).

Although earlier reports suggested that adiponectin is produced and secreted by the human placenta (23,24), more recent studies show that adiponectin mRNA is not expressed in the placenta (25,26). However, the placenta is a likely target for circulating adiponectin because AdipoR2 mRNA is expressed in the human placenta (23,27), and AdipoR2 protein has been localized to the syncytiotrophoblast (23). Both AdipoR1 and AdipoR2 proteins were expressed in cytotrophoblast cells freshly isolated from human placenta as well as in human primary trophoblast cells in culture (28). Apart from reports that adiponectin stimulates placental cytokine production (29), reduces the gene expression of GLUT isoform 3 and lipoprotein lipase in rat placenta (23), and inhibits endocrine function (28), effects of adiponectin on placental function remain unknown.

There are many observations in the literature implicating changes in placental amino acid transport in the regulation of fetal growth (30). For example, placental system A amino acid transport is downregulated prior to the onset of impaired fetal growth in a rat model of intrauterine growth restriction (31). We recently reported markedly increased placental system A amino acid transport capacity associated with fetal overgrowth in mice fed a high-fat diet (32). Amino acid transport system A is ubiquitously expressed and mediates the cellular uptake of small, neutral amino acids by cotransporting sodium.

There are three isoforms of system A: SNAT1, SNAT2, and SNAT4 (30). In placental cells, the system A amino acid transporter is regulated by several hormones including insulin, cortisol, and leptin (30). Another key amino acid transporter is system L, which transports large neutral amino acids in a sodium-independent manner (33,34). Regulation of system L is dependent on which light chain is present, and studies show that regulation of large neutral amino acid transporter 1 involves protein kinase C or intracellular calcium concentrations (34). To the best of our knowledge, the regulation of amino acid transporter function by adiponectin has not been studied in any tissue. Interestingly, administration of gAd in pregnant rats was recently reported to decrease placental gene expression of GLUT isoform 3 and lipoprotein lipase (23), compatible with a role of maternal adiponectin in the regulation of placental nutrient transport.

The aim of this study was to determine the effects of gAd and fAd, with or without insulin, on intracellular signaling and amino acid transport in cultured primary human trophoblast cells. We tested the hypothesis that adiponectin downregulates placental amino acid transport mediated by inhibition of insulin signaling.

**RESEARCH DESIGN AND METHODS**

**Cytotrophoblast isolation.** Isolation of cytotrophoblasts from normal-term placentas was based on the protocol published by Kliman et al. (35) and performed as we reported previously (36,37). Cells were plated on 35-mm culture dishes (5 × 10⁶ cells/dish) and six-well plates (2 × 10⁵ cells/well) in 5% CO₂, 95% atmosphere air at 37°C. Media were changed daily for 90 h. At 66 h after plating, cells were exposed to control media or control media plus gAd or fAd for 24 h. A subset of cells was pretreated with 1 nmol/l insulin for 4 h, gAd or fAd was subsequently added, and cells were incubated for an additional 20 h. At 90 h after plating, cells were used in amino acid uptake assays or expression studies, and culture media were collected for cytokine analysis. The concentration of insulin used corresponds to normal postprandial insulin levels in pregnant women (38).

**Small interfering RNA.** Dharmafect2 transfection reagent (Thermo Scientific; Rockford, IL) and small interfering RNA (siRNA), targeted against glyceraldehyde-3-phosphate dehydrogenase, signal transducer and activator of transcription 3 (STAT3), or a scrambled sequence, were added to cells after 20 h in culture, incubated for 24 h, and removed, and fresh media were added to wells (36). After a total of 66 h in culture, cells were treated with gAd or control media for 24 h and used in the amino acid uptake assay.

**Amino acid uptake assay.** The activity of the system A and L amino acid transporters was measured in cultured trophoblast cells (23). Cells were divided into two groups and incubated with 0.5 mmol/l unlabeled leucine in the presence or absence of 5 μmol/l [3H]-labeled leucine. The activity of the system A and L amino acid transporters is reported as nanomoles of [3H]-leucine uptake per 10⁵ cells/h. At the end of the incubation period, each well was washed twice with cold media, and cells were scintillation counted to determine cellular uptake.

**Western blot.** Protein expression of the system A transporter isoforms SNAT1, SNAT2, and SNAT4; phospho-AMPK Thr172 and AMPK; STAT3; phospho-STAT3 Tyr705; suppressor of cytokine signaling 3 (SOCS3); PPARα; phospho-PPARα Ser211; insulin receptor substrate (IRS-1; Tyr612); α-actin, and β-actin was analyzed using Western blotting. A polyclonal SNAT2 antibody was generated in rabbit by Dr. P.D. Prasad (Medical College of Georgia), and affinity-purified polyclonal anti-SNAT1 and -SNAT4 antibodies were generated in rabbits by Eurogentec (Seraing, Belgium). Antibodies targeted against phospho-AMPK Thr172, phospho-AMPK, AMPK, SOCS3, and STAT3 were purchased from Cell Signaling, and anti–β-actin antibodies were obtained from Sigma. Protein concentrations were determined by Bradford assay, and Western blotting was performed as previously described (30).

**Real-time PCR.** After exposure of syncytiotrophoblast cells to insulin (1 nmol/l), globular adiponectin (5 μg/ml), full-length adiponectin (5 μg/ml), or control media for 24 h, total RNA was isolated using TRizol reagent (Invitrogen, Carlsbad CA) and reverse transcribed using a Quantitect Reverse Transcription kit (Qiagen, Valencia, CA). Proprietary Quantitect Primer Assays for SNAT1, SNAT2, and SNAT4 and succinate dehydrogenase were obtained from Qiagen. Quantitative PCR was performed in triplicate in 96-well plates following the standard Quantitect SYBR PCR protocol in a 7800 (Applied Biosystems, Foster City, CA). For analysis, SNAT1, SNAT2, and SNAT4 were standardized against succinate dehydrogenase expression. As negative controls, preparations lacking RNA were used. RNAs were assayed from six independent biological replicates. The RNA levels are expressed as a ratio, using the comparative cycle threshold method for comparing relative expression results between treatments in real-time PCR.

**Cytokine enzyme-linked immunosorbent assays.** Syncytiotrophoblast cell culture media were collected after 24-h incubation with 5 μg/ml gAd or fAd. Interleukin-6 (IL-6) and tumor necrosis factor-α concentrations in media were determined using colorimetric ELISA (Thermo Scientific), following instructions provided by the manufacturer.

**Data presentation and statistics.** Data are presented as means ± SEM. Statistical significance of differences between control and treated cells was assessed using repeated-measures ANOVA or Student t test. A P value <0.05 was considered significant. n = number of individual placentas that cells were isolated from in each treatment group. Experiments were run on duplicate sets of cells from each placenta.

**RESULTS**

The effect of adiponectin on amino acid uptake in cultured human primary trophoblast cells was highly dependent on the presence or absence of insulin. In the absence of insulin, gAd significantly (P < 0.05 repeated-measures ANOVA [RMANOVA], n = 6) increased system A amino acid transporter activity, as measured by sodium-dependent methylaminoisobutyric acid (MeAIB) uptake (Fig. 1A). However, system L amino acid transport activity was not altered by gAd (Fig. 1C). Both MeAIB and leucine...
uptake in fAd-treated cells remained at control levels (Fig.
1B and D). Insulin in concentrations corresponding to
postprandial levels in pregnant women (38) markedly
increased (P < 0.05, RMANOVA, n = 6) system A transport
activity (Fig. 2A and B), in agreement with previous
studies (39,40), with no effect on system L (data not
shown). gAd did not modify the insulin-stimulated system
A amino acid transport activity (Fig. 2A). However, fAd
abolished the insulin-stimulated system A activity (Fig.
2B). Because altered mRNA and protein expression of
specific SNAT isoforms, in particular SNAT2, constitutes a
key mechanism by which placental system A activity is
regulated (32,36), we determined the gene and protein
expression of SNAT isoforms in response to insulin, gAd,
and fAd. SNAT1, SNAT2, and SNAT4 RNA expression
levels were not altered by gAd (Fig. 2C). In contrast,
insulin significantly increased mRNA expression of all
SNAT7 isoforms (Fig. 2C). Furthermore, in the presence of
insulin, fAd significantly decreased the expression levels of
SNAT2 and SNAT4 mRNA, but not SNAT1 RNA, com-
pared with both fAd and insulin treatment alone (Fig. 2C).
Indeed, SNAT1, SNAT2, and SNAT4 mRNA levels after fAd
treatment in the presence of insulin were not significantly
different from control cells (Fig. 2C). A significant increase
in SNAT2 protein expression was observed after insulin,
gAd, and insulin/gAd incubations (Fig. 2D and E). Whereas
fAd alone had no effect on SNAT2 protein expression, fAd
completely reversed the insulin-stimulated increase in
SNAT2 expression (Fig. 2E).
AMPK is one of the key mediators of adiponectin
signaling in muscle and liver (15,16). We determined the
effect of gAd and fAd on AMPK activation, which is
dependent on the phosphorylation of the Thr172 residue
(41). Incubation with gAd alone for 24 h increased the
level of phospho-AMPK, but this was not observed in the
presence of insulin (Fig. 3). On the other hand, fAd did not
alter the phospho-AMPK expression, in the presence or
absence of insulin. fAd alone significantly increased the
total expression of AMPK, but this effect was lost in the
presence of insulin (Fig. 3).

It is well established that adiponectin may affect the
synthesis/release of proinflammatory cytokines in other
tissues (42–44). Furthermore, Lappas et al. (29) showed an
increase in cytokine secretion after exposure of placental
villous fragments to gAd. Because we recently reported
that IL-6 and TNF-α stimulate system A transporter activity
in cultured primary human trophoblast cells (36), it is
possible that the effects of gAd on system A activity may
be mediated by the release of proinflammatory cytokines.
We demonstrated that gAd significantly increased the
levels of IL-6 and TNF-α produced by the trophoblast cells
(Fig. 4A and B). In contrast, fAd dramatically reduced the
levels of IL-6 produced, whereas TNF-α levels were in-
creased (Fig. 4C and D).

STAT3 is a key component in the IL-6 signaling pathway
(45) and the stimulating effect of IL-6 on system A activity
in cultured human primary trophoblast cells is mediated
by STAT3 activation (36). To investigate whether the
effects of gAd (without insulin) on system A transport
were mediated via STAT3, we transfected cultured pri-
mary human trophoblast cells with siRNA targeting
STAT3, resulting in a 70% reduction in STAT3 protein
expression, (36). The significant stimulation of MeAIB

FIG. 1. Sodium-dependent 14C-MeAIB (A and B) and BCH-inhibitable 3H-leucine (C and D) uptake after incubation of cultured trophoblast cells
in control media, gAd (A and C), or fAd (B and D) for 24 h. Data are mean ± SEM for cells isolated from six different placentas. gAd significantly
(P < 0.05) stimulated MeAIB uptake in a dose-dependent manner (RMANOVA with post hoc tests, *P < 0.05, **P < 0.01).
uptake observed after gAd treatment alone was abolished in cells in which STAT3 was silenced (Fig. 5). These results clearly demonstrate that STAT3 is critical in mediating the effect of gAd on system A amino acid transport activity.

One key mechanism involved in the insulin-sensitizing effect of adiponectin in liver and muscle is the ability to enhance insulin-stimulated AKT phosphorylation (46). To investigate possible interactions between adiponectin and insulin signaling in trophoblast cells, we measured protein expression of phospho-AKT Ser473 and phospho-AKT Thr308 after incubation in insulin with or without adiponectin. Incubation of cultured primary trophoblast cells with insulin significantly increased the levels of phospho-AKT Ser473 and phospho-AKT Thr308 (Fig. 6). This observation is consistent with the enhanced insulin-stimulated AKT phosphorylation (46), and we explored the possibility that fAd alters the activation status of IRS-1 by determining the expression of the p85 subunit of phosphatidylinositol 3-kinase (47), phosphatase and tensin homolog (48), SOCS3 (49), and phospho-STAT3 (50), which all attenuate insulin signaling. However, in our study, protein expression of phosphatase and tensin homolog, SOCS3, phospho-STAT3, and p85 was not different after exposure to fAd plus insulin compared with insulin alone (data not shown).

Adiponectin has been shown to activate IRS-1 (46), and we explored the possibility that fAd alters the activation status of IRS-1 by determining the expression of IRS-1 in placenta.
IRS-1 phosphorylated at Tyr612. In cells treated with insulin plus fAd, phosphorylation of IRS-1 at Tyr612 was significantly reduced compared with insulin-treated cells (Fig. 7A and B). Because IRS-1 is activated when phos-

![Graph showing protein expression normalized to β-Actin](image)

**FIG. 3.** Summary of phospho-AMPK and AMPK protein expression after incubation of cultured trophoblast cells with control media, insulin (1 nmol/l), gAd (5 µg/ml), or fAd (5 µg/ml) for 24 h. Subsets of cells were pre-treated with insulin (1 nmol/l) for 4 h and then exposed to 5 µg/ml gAd (IgAd) or fAd (IfAd) for an additional 20 h. $n = 5$ placentas for each treatment. Phospho-AMPK expression was significantly increased by gAd (RMANOVA, $P < 0.01$). AMPK expression was significantly increased by fAd (RMANOVA, $P < 0.002$). Tukey-Kramer multiple comparisons post tests, *$P < 0.05$.

![Graph showing MeAIB uptake](image)

**FIG. 5.** A: Representative Western blot showing reduction in protein expression of STAT3 compared with control (C) using three unique siRNAs (1, 2, 3) targeted against STAT3. B: System A activity in control, siRNA STAT3 knockdown, and gAd-treated cells. Data are mean ± SEM for six placentas. Knocking down STAT3 significantly (RMANOVA, $P = 0.002$) reduced gAd stimulation of system A activity. Tukey-Kramer multiple comparisons post tests, **$P < 0.01$.

![Graph showing IL-6 secretion](image)

**FIG. 4.** A: IL-6 secretion was significantly (Student paired $t$ test, $P < 0.01$) increased after incubation of cultured trophoblast cells with gAd (1 µg/ml). Data are mean ± SEM for six placentas. B: TNF-α secretion was significantly (Student paired $t$ test, $P < 0.01$) increased after incubation of cultured trophoblast cells with gAd (1 µg/ml). Data are mean ± SEM for six placentas. C: IL-6 secretion was significantly (RMANOVA, $P < 0.01$) reduced in a dose-dependent manner after incubation of cultured trophoblast cells with fAd (1 µg/ml). Data are mean ± SEM for six placentas. D: TNF-α secretion was significantly (RMANOVA, $P < 0.01$) increased in a dose-dependent manner after incubation of cultured trophoblast cells with fAd. Data are mean ± SEM for six placentas. **$P < 0.01$. 

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It is well established that insulin stimulates placental system A amino acid transport (39,40); however, the underlying mechanism has not previously been explored. We found that physiological levels of insulin increased the mRNA expression of all system A isoforms. In contrast, only SNAT2 was upregulated at the protein level, indicating that changes in SNAT2 protein expression are important in mediating the regulation of system A activity by insulin. These findings are in line with observations that SNAT2 appears to be a highly regulated SNAT isoform both in the placenta (31,32,55,56) and in other tissues and cells such as the mammary gland (56) and 3T3-L1 adipocytes (57).

Incubation of cultured human primary trophoblast cells in gAd in the absence of insulin increased AMPK phosphorylation, in agreement with previous studies in other tissues (15,16). Silencing of STAT3 completely abolished the stimulation of system A amino acid transporter activity by gAd. These data demonstrate that STAT3 activation is critical in mediating the stimulating effect of gAd on system A activity in the absence of insulin, findings that are in agreement with studies showing that STAT3 mediates the cellular effects of gAd in mouse cardiac fibroblast cells (58). STAT3 constitutes a key component in IL-6 signaling (45), and we show that gAd treatment enhanced trophoblast IL-6 secretion, observations that reflect a report by Lappas et al. demonstrating increased IL-6 production in placental villous fragments treated with gAd (29). Furthermore, we recently reported that IL-6 increases system A amino acid transport activity in cultured human primary trophoblast cells (36). Collectively, these observations suggest that gAd stimulates system A activity in cultured trophoblast cells by releasing IL-6, resulting in STAT3 activation. However, it cannot be excluded that mediators other than IL-6 could have contributed to the STAT3-mediated stimulation of system A in response to gAd. The molecular mechanisms linking AMPK activation, IL-6 release, and STAT3 phosphorylation in trophoblast cells remains to be established but may involve mitogen-activated protein kinase and nuclear factor-κB signaling as shown in other tissues (42–44,59).

The effect of gAd on trophoblast signaling and amino acid transport was distinctly different in the presence of physiological concentrations of insulin compared with incubations with gAd alone. Indeed, when insulin was present, gAd did not increase AMPK phosphorylation. Furthermore, gAd failed to enhance insulin-stimulated AKT phosphorylation. This is in contrast to effects in C2C12 myotubes in which gAd further enhanced insulin-stimulated AKT phosphorylation (46), but is consistent with effects in HeLa cells (46) and L6 muscle cells (60). Thus, the effects of gAd on the insulin-signaling pathway are tissue specific, and our data suggest that gAd is not an insulin sensitizer in human primary trophoblast cells.

Unlike gAd, fAd treatment caused a reduction in IL-6 secretion, consistent with an anti-inflammatory role for fAd in the placenta similar to that seen in monocytic cells (42,44). In contrast to the lack of effect of gAd treatment on insulin-stimulated cells, fAd treatment abolished the stimulation of system A transport activity and SNAT2 expression elicited by insulin. Importantly, our results demonstrate that this effect is mediated via cross-talk between the full-length adiponectin signaling pathway and the insulin-signaling pathway. In trophoblast cells stimulated by insulin, fAd significantly reduced the phosphorylation of both IRS-1 and AKT.

**DISCUSSION**

We demonstrate that, in contrast to the insulin-sensitizing actions of adiponectin in liver and muscle, fAd attenuates insulin signaling in primary human trophoblast cells. As a result, fAd inhibits insulin-stimulated amino acid transport. To the best of our knowledge, this is the first report of regulation of amino acid transport by adiponectin, in any tissue.
attenuation of placental insulin signaling and trophoblast and physiological concentrations of insulin, we propose that the altered trophoblast function in the presence of physiological ranges, which contributes to the physiological relevance of our results. Because fAd was the only form of adiponectin that altered trophoblast function in the presence of physiological concentrations of insulin, we propose that the attenuation of placental insulin signaling and trophoblast amino acid transport by fAd constitutes the important biological effect in vivo.

Our data show that fAd attenuated insulin signaling in primary human trophoblast cells and reversed insulin-stimulated system A activity. Interestingly, McDonald and Wolfe (28) recently reported that adiponectin inhibits endocrine functions of cultured human primary trophoblast cells, as evidenced by a decreased synthesis of human chorion gonadotropin and human placental lactogen. These authors did not identify the mechanisms involved; however, it is possible that inhibition of insulin signaling, as demonstrated in the current study, may mediate the inhibitory effect of adiponectin on trophoblast endocrine function because it is well established that insulin stimulates trophoblast production of hormones, including human placental lactogen (64) and human chorion gonadotropin (65).

Insulin had a marked effect on the response of cultured human trophoblast cells to gAd and fAd. There may be a multitude of mechanisms underlying the regulation of adiponectin responsiveness by insulin. Human trophoblast cells have been shown to express functional AdipoR1 and AdipoR2 (28). Because AdipoR1 binds primarily gAd and AdipoR2 binds fAd with higher affinity than gAd, it is possible that insulin affects the responsiveness to adiponectin by regulating the relative abundance of the two adiponectin receptors in trophoblast cells. In support of this hypothesis, insulin increased AdipoR2 expression threelfold in muscle cells while reducing AdipoR1 expression by 50% (60), which was associated with an increased sensitivity to fAd and resistance to the effects of gAd.
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(60,66). Considering that both AdipoR1 and AdipoR2 are believed to mediate cellular effects by promoting increased insulin sensitivity, it may be expected that deletion of the receptors would result in insulin resistance. However, deletion of AdipoR2 in transgenic mice caused enhanced insulin sensitivity, rather than insulin resistance (67–69). One explanation for the unexpected phenotype of the AdipoR2 knockout mouse could be activation of AdipoR2 inhibits insulin signaling in some tissues, possibly including the placenta. Emerging evidence shows that downstream signaling of the adiponectin receptors involves APPL1 (adaptor protein containing pleckstrin homology domain) (70). Indeed, APPL1 associates with the adiponectin receptors and mediates adiponectin signaling and its effects on metabolism (71). APPL1 also functions in the insulin-signaling pathway and is an important mediator of adiponectin-dependent insulin sensitization in skeletal muscle (70). Chronic insulin treatment results in translocation of this signaling molecule to the nucleus, making it unavailable for downstream propagation of the adiponectin signal (70). Therefore, it is possible that the inhibitory effect of insulin on gAd AMPK activation in primary trophoblast cells is mediated through APPL1 translocation to the nucleus. Recently, APPL2, an isoform of APPL1, was identified (72) and shown to suppress adiponectin and insulin signaling by sequestrating APPL1. We speculate that differences between placenta and muscle in the expression and/or function of APPL1 and APPL2 result in distinct responses to adiponectin.

In conclusion, fAd inhibits insulin-stimulated trophoblast system A amino acid transport. Our data indicate that the underlying mechanism involves activation of PPARα, which inhibits IRS-1 and AKT phosphorylation, resulting in reversal of insulin-stimulated SNAT2 expression and system A activity. Fetal growth is highly dependent on the capacity of the placenta to transport amino acids (8). Because insulin and IGF-I are well-established stimulators of placental nutrient transporters and fetal growth, the finding that fAd attenuates placental insulin signaling and amino acid transport may have important implications for placental nutrient transport and fetal growth in pregnancies associated with altered maternal adiponectin levels.

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