Adiponectin in glucose metabolism
Blumer, R.

Citation for published version (APA):
Regulation of adiponectin secretion by insulin and amino acids in 3T3-L1 adipocytes

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Metabolism 2008, in press
Abstract

Objective:
Adiponectin is a fat cell-derived hormone with insulin-sensitizing properties. Low plasma adiponectin levels are associated with insulin resistance as found in obesity. One of the mechanisms for this finding is hampered insulin signaling via phosphatidylinositol 3-kinase (PI3K) with concomitant decreased adiponectin secretion. Because insulin can also stimulate signaling at the level of mammalian target of rapamycin (mTOR) by a mechanism that is dependent on the presence of amino acids, the role of mTOR signaling in adiponectin secretion was studied. In view of the vesicular nature of adiponectin secretion, the role of lysosomes was explored as well.

Methods:
In 3T3-L1 adipocytes, by selective stimulation and/ or inhibition of the PI3K- and the mTOR- signaling pathways, we studied their effects on adiponectin production and secretion. The role of lysosomes was studied by administration of several regulators of lysosomal function. Adiponectin was measured by ELISA.

Results:
In 3T3-L1 adipocytes, both insulin and amino acids stimulated adiponectin secretion. The stimulation by insulin was PI3K dependent but mTOR independent. The stimulation by amino acids was independent of both PI3K and mTOR. Whereas the effect of insulin via PI3K was mainly on adiponectin secretion from adipocytes, the effect of amino acids was predominantly due to their role as substrates for adiponectin synthesis. The acidotropic agents ammonia and methylamine, but not the lysosomal protease inhibitor leupeptin and the autophagy inhibitor 3-methyladenine, strongly inhibited adiponectin secretion and increased the intracellular adiponectin pool.

Conclusions:
Adiponectin production is substrate driven. PI3K and an acidic lysosomal pH, but not amino acid-mediated mTOR signaling or lysosomal breakdown are involved in adiponectin secretion.
Chapter 2

Introduction

Insulin resistance is a major risk factor for the development of type 2 diabetes and has frequently been associated with obesity. In addition to being a fat storage depot, adipose tissue has been shown to synthesize and secrete several biologically active molecules that influence glucose metabolism. Among these adipocytokines is adiponectin, a relatively abundant circulating plasma protein, which is produced and secreted exclusively by adipocytes. In animal experiments, administration of adiponectin ameliorates glucose metabolism by enhancing glucose uptake and suppressing hepatic glucose output. Probably, these effects occur via AMP-activated protein kinase (AMPK)-dependent stimulation of fat oxidation.

Plasma concentrations of adiponectin are low in insulin resistant patients with obesity and type 2 diabetes. The factors responsible for the dysregulation of adiponectin levels in these subjects have not yet been fully determined. Since these subjects are insulin resistant, insulin itself could be involved in the regulation of adiponectin production. In line with this, most, but not all studies reported an increase in adiponectin gene expression in adipocytes in response to insulin. Moreover, the insulin-stimulated increase in adiponectin secretion in 3T3-L1 adipocytes is likely mediated by the PI3K-dependent signaling pathway, since selective inhibition of this pathway prevented the effect of insulin. Apart from the involvement of the insulin/PI3K pathway in the regulation of adiponectin, two other main signal transduction pathways regulated by insulin, could be involved as well: the Mitogen-activated protein kinase- (MAPK) and the mTOR pathway. MAPK does not seem to regulate the stimulation of adiponectin secretion by insulin. The effect of the mTOR signaling pathway has not been considered so far. This is surprising because amino acid-dependent signaling synergizes with insulin at the level of mTOR and it is generally accepted that mTOR signaling controls protein levels via modulation of both protein translation and (autophagic) degradation. The nutrient-sensing mTOR pathway has also been implicated in the regulation of leptin production by adipocytes, indicating that this pathway is important in the regulation of hormones, at least those produced by adipose tissue.

In the present study carried out with 3T3-L1 adipocytes, we tested whether, in addition to insulin/PI3K signaling, the mTOR pathway is involved in the regulation of adiponectin production and secretion. Because of the known vesicular nature of adiponectin secretion, we also investigated whether autophago-lysosomal breakdown of adiponectin plays a negative role in this process.
Materials and methods

Materials
Insulin, PD98059, rapamycin, leupeptin, 3-methyladenine, methylamine, cycloheximide, AICAR (5-aminomidazole-4-carboxamide riboside) and the chemicals for enhanced chemiluminescence (ECL) were from Sigma (St. Louis, MO, USA). LY294002 was obtained from Biomol (Plymouth Meeting, PA, USA). Complete protease inhibitor cocktail tablets were from Roche Diagnostics (Almere, The Netherlands). Restore Western Blot Stripping Buffer was from Thermo Fisher Scientific Inc. (Rockford, IL, USA). Phosphospecific anti-PKB (Thr\textsuperscript{308}), phosphospecific anti-p44/p42 MAPK (Thr\textsuperscript{202}/Tyr\textsuperscript{204}), phosphospecific anti-AMPK (Thr\textsuperscript{172}), phosphospecific anti-p70S6 kinase (Thr\textsuperscript{389}), anti-PKB and anti-MAPK were from Cell Signaling Technology Inc. (Leusden, The Netherlands). Goat anti-rabbit-HRP was from Biorad (Hercules, CA, USA). Cell-culture reagents were from Gibco BRL Life technologies (Paisley, Scotland). LY294002, rapamycin and PD98059 were dissolved in DMSO; the final DMSO concentration in the incubations did not exceed 0.5% (v/v). Controls were carried out with DMSO alone. All other chemicals were obtained from Sigma.

Cell culture and differentiation
3T3-L1 adipocytes (American Type Culture Collection, Rockville, USA) were pre-cultured at 37 °C under a 10% CO\textsubscript{2} air atmosphere in Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with 10% foetal calf serum and 1% penicillin/streptomycin (100 units/ml). Confluent cells were differentiated in 12-wells plates by culturing in DMEM containing 10% foetal calf serum, 1% penicillin/streptomycin, 0.5mM 3-Isobutyl-1-methylxanthine (IBMX), 1μM dexamethasone (DEX) and 100nM insulin. After 2 days, medium was replaced by the same medium except for IBMX, followed by replacement of the medium after another 2 days omitting IBMX and DEX. Cell medium was refreshed every 2 days until cells were fully differentiated.

Experiments
Prior to experimentation, adipocytes were washed 3 times with minimal medium (HBSS) containing 5mM glucose, supplemented with 45 mM bicarbonate, 10 mM Na\textsuperscript{+}-HEPES, pH 7.4 and 0.1% (m/v) BSA. Subsequently, adipocytes were pre-incubated for 3h in minimal medium. Adipocytes were then incubated for 24h in minimal medium containing the appropriate treatments as described in the results section. Inhibitors (LY294002, PD98059 and rapamycin) were added 30 minutes before insulin and/or amino acid
In-vitro effects of insulin and amino acids on adiponectin

Chapter 2

After 24h of incubation, medium was collected and stored at -80°C in the presence of a protease inhibitor cocktail until further analysis. Subsequently, cells were quickly washed 3 times with HBSS and lysed in 300μl ice-cold lysis buffer (20 mM Tris (pH 7.5), 50 mM NaCl, 250 mM sucrose, 50mM NaF, 5mM Na₄P₂O₇, 1mM dithiothreitol (DTT), 1,0% (v/v) Triton X-100), supplemented with protease inhibitor cocktail tablets (1 tablet per 20 ml lysis buffer). Cell lysate was cleared by centrifugation in a microcentrifuge for 15 minutes at 4°C and stored at – 80 °C.

The composition of the complete mixture of all amino acids (4aa) used in the experiments was such that the concentration of each of the amino acids was present at a concentration 4 times that found in fasted rat plasma. The composition of the 1aa mixture was, except for a leucine concentration of 250 μM, exactly as described before 20; (in μM): asparagine 60, isoleucine 100, leucine 250, lysine 300, methionine 40, phenylalanine 50, proline 100, threonine 180, tryptophan 70, valine 180, alanine 400, aspartate 30, glutamate 100, glutamine 350, glycine 300, cysteine 60, histidine 60, serine 200, tyrosine 75 and arginine 100.

Immunoblotting

Cell protein was determined by the method of Lowry 21 and 30 μg of protein was separated by SDS-PAGE. After separation, a standard Western blotting procedure was performed and the polyvinylidene-fluoride blots were incubated with appropriate antibodies. To ensure equal loading, phospho-blots were stripped for 15 minutes at room temperature with 30 ml of Restore Western Blot Stripping Buffer and incubated with antibodies against total PKB and MAPK. The proteins were visualised by ECL.

Analysis of adiponectin levels

Quantification of adiponectin protein levels was performed using ELISA from R&D Systems (Abingdon, UK).

Statistical analysis

The adiponectin levels of individual treated cell cultures were expressed as ng/ml per 24h. Results are shown as mean ± SD of 4 to 6 experiments with 3 repeats of each condition per experiment. Comparisons between various conditions were performed using an unpaired t-test. Probability values of < 0.05 were considered statistically significant. SPSS statistical software version 12.0.1 (SPSS Inc, Chicago, IL, USA) was used to analyze data.
Results

Insulin- and amino acid-dependent signaling

The effects of insulin and amino acids on the activation of the PI3K-, MAPK- and mTOR pathways are shown in Fig. 1. The efficacy of LY294002, PD98059 and rapamycin as inhibitors of the PI3K-, MAPK- and mTOR pathways, respectively, was verified. PKB phosphorylation at Thr^{308}, which is essential for PKB activity \(^{22}\), was stimulated by insulin in a LY294002-sensitive manner (Fig. 1A). In contrast, PD98059 did not affect the stimulation of PKB phosphorylation by insulin. MAPK phosphorylation at Thr^{202}/Yr^{204} was stimulated by insulin and inhibited by PD98059, but not by LY294002, as expected (Fig. 1B). Rapamycin, which specifically inhibits mTOR, had no effect on insulin-stimulated PKB- and MAPK phosphorylation (Fig. 1A and B). In contrast to the effect of insulin, exposure to amino acids did not result in phosphorylation of PKB at Thr^{308} (Fig. 1C).

Amino acids stimulated phosphorylation of p70S6 kinase at Thr^{389}, which is most critical for kinase function \(^{23}\), in a concentration-dependent manner (Fig. 1D). Insulin did not affect p70S6 kinase phosphorylation. LY294002 and rapamycin completely prevented phosphorylation of p70S6 kinase under all conditions. None of these inhibitors stimulated phosphorylation of AMPK (Fig. 1E), indicating that they did not affect the cellular energy state. AICAR, an activator of AMPK phosphorylation \(^{24, 25}\), was used as a positive control.

![Fig. 1. Effects of insulin and amino acids on signaling. Differentiated 3T3-L1 adipocytes were starved for 3h on minimal medium, followed by 24h of incubation with the additions indicated. Lysates were separated by SDS-PAGE, immunoblotted with anti-phospho-PKB (Thr^{308}), anti-phospho-MAPK (Thr^{202}/Yr^{204}), anti-phospho-p70S6 kinase (Thr^{389}), anti-phospho-AMPK (Thr^{172}), anti-PKB or anti-MAPK antibodies and visualised by ECL. Cell integrity was analysed by light microscopy. ctl, control (minimal medium); ins, insulin (100nM); ly, LY294002 (100 μM); pd, PD98059 (25μM); ra, rapamycin (100 nM); ch, cycloheximide (50μM); AICAR, 5-aminoimidazole-4-carboxamide riboside (250μM).]
Insulin and amino acids stimulate adiponectin secretion

Adiponectin secretion into the culture media of 3T3-L1 adipocytes was linear with time for at least 24h and markedly stimulated by insulin (100nM) and 4aa. Insulin stimulated adiponectin secretion by 37% (control 35 ng/ml ± 3.5 vs. insulin 48 ng/ml ± 7.4; p<0.0001) and amino acids stimulated adiponectin secretion by 66% (control 35 ng/ml ± 3.5 vs. amino acids 58 ng/ml ± 7.0; p<0.0001) (Fig. 2) compared to control.

Insulin-stimulated adiponectin secretion is PI3K dependent but MAPK independent

The stimulation by insulin was prevented by the PI3 kinase inhibitor LY294002, but not by the MAPK inhibitor PD98059. Both compounds slightly inhibited basal adiponectin secretion in the absence of insulin (Fig. 3A).

Insulin-stimulated adiponectin secretion is largely independent of protein synthesis

In order to determine whether protein synthesis was involved in adiponectin secretion, 3T3-L1 adipocytes were incubated in the presence of the protein synthesis inhibitor cycloheximide. After 24h, basal adiponectin secretion was significantly decreased when cycloheximide was added (control 35 ng/ml ± 3.5 vs. cycloheximide 14 ng/ml ± 1.0; p<0.002, n=4). In the presence of cycloheximide, adiponectin secretion was still stimulated by insulin in a PI3K-dependent manner (Fig. 3B). As in the absence of cycloheximide, PD98059 inhibited basal adiponectin secretion in its presence, but did not affect the stimulation by insulin (Fig. 3B). Cycloheximide incubation did not affect cell integrity as

Light microscopy analysis of the cells after 24h also indicated that cell integrity was not influenced by these compounds (Fig. 1F).
indicated by the AMPK phosphorylation state and by light microscopy analysis (Fig. 1E and 1F).

**Intracellular adiponectin**

Although insulin increased adiponectin secretion (Fig. 3A), it had no significant effect on intracellular adiponectin levels (Fig. 3C). In the absence of insulin, inhibition of the PI3K pathway slightly reduced intracellular adiponectin. In contrast, inhibition of the MAPK pathway had no effect on intracellular adiponectin. When protein synthesis was blocked by cycloheximide, intracellular levels of adiponectin dropped by 73% compared to control (control 33 ng/ml ± 0.6 vs. cycloheximide 9 ng/ml ± 0.5; p<0.002, n=4) and were reduced by insulin, but not significantly affected by LY294002 or PD98059 (Fig. 3D).

**Amino acid-stimulated adiponectin production is independent of the PI3K, MAPK and mTOR pathways**

To further investigate the stimulation of adiponectin secretion by amino acids, 3T3-L1 cells were incubated in the absence or presence of amino acids (4aa), in combination with insulin (Fig. 4A). After 24h of incubation, adiponectin secretion was increased by
66% (p<0.0001) with 4aa and by 57% with 1aa (p<0.0001) (data of 1aa not shown). Insulin had no further effect on adiponectin-stimulated secretion when combined with 4aa. The stimulation of adiponectin release by amino acids was largely insensitive to LY294002 (Fig. 4A) and also not affected by PD98059 (data not shown). Intracellular adiponectin levels were enhanced by amino acids in a PI3K-independent fashion (Fig. 4B). In the presence of cycloheximide, the stimulation of adiponectin secretion by amino acids, but not by insulin, was lost (Fig. 4C).

The involvement of the mTOR pathway in adiponectin secretion and production was tested by incubating 3T3-L1 adipocytes with rapamycin. Basal adiponectin secretion was decreased by 16% (p<0.0007) with rapamycin. The stimulation by insulin and amino acids remained largely unaffected by rapamycin incubation (Fig. 4A). Likewise, the increase in intracellular adiponectin by amino acids was largely unaffected in the presence of rapamycin (Fig. 4B).

Fig. 4. Involvement of the PI3K- and the mTOR pathway in the stimulation of adiponectin secretion by insulin and amino acids. Differentiated 3T3-L1 adipocytes were starved for 3h on minimal medium, followed by 24h of incubation with the additions indicated. Results are expressed as mean ± SD of 4 to 6 experiments with 3 repeats of each condition per experiment. ctl, control (minimal medium); ins, insulin (100nM); ly, LY294002 (100 μM); ra, rapamycin (100nM); CH, cycloheximide (50μM).
Effect of autophagy on adiponectin secretion

Adiponectin is known to be secreted in vesicles \(^{13}\). As secretion is the result of both the rate of protein synthesis and degradation, the effect of inhibition of lysosomal function was studied. 3-Methyladenine, the classical inhibitor of autophagy \(^{26}\), neither affected the secretion of adiponectin (Fig. 5A) nor its intracellular levels (Fig. 5B). Leupeptin, a lysosomal protease inhibitor, had no effect either. Interestingly, methylamine and NH\(_4\)Cl, acidotropic agents that increase the pH of acidic compartments, including the lysosomes, strongly decreased adiponectin secretion (by 47 and 49\% (p<0.00004), respectively; Fig. 5A), and increased the intracellular adiponectin levels (by 41 and 25\% (p<0.004), respectively; Fig. 5B). None of these chemical compounds affected cell integrity, as indicated by the AMPK phosphorylation state and by light microscopy analysis (Fig. 1E and 1F).

Fig. 5. Effect of inhibition of lysosomal function on adiponectin secretion. Differentiated 3T3-L1 adipocytes were starved for 3h on minimal medium, followed by 24h of incubation with the additions indicated. Results are expressed as mean ± SD of 3 to 5 experiments with 3 repeats of each condition per experiment. ctl, control (minimal medium); ra, rapamycin (100 nM); 3-MA, 3-methyladenine (10mM); leup, leupeptin (200μM); ma, methylamine (5mM); NH\(_4\)Cl, ammonium chloride (5mM).

Discussion

Plasma adiponectin concentrations are low in subjects with insulin resistance \(^7\). Hampered insulin signaling has been implicated to be involved in the reduced levels of this adipocytokine. Studies performed with 3T3-L1 adipocytes have shown that insulin stimulates adiponectin secretion via PI3K-dependent signaling \(^{13, 14}\). The present study confirms this: inhibition of the PI3K pathway by LY294002 administration completely inhibited the insulin-mediated increase in adiponectin secretion. In the presence of insulin, adiponectin levels in the medium of 3T3-L1 adipocytes increased, but intracellular adiponectin remained unaffected. This suggests that insulin stimulates both the
synthesis and secretion of adiponectin. Indeed, when protein synthesis was blocked by cycloheximide, insulin still increased the appearance of adiponectin in the medium and decreased its intracellular level.

The inhibition of basal adiponectin production by LY294002 is in contrast with 13, but in agreement with the effect of wortmannin, another PI3K-inhibitor, on adiponectin mRNA 14. The reason for these slight differences in results is not entirely clear but may be related to slight differences in experimental conditions and in the duration of the experimental period (2h in 13 vs. 24h in the present study).

Apart from the involvement of the insulin/PI3K signaling pathway in the regulation of adiponectin secretion, little information is available about other possible insulin-dependent pathways that may influence adiponectin production and secretion. Confirming an earlier study 14, inhibition of the MAPK pathway had no effect on the stimulation of adiponectin secretion by insulin. This is consistent with observations in models of insulin resistance in which the activity of the PI3K signaling pathway is decreased but the MAPK pathway still active 27-29.

In studies on the regulation of adiponectin secretion, the effect of amino acids and of amino acid-dependent signaling has not been considered so far. This is surprising as amino acid-dependent signaling synergizes with insulin at the level of mTOR and it is generally known that the mTOR signaling cascade regulates both the synthesis and autophagic degradation of proteins 15-18. The synergy between insulin and amino acids with regard to mTOR downstream signaling, first reported 30 for hepatocytes and later confirmed for other cell types 18, was also apparent in our experiments: insulin alone did not affect p70S6 kinase phosphorylation, but promoted the effect of amino acids. In agreement with the literature, amino acids did not affect the phosphorylation of PKB 15, 16, 18. However, the stimulation of adiponectin secretion by amino acids was largely insensitive to rapamycin and also to LY294002. This suggests that the stimulation of adiponectin secretion by amino acids was largely unrelated to their ability to stimulate signaling. Because adiponectin secretion by amino acids was completely prevented by cycloheximide, the stimulation of adiponectin production by amino acids must have largely been due to their role as substrates for protein synthesis, and thus for adiponectin synthesis.

It was shown recently that overactivation of mTOR signaling by amino acids, inhibits insulin signaling upstream of mTOR by a negative feedback effect, because of p70S6 kinase-dependent phosphorylation of IRS1, which results in decreased activity of PI3K 31, 32. If this were the case, we would have expected a decrease in adiponectin secretion by high concentrations of amino acids in the presence of insulin, but this was not observed.
Additionally, we did not find a decrease in insulin-stimulated PKB phosphorylation after a 24h exposure of the cells to high amino acid concentrations. This demonstrates that the feedback effect was not observed under our experimental conditions.

Although, in principle, autophagic degradation of adiponectin could have been involved in negative control of adiponectin secretion, our data strongly indicate that this was not the case. Inhibition of the autophago-lysosomal pathway by the autophagy inhibitor 3-methyladenine and by the lysosomal protease inhibitor leupeptin had no effect. These findings are consistent with the fact that mTOR activity, which controls autophagy 15-18, did not play a major role in adiponectin secretion. Interestingly, both ammonia and methylamine, acidotropic agents which increase the pH of acidic compartments, including lysosomes, strongly inhibited export of adiponectin from the adipocytes. Because adiponectin is secreted via a vesicular pathway 13, 33, this strongly suggests that an acidic interior of these vesicles is required for adiponectin secretion, in analogy with other exocytotic processes, such as the release of insulin by pancreatic islets 34.

A potential limitation of the present study could be that the function of 3T3-L1 adipocytes alters after 24h of incubation in minimal medium. Additionally, prolonged administration of several stimulators and inhibitors could have resulted in toxicity and consequently in changes in adiponectin levels. We believe these possible distorting factors are unlikely to play a role. Firstly, our results regarding the role of the insulin/PI3K signaling pathway in adiponectin secretion are in agreement with the literature 13, 14 as are the results (Fig. 1) on the inhibition and stimulation of the phosphorylation of PKB, MAPK and p70S6K 14, 18, 35. Secondly, toxicity would have resulted in lysis of the adipocytes with increased medium adiponectin levels as the consequence. This contrasts with the observed decrease in medium adiponectin levels in the present study after addition of e.g. LY294002, PD98059, methylamine and NH₄Cl. Light microscopy also failed to detect cell lysis (Fig. 1F). Finally, AMP-activated protein kinase was not phosphorylated after 24h of incubation in minimal medium whether in the absence or presence of the various inhibitors, indicating that the adipocyte energy level was not affected (Fig 1E).

In conclusion, our data indicate that amino acid-dependent mTOR-mediated signaling does not regulate adiponectin secretion. Whereas insulin stimulates the secretion of adiponectin in a PI3K-dependent manner, amino acids stimulate adiponectin production and secretion largely by virtue of their role as substrates for adiponectin synthesis. A minor role of mTOR signaling cannot be entirely excluded, however. The lysosomes do not regulate adiponectin secretion. An acidic intravesicular pH is required for efficient adiponectin secretion.
Acknowledgements

This work was supported by a grant of the Dutch Diabetes Foundation (DFN 2003.11.008).
The technical assistance by A. Strijland was greatly acknowledged.

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