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Metformin reduces adiponectin protein expression and release in 3T3-L1 adipocytes involving activation of AMP activated protein kinase

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Abstract

The drugs troglitazone and metformin are used to reduce the degree of insulin resistance in type 2 diabetes. Both compounds act through different mechanisms which might include opposing effects on the production of adiponectin, an insulin-sensitizer released by adipocytes. This study compared the effects of troglitazone and metformin on adiponectin production by 3T3-L1 adipocytes during 48 h culture. Troglitazone increased adiponectin mRNA and protein expression as well as release, whereas metformin did not affect transcription but reduced protein expression and release. The effect of metformin was also seen with phenformin, and with low-glucose culture, all conditions with a reduced mitochondrial activity and an activated AMP activated protein kinase (AMPK). Addition of the AMPK activator 5-aminoimidazole-4-carboxamide-riboside (AICAR) also caused a decrease in adiponectin protein expression. These data indicate that metformin and troglitazone exert opposing effects on adiponectin expression and release by differentiated 3T3-L1 adipocytes. The metformin-induced suppression involves an activation of AMP activated protein kinase.

Keywords: Adiponectin; Metformin; AMP activated protein kinase; Glucose; Adipocyte; Diabetes

1. Introduction

Metformin and/or troglitazone are commonly used in the treatment of type 2 diabetes and mediate their insulin-sensitizing actions via distinct metabolic pathways (Lenhard et al., 1997; Ciaraldi et al., 2002). In adipocytes they seem to exert different effects. Troglitazone, a member of the thiazolidinediones (TZD), seems to enhance anabolic pathways, leading to an increase in adipocyte size, while metformin appears to stimulate catabolic pathways, which results in a reduced adipocyte size (Lenhard et al., 1997; Ciaraldi et al., 2002). It is possible that both agents cause differences in production of adiponectin, an adipocyte specific hormone that increases insulin sensitivity and that is found to be reduced in type 2 diabetes (Arita et al., 1999; Hotta et al., 2000; Weyer et al., 2001).

It is presently unclear whether metformin can directly influence adiponectin production and release in adipocytes, and the role played by its target, AMP activated protein kinase (AMPK). Whereas the peroxisome proliferator activated receptor (PPAR)-γ-stimulating thiazolidinediones, including troglitazone and rosiglitazone, have been shown to increase adiponectin gene expression and circulating hormone levels (Maeda et al., 2001; Yu et al., 2002; Phillips et al., 2003), the effects of AMPK activators are less well understood. Although metformin was found to have no effect on adiponectin levels (Phillips et al., 2003; Tiikkainen et al., 2004), recent data showed that adiponectin gene expression is stimulated by AMPK activation with an AMP mimetic (Lihn et al., 2004).

We hypothesized that pharmacological AMPK activators may inhibit adiponectin protein synthesis or release in adipocytes, potentially explaining apparent contradictions in the literature. To investigate this, we compared the in vitro

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effects of AICAR, metformin, phenformin and troglitazone on the expression and activation of AMPK and on adiponectin mRNA and protein expression as well as release in mature 3T3-L1 adipocytes. Data obtained support translational regulation of adiponectin expression by pharmacological agents used to enhance insulin sensitivity of peripheral tissues in type 2 diabetes, and point towards a key role of AMPK activation in this process.

2. Materials and methods

2.1. Chemicals

Glucose (Sigma, St. Louis, USA), AICA-riboside (5-aminoimidazole-4-carboxamide-riboside, Sigma, St. Louis, USA), metformin (1,1-dimethylbiguanide, Sigma, St. Louis, USA), phenformin (Phenethylbiguanide, Sigma, St. Louis, USA), human insulin (Actrapid HM Penfill, Novo Nordisk, Brussels, Belgium), murine tumor necrosis factor (TNF)-α (Peprotech, Rocky Hill, NJ, USA), troglitazone (Sankyo, Shinagawa-ku, Tokyo), dexamethasone (Sigma, St. Louis, USA), and isobutylmethylxanthine (IBMX, Janssen Chimica, Beerse, Belgium) were used in 3T3-L1 adipocyte cell cultures as indicated.

2.2. Cell culture, adiponectin content and release measurements

3T3-L1 cells were cultured and differentiated in 6-well (for RNA isolation) or 24-well (all other experiments) tissue culture plates (Falcon, BD Biosciences, Erembodegem, Belgium). Preadipocytes were maintained as fibroblasts (passage 7–13) in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (vol/vol) newborn calf serum and antibiotics. Differentiation was initiated using DMEM medium complemented with 10% (vol/vol) heat-inactivated fetal calf serum, 670 nM insulin (Actrapid HM Penfill, Novo Nordisk, Brussels, Belgium), 25 nM dexamethasone (Sigma, St. Louis, USA), 500 μM isobutylmethylxanthine (IBMX, Janssen Chimica, Beerse, Belgium) and antibiotics, as previously described (Hresko et al., 1998). After 4 days of differentiation, the cells were cultured in DMEM, 10% (vol/vol) fetal calf serum and antibiotics, and medium was changed every two days. At 10 days post-induction of differentiation, cells were used for experimentation. The differentiation status of the cells was ascertained by in situ staining with oil-red-O (1-8-[4 (Dimethylphenylazo) dimethylphenylazo]-2-naphthalenol) (Green and Kehinde, 1974), which accumulates in the intracellular fat droplets of adipocytes. Terminal differentiation of 3T3-L1 cells was marked by the accumulation of dye in large cytoplasmic vesicles as observed by light microscopy after staining.

Adiponectin release measurements with differentiated 3T3-L1 adipocytes consisted of washing cells twice with serum-free DMEM, followed by culturing in 1 ml of this medium with specified chemicals for the indicated periods. Measurement of adiponectin release in the culture medium was based on immunoblotting of adiponectin in medium samples. The results were normalized to the control condition in each experiment; there was <20% variation among results obtained in different experiments. Glucose deprivation experiments were performed in serum- and glucose-free DMEM (Gibco BRL), supplemented with the indicated concentrations of glucose.

2.3. Northern blot analysis

Total RNA was isolated from 3T3 adipocytes using Trizol reagent (Life Technologies, Merelbeke, Belgium). Ten micrograms total RNA per sample was loaded on an agarose gel (1.5%) and separated by electrophoresis. The RNA was transferred to nylon membranes (GeneScreen, NEN Dupont, Boston, USA) as previously described (Huypens et al., 2000). Blots were UV-cross-linked using a StratalinkerTM1800 (Stratagene, La Jolla, USA) and hybridised with ^{32}P-labelled cDNA probes corresponding to mouse adiponectin (accession number AF304466) and mouse beta actin (accession number X03672). The adiponectin cDNA fragment was

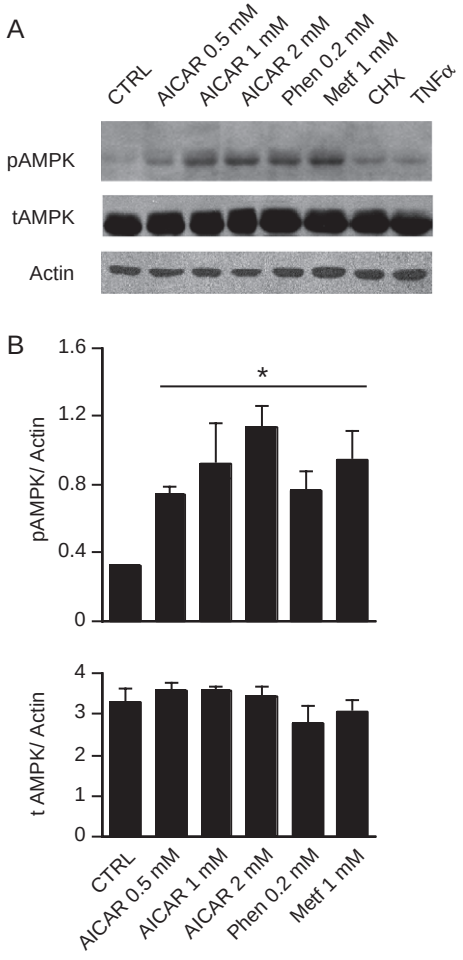

Fig. 1. Effect of pharmacological AMPK-activators on phospho-AMPK and total AMPK protein in 3T3-L1 adipocytes. Differentiated adipocytes were exposed to the indicated concentrations of the AMPK-activators AICA-riboside (AICAR), metformin (Metf), and phenformin (Phen), to cycloheximide (CHX, 5 μg/ml) or tumor necrosis factor-alpha (TNF-α, 500 U/ml), or they were left untreated (CTRL) for 48 h. The cells were then harvested and protein extracted. (A) Proteins (20 μg/lane) were separated on a 10% polyacrylamide gel. After transfer to a nitrocellulose membrane, polyclonal sera specific for phospho-AMPK (pAMPK), total AMPK (tAMPK), or beta actin were applied overnight, and detection was carried out as described in Materials and methods. The results shown are representative of four experiments. (B) Signals for pAMPK, tAMPK, and beta actin obtained in A, were quantified for the indicated experimental conditions, and pAMPK and tAMPK expressed as a ratio, relative to the actin signal obtained for each sample. The data represent the mean ± S.E.M. of four independent experiments (*P < 0.05 vs. CTRL, by ANOVA).

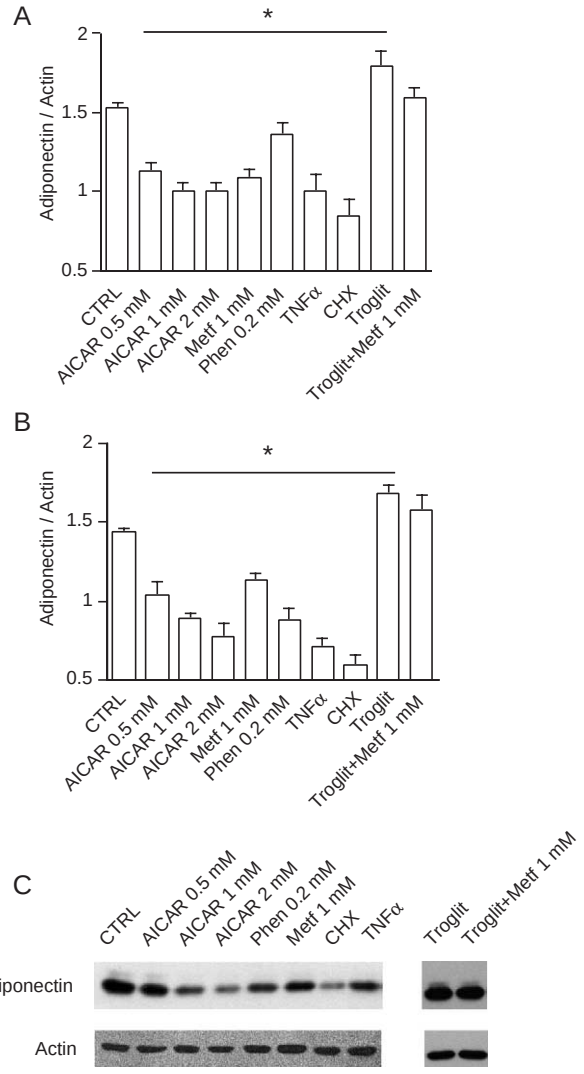

Fig. 2. Effect of pharmacological AMPK-activators on adiponectin protein content in 3T3-L1 adipocytes. Differentiated adipocytes were exposed for either 24 or 48 h to the indicated concentrations of the AMPK-activators AICA-riboside (AICAR), metformin (Metf), and phenformin (Phen), or to cycloheximide (CHX, 5 μg/ml), tumor necrosis factor-alpha (TNF-α, 500 U/ml), troglitazone (Troglit 10 μM), metformin (1 mM)+troglitazone (10 μM), or they were left untreated (CTRL). Proteins were prepared, applied (5 μg of protein/lane), and separated as in Fig. 1. The membranes were incubated with a monoclonal antibody against adiponectin, or with anti-beta actin antibody. The data are expressed as the ratio of the signals measured for adiponectin and actin after 24 h of culture (A) or 48 h of culture (B) in the presence of the test substances, and represent the mean±S.E.M. of 6–13 experiments (all conditions except troglitazone+metformin were significantly different from control, *$P<0.05$ vs. CTRL, by ANOVA). A representative blot of a 48-h culture is shown (C).

prepared by RT-PCR (forward primer: 5′-TGATGGCAGAGATGGCA and reverse primer 5′GAGGCCTGGTCCACATT) and purified on Qiaquick PCR purification columns (Westburg, Leusden, The Netherlands). DNA sequences of adiponectin and actin PCR products were verified by direct sequencing (ABI Prism 310; Perkin Elmer Cetus, Emeryville, California, USA). ^{32}P incorporation into cDNA probes was accomplished using the random labeling method (Amersham, UK). After hybridization with the adiponectin probe (4 h) and autoradiographic exposure, membranes were stripped and re-hybridized (4 h) with the beta actin cDNA probe. The autoradiographic signals were quantified using NIH Image 1.60 software.

2.4. Western blot analysis

3T3 adipocytes were washed with phosphate buffered saline (PBS), harvested with a cell scraper, and lysed in RIPA buffer containing protease inhibitors (Heimberg et al., 2000). Five, 10 or 25 μg of protein was mixed with sample buffer (final concentrations: 5% sodium dodecyl phosphate (SDS), 0.05% w/v bromophenol blue, 10% glycerol, 5% β-mercaptoethanol in 80 mM Tris HCl pH 6.8) and proteins were resolved by SDS-PAGE on 10% (w/v) (acrylamide:bisacrylamide, 37.5:1) gels. Standard procedures were used for transferring proteins from the gels to nitrocellulose membranes (Schleicher and Schuell, Dassel, Germany) and immunodetection of specific proteins. Primary antibodies raised against adiponectin (Chemicon International, Temecula, CA, USA), phospho-(Thr 172)-AMPK (Cell Signaling Technology, Upstate, Lake Placid, NY, USA), total AMPK (Cell Signaling) and beta actin (Santa Cruz Biotechnology, CA, USA) were applied overnight at 4 °C; horseradish peroxidase-linked secondary antibodies (Amersham, Piscataway, NJ, USA) were applied for 1 h at room temperature. Immunoreactive proteins were visualized using enhanced chemiluminescence (ECL, Amersham) and the band intensities were quantified using NIH Image 1.60 software.

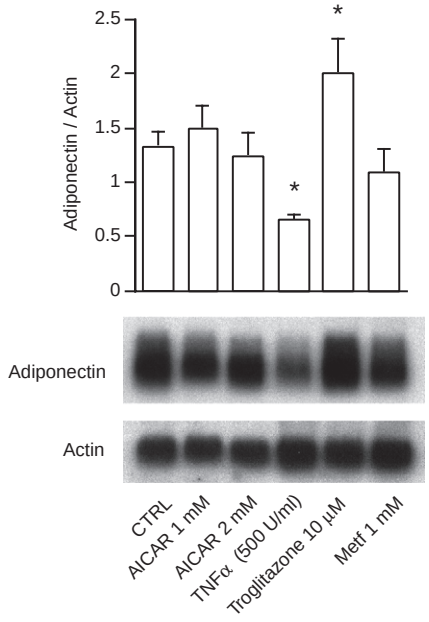

Fig. 3. Effect of AMPK-activators on adiponectin mRNA expression in 3T3-L1 adipocytes. Differentiated 3T3-L1 adipocytes were cultured for 24 h in the indicated concentrations of AICA-riboside (AICAR), metformin (Metf), troglitazone, TNF-α or in control medium (CTRL), and their RNA was extracted. Total RNA (10 μg) was submitted to gel eletrophoresis, transferred to nitrocellulose and hybridized with cDNA probes specific for adiponectin- and beta actin mRNA, as described in the Materials and methods. Data are expressed as the ratio of adiponectin-mRNA/actin-mRNA and represent the mean±S.E.M. of three experiments. Conditions showing statistically significant differences with results in control (CTRL) are indicated by an asterisk ($P<0.05$, by ANOVA). Representative Northern blotting results are shown.

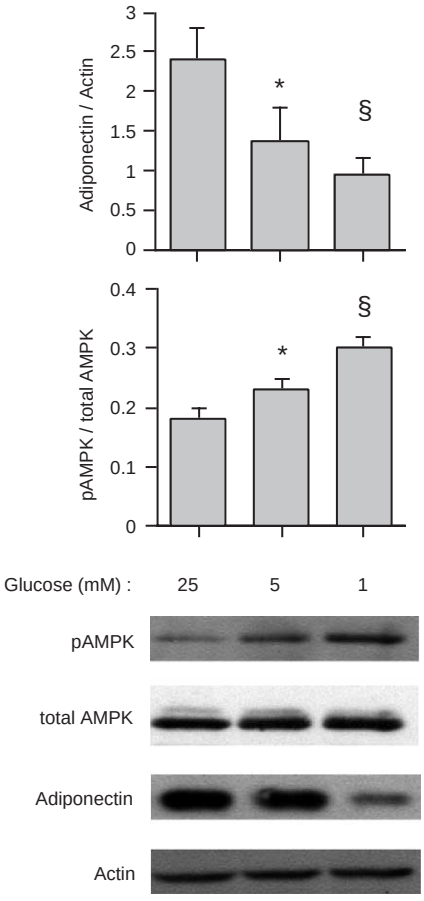

Fig. 4. Effect of glucose concentration on AMPK-phosphorylation and adiponectin protein content in 3T3-L1 adipocytes. Adiponectin protein expression and AMPK-phosphorylation were determined by Western blotting using lysates of 3T3-L1 adipocytes that had been cultured for 48 h in the indicated glucose concentrations. Five micrograms protein was electrophoresed and blotted for detection of adiponectin; 20 μg protein was used in the case of phospho-AMPK (pAMPK), total AMPK, and beta-actin (Actin). Immunoblotting was performed using specific antibodies as in Figs. 1 and 2. Data are expressed as the ratio of adiponectin/actin and pAMPK/total AMPK, respectively. The results shown represent the mean ± S.E.M. of four experiments (*$P<0.05$, §$P<0.01$ vs. 25 mM glucose, by ANOVA).

2.5. Statistical analysis

Results are expressed as mean ± S.E.M. with the number of independent experiments indicated in the figure legends. Statistical significance of differences between controls and experimental conditions was assessed by analysis of variance (ANOVA), with post hoc Fisher test, taking P-values <0.05 to indicate significant differences.

3. Results

3.1. Effects of AICAR, phenformin and metformin, and troglitazone on AMPK and adiponectin protein expression in 3T3-L1 adipocytes

Culture of 3T3-L1 adipocytes in presence of AICAR (0.5–2 mM), metformin (1 and 2 mM), and phenformin (0.2 mM) over 48 h did not influence their AMPK protein expression but increased its phosphorylation level (Fig. 1). These same conditions resulted in a reduction of adiponectin protein expression measured after culture for 24 and 48 h (Fig. 2). The reduction in adiponectin content by AICAR (47%, $P<0.01$) was similar to that by tumor necrosis factor (TNF)-α (500 U/ml) and more pronounced than that by metformin (at 1 mM reduced by 22%; $P<0.01$). The protein synthesis inhibitor, cycloheximide (5μg/ml), also markedly decreased the adiponectin content, suggesting that adiponectin content strongly depends on de novo protein synthesis in these cells (Fig. 2). The peroxisome proliferator activated receptor (PPAR)-γ agonist troglitazone (10 μM) did not modulate AMPK (result not shown), but significantly increased the adiponectin content, and prevented the metformin-mediated down-regulation of adiponectin proteins in 3T3 adipocytes (Fig. 2).

3.2. Effects of AMPK activators and troglitazone on adiponectin gene expression

Northern blot analysis showed that culture in the presence of AICAR (2 mM) or metformin (1 mM), did not significantly alter adiponectin mRNA expression in differentiated 3T3-L1 cells (Fig. 3), whereas addition of TNF-α (500 U/ml) decreased the number of adiponectin gene transcripts.

On the other hand, addition of troglitazone (10 μM) significantly increased adiponectin mRNA levels.

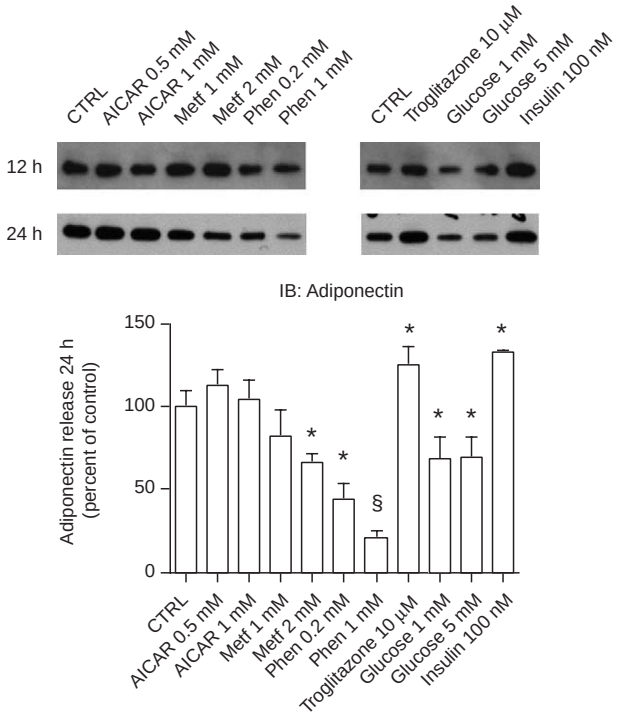

Fig. 5. Western blot analysis of released adiponectin protein. Differentiated 3T3-L1 adipocytes were cultured in the indicated conditions for either 12 or 24 h as described in the Materials and methods. Ten microliters medium was taken from the cultures for detection of released adiponectin by Western blotting using a monoclonal antibody (as in Fig. 2). Representative blots are shown for each time point. Quantification of the results for the 24-h release was carried out using densitometric scanning of the protein signals. The data are expressed as a percentage, relative to the adiponectin release measured in the control condition (CTRL, 25 mM glucose). The results represent the mean ± S.E.M. of three experiments (*$P<0.05$, §$P<0.01$ vs. CTRL, by ANOVA).

3.3. Effect of glucose-deprivation on AMPK and adiponectin

Culture at low glucose (1 and 5 mM) for 48 h resulted in a significant increase in phospho-AMPK levels, whereas total AMPK levels remained unchanged (Fig. 4). Under the same conditions, a reduced cellular adiponectin content was observed (Fig. 4).

3.4. Release of adiponectin into cell culture medium

Culture with metformin and phenformin resulted in lower adiponectin levels in the medium whereas troglitazone resulted in higher medium levels (Fig. 5). Adiponectin release was also lowered in cultures at low glucose concentration (1 or 5 mM) as compared to 25 mM glucose (Fig. 5).

4. Discussion

Adiponectin influences glucose and fatty acid metabolism through an insulin-sensitizing effect that appears to be mediated through activation of AMPK in liver, muscle and adipocytes (Yamauchi et al., 2002; Berg et al., 2001; Wu et al., 2003). Its circulating levels are reduced in patients with type 2 diabetes or with obesity, which may partially explain the insulin resistance in these patients (Weyer et al., 2001; Hotta et al., 2000; Arita et al., 1999). Both conditions are characterized by elevated concentrations of TNF-α, a cytokine that was found to lower adiponectin expression in adipocytes (Kappes and Loffler, 2000). Treatment with PPAR-γ agonists of the thiazolidinedione family increased expression of adiponectin (Maeda et al., 2001; Yu et al., 2002; Phillips et al., 2003), as part of a broader anabolic effect on adipocytes (Lenhard et al., 1997; Ciaraldi et al., 2002). Thus the insulin-sensitizing effect of troglitazone appears mediated through an elevation in adiponectin release (Maeda et al., 2001; Yu et al., 2002; Phillips et al., 2003), and associated to an increase in lipogenesis and in adipocyte size (Lenhard et al., 1997; Ciaraldi et al., 2002). In contrast, metformin increases lipolysis and reduces triglyceride stores in adipocytes, whereas its insulin-sensitizing effect appears to be mediated through AMPK activation, not PPAR-γ induction (Lenhard et al., 1997; Zhou et al., 2001; Ciaraldi et al., 2002). The AMPK stimulator AICAR has been reported as a potent inhibitor of adipogenesis and of PPAR-γ expression during 3T3-L1 differentiation (Habinowski and Witters, 2001). These studies have thus indicated differences in the mechanisms of action of PPAR-γ ligands and AMPK-activators in insulin sensitization at the level of adipocyte metabolism and differentiation. The present study extends these findings showing that the two classes of anti-diabetic drugs have opposing effects on adiponectin protein expression and release in differentiated adipocytes, which may help to clarify their pharmacological action. While positive effects of troglitazone on adiponectin expression in mature adipocytes have been described (Phillips et al., 2003), it was unknown whether AMPK stimulatory drugs directly affect the adiponectin protein level in adipocytes. The present study shows that prolonged exposure of 3T3-L1 adipocytes to AICAR, metformin, phenformin or low glucose concentrations stimulates their AMPK activity (Figs. 1 and 4), and was associated with a profound reduction in the adiponectin protein content in these cells (Figs. 2 and 4). Moreover, addition of biguanides or culture at low glucose also diminished adiponectin release (Fig. 5). Together, these data indicated an inhibition of adiponectin production and release through AMPK activation. The inhibitory effects of AMPK activation appeared mediated at the post-transcriptional level since adiponectin mRNA levels were not affected by metformin or AICAR (Fig. 3). The latter observation can not be readily reconciled with data showing that adiponectin gene expression was stimulated by AICAR in human subcutaneous adipose tissue (Lihn et al., 2004). However, it should be noted that in this tissue AICAR also lowered the production of TNF-α, a potent inhibitor of adiponectin expression (Kappes and Loffler, 2000; Lihn et al., 2004), which may explain the differences with 3T3-L1 cells. In addition, our data demonstrate a direct negative action of AICAR on adiponectin protein expression, which was not previously investigated.

It was recently suggested that modulation of adipocyte function including upregulation of adiponectin synthesis and secretion, is an important mechanism by which thiazolidinediones improve insulin action (Yu et al., 2002; Phillips et al., 2003; Tiikkainen et al., 2004). Upregulation of adiponectin was not seen with the antidiabetic biguanide metformin (Phillips et al., 2003; Tiikkainen et al., 2004). In combination with thiazolidinediones, metformin is a more efficient treatment strategy for improving insulin sensitivity, glycemic control, and beta cell function, as compared to metformin-monotherapy (Fonseca et al., 2000; Bailey and Day, 2004; Strowig et al., 2004). Our data show that the suppressive effects of metformin on production of the insulin sensitizing hormone adiponectin do not occur in the presence of troglitazone, illustrating that both AMPK- and PPAR-γ stimulating compounds exert opposing effects on adiponectin production and release in adipocytes. This finding may help to explain differences in effectiveness of their single and combined use in antidiabetic drug therapy.

In conclusion, metformin suppresses adiponectin production and release in differentiated adipocytes. This effect occurs through post-transcriptional mechanisms, involves the stimulation of AMP activated protein kinase, and is prevented by troglitazone.

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