Adiponectin gene expression is inhibited by β-adrenergic stimulation via protein kinase A in 3T3-L1 adipocytes

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Abstract Recently, it has been shown that the fat-derived factor adiponectin is downregulated in insulin resistance and obesity and that replenishment of this adipocytokine reverses insulin resistance in mice. Growing evidence, on the other hand, suggests that raised levels of catecholamines due to increased activity of the sympathetic nervous system are an integral part in the development of insulin resistance. To clarify whether catecholamines might exert their insulin resistance-inducing effects at least partly via downregulation of adiponectin gene expression, 3T3-L1 adipocytes were treated with isoproterenol, and adiponectin mRNA was measured by quantitative real-time reverse transcription-polymerase chain reaction. In fact, isoproterenol treatment reduced the level of adiponectin mRNA by about 75% in a dose-dependent fashion with significant inhibition detectable at concentrations as low as 10 nM isoproterenol. Furthermore, the inhibitory effect of isoproterenol was almost completely reversed by pretreatment of 3T3-L1 cells with the β-adrenergic antagonist propranolol and the protein kinase A (PKA) inhibitor H-89. Moreover, the effects of isoproterenol could be mimicked by stimulation of stimulatory guanine nucleotide-binding (G_S)-proteins with cholera toxin and adenylyl cyclase with forskolin. Thus, our results suggest that adiponectin gene expression is severely suppressed by β-adrenergic agents via activation of a G_S-protein–PKA-dependent pathway. The data support a possible role of adiponectin in catecholamine-induced insulin resistance. © 2001 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.

Key words: Adiponectin; β-Adrenergic receptor; Insulin resistance; Obesity; 3T3-L1 adipocyte

1. Introduction

Insulin resistance is a major risk factor for diabetes and cardiovascular disease. The factors causing insulin resistance are still not completely understood. However, progress has been made during the last 10 years [1]. It has become apparent that several adipocyte-secreted factors such as free fatty acids (FFA) [2], tumor necrosis factor (TNF) α [3], angiotensin [4],

plasminogen-activator inhibitor-1 (PAI-1) [5], and resistin [6] can decrease insulin sensitivity in vivo and in vitro. Most recently, two groups showed independently for the first time that replenishment of adiponectin, also called Acrp30, GBP28 or AdipoQ, increases insulin sensitivity in different murine models of insulin resistance in vivo [7,8].

Adiponectin was originally identified independently by four different groups and has been shown to be a protein induced during adipogenesis [9–12]. Hu et al. initially demonstrated that adiponectin was decreased in murine models of obesity and insulin resistance [11]. Recently, these studies were confirmed in humans where adiponectin is also suppressed in insulin-resistant and obese states [13,14]. First evidence that adiponectin is not simply a factor passively regulated by insulin resistance and obesity, but actively influencing these states came from studies by Fruebis et al. [15]. They could demonstrate that a proteolytic cleavage product of adiponectin increased fatty acid oxidation in muscle and caused weight loss in mice [15]. Further support for an important role of adiponectin in the pathogenesis of insulin resistance and obesity comes from two independent genetic studies. Thus, Vionnet et al. mapped a diabetes-susceptibility locus in a native French cohort to human chromosome 3q27 where the gene encoding adiponectin is located [16]. Furthermore, Kissebah et al. demonstrated a quantitative-trait locus on 3q27 strongly linked to the metabolic syndrome in European individuals [17].

Together these data suggest that adiponectin is an important modulator of insulin sensitivity in vivo. Furthermore, it appears possible that various factors might increase or decrease insulin sensitivity at least partly by up- or downregulation of adiponectin. In accordance with this view, Berg et al. could demonstrate that thiazolidinedione treatment which is known to improve insulin sensitivity increased adiponectin expression in mice [8]. Growing evidence suggests that increased activity of the sympathetic nervous system contributes to insulin resistance [18–21]. Therefore, we hypothesized that catecholamines might induce insulin resistance at least partly by downregulation of adiponectin gene expression.

In the current study, we examined the effect of isoproterenol on adiponectin mRNA expression in 3T3-L1 adipocytes in vitro. In accordance with our hypothesis, we demonstrate for the first time that isoproterenol inhibits adiponectin gene expression. Furthermore, we show that this inhibitory effect is mediated via β-adrenergic receptors, stimulatory guanine nucleotide-binding (G_S)-proteins and protein kinase A (PKA).

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2. Materials and methods

2.1. Materials

Isoproterenol, propranolol, phentolamine, cholera toxin, forskolin, PD 98059, SB 203580, and H-89 were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Cell culture reagents were obtained from Life Technologies, Inc. (Grand Island, NY, USA), oligonucleotides from MWG-Biotech (Ebersberg, Germany).

2.2. Cell culture and differentiation

3T3-L1 adipocytes (American Type Culture Collection, Rockville, MD, USA) were grown at 37°C in 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) containing 25 mM glucose, 10% fetal bovine serum, and antibiotics (culture medium). At confluence preadipocytes were cultured for 2 days in culture medium further supplemented with 1 μM insulin, 0.5 mM isobutylmethylxanthine and 0.1 μM dexamethasone. After additional 2 days in culture medium with 1 μM insulin, cells were grown for 4 to 8 days in culture medium. At the time of the experiments more than 90% of the cells had accumulated fat droplets. Adipocytes were maintained for 6 h in serum-free medium before various effectors were added as described in the figure legends.

2.3. Analysis of adiponectin gene expression

Measurement of adiponectin gene expression was performed by quantitative real-time reverse transcription-polymerase chain reaction (RT-PCR) in a fluorescent temperature cycler (LightCycler, Roche Molecular Biochemicals, Mannheim, Germany) as previously described [22]. Briefly, total RNA was isolated from 3T3-L1 adipocytes using TRIzol reagent (Life Technologies, Inc., Grand Island, NY, USA) and 1 μg of total RNA was reverse transcribed using standard reagents (Life Technologies, Inc., Grand Island, NY, USA). 10% of each RT reaction was amplified in a 20 μl PCR containing 3 mM $MgCl_2$, 0.5 μM of each primer and 1× LightCycler DNA Master SYBR Green I mix (Roche Molecular Biochemicals, Mannheim, Germany). Samples were loaded into capillary tubes and incubated in the LightCycler for an initial denaturation at 94°C for 30 s, followed by 40 cycles, each cycle consisting of 95°C for 0 s, 61°C for 7 s, and 72°C for 10 s (adiponectin) or 94°C for 5 s, 60°C for 7 s, and 72°C for 21 s (β-actin). The following oligonucleotide primers were used: adiponectin (accession number U37222) AAGGACAAGGCCGTTCTCT (sense) and TATGGGTAGTTGCAGTCAGTTGG (antisense); β-actin (accession number X03672) CCAGGGTGTGATGGTGGGA-ATG (sense) and CGCACGATTTCCCTCTCAGCTG (antisense). Cycle-to-Cycle SYBR Green I fluorescence emission readings were monitored. Quantification was done by using the second derivative maximum method of the LightCycler Software (Roche Molecular Biochemicals, Mannheim, Germany) which determines the crossing points of individual samples by an algorithm identifying the first turning point of the fluorescence curve. This turning point corresponds to the first maximum of the second derivative curve and correlates inversely to the log of the initial template concentration. Adiponectin mRNA levels were normalized to those of β-actin.

To confirm amplification of specific transcripts, melting curve profiles were produced (cooling the sample to 68°C and heating slowly to 95°C with continuous measurement of fluorescence) at the end of each run. The specificity of the PCR was further verified by subjecting the amplification products to electrophoresis on a 1.5% agarose gel followed by staining with ethidium bromide.

2.4. Statistical analysis

Results are shown as mean ± S.E.M. For analysis of differences between various cell treatments, unpaired Student's t-test was used. P values < 0.05 are considered significant, < 0.01 highly significant.

3. Results

3.1. Measurement of adiponectin and β-actin mRNA expression

First, the reliability of adiponectin and β-actin quantification by real-time RT-PCR was tested. For this purpose, increasing amounts of total cellular RNA from differentiated 3T3-L1 cells were reverse transcribed and analyzed using specific primer pairs for the two genes (Fig. 1A,B). Linearity
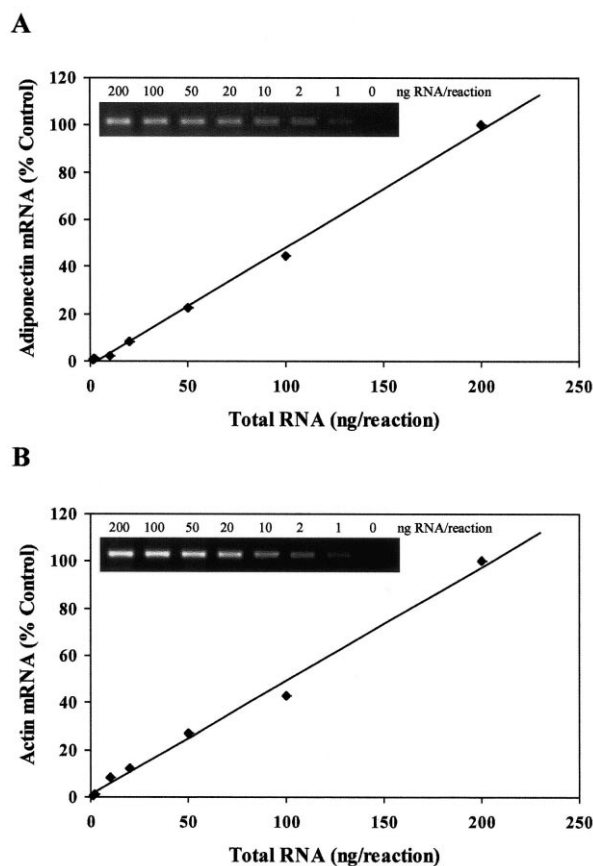

Fig. 1. Quantification of adiponectin mRNA in 3T3-L1 adipocytes. Total RNA from fully differentiated 3T3-L1 adipocytes was isolated and subjected to quantitative real-time RT-PCR with primers specific for adiponectin (A) and β-actin (B) as described in Section 2. Data are expressed relative to adiponectin and β-actin mRNA expression measured with 200 ng RNA (= 100%). Inset: Agarose gel electrophoresis of the PCR products at cycle 21 (A) and 26 (B), respectively.

between total RNA used per reaction and amount of mRNA measured by the LightCycler software was obtained in the range between 1 and 200 ng total RNA for both, adiponectin (Fig. 1A) and β-actin (Fig. 1B).

3.2. Isoproterenol inhibits adiponectin gene expression

β-Adrenergic stimulation has long been known to induce insulin resistance [23,24]. To determine whether this effect might at least partly be explained by decreased adiponectin gene expression, fully differentiated adipocytes were incubated with increasing concentrations of isoproterenol for 16 h. Treatment of 3T3-L1 cells with 10 μM isoproterenol reduced the levels of adiponectin mRNA normalized to β-actin dramatically by about 75% as compared to untreated controls ($P < 0.01$) (Fig. 2). This effect was dose-dependent with a 65% inhibition already detectable at concentrations as low as 10 nM isoproterenol ($P < 0.01$) (Fig. 2).

3.3. The inhibitory effect of isoproterenol on adiponectin mRNA is mediated via β-adrenergic receptors and PKA

To verify that inhibition of adiponectin gene expression by isoproterenol is, in fact, mediated via β-adrenergic receptors, fully differentiated adipocytes were pretreated with selective antagonists of α- (phentolamine, 100 μM) and β- (proprano-
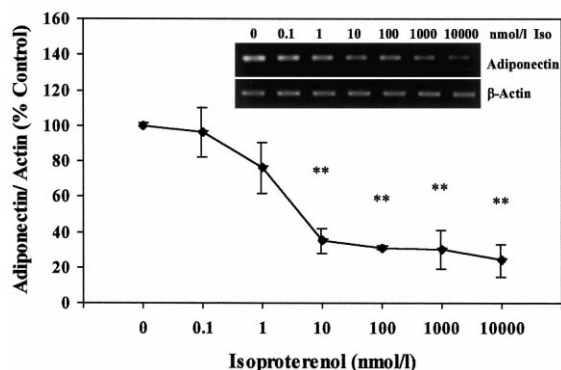
Fig. 2. Dose-dependent inhibition of adiponectin gene expression by isoproterenol. Fully differentiated 3T3-L1 cells were serum-starved for 6 h before various concentrations of isoproterenol (Iso) were added for 16 h. Total RNA was subjected to quantitative real-time RT-PCR to determine adiponectin mRNA levels as described in Section 2. Adiponectin gene expression normalized to β-actin mRNA levels is expressed relative to untreated control cells (= 100%). Inset: Agarose gel electrophoresis of the PCR products at cycle 21 (adiponectin) and 26 (β-actin), respectively. Results are the means ± S.E.M. of two to four independent experiments. **$P < 0.01$ comparing isoproterenol-treated with non-treated cells.

lol, 100 μM) adrenergic receptors for 1 h before isoproterenol (10 μM) was added for 16 h. Adiponectin mRNA significantly increased to about 70% of the level observed in untreated control cells when 3T3-L1 adipocytes were pre-incubated with propranolol before isoproterenol stimulation ($P < 0.05$) (Fig. 3). Phentolamine, on the other hand, rather enhanced the inhibitory effect of isoproterenol on adiponectin gene expression (Fig. 3). Taken together, these results suggest that isoproterenol inhibits adiponectin gene expression via β-adrenergic receptors.

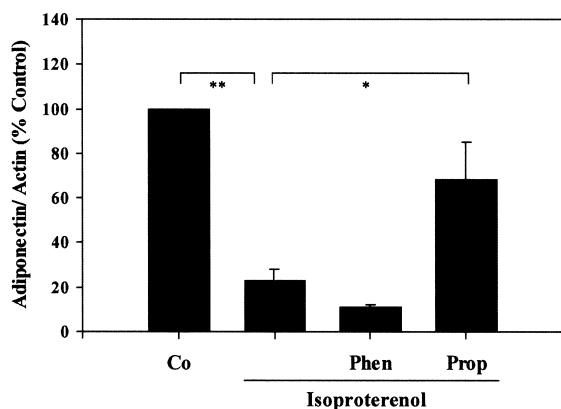Typically, stimulation of G_S-proteins leads to activation of G_S-proteins which, in turn, activate adenylyl

Fig. 3. Inhibition of adiponectin gene expression by isoproterenol is mediated via β-adrenergic receptors. After 3T3-L1 cells were serum-starved for 5 h, adipocytes were cultured in the presence or absence of phentolamine (Phen, 100 μM) or propranolol (Prop, 100 μM) for 1 h before isoproterenol (10 μM) was added for 16 h. Total RNA was extracted and subjected to quantitative real-time RT-PCR to determine adiponectin mRNA levels normalized to β-actin expression as described in Section 2. Data are expressed relative to non-treated control (Co) cells (= 100%). **$P < 0.01$ comparing isoproterenol-treated with non-treated cells; *$P < 0.05$ comparing isoproterenol-treated with propranolol-pretreated adipocytes.
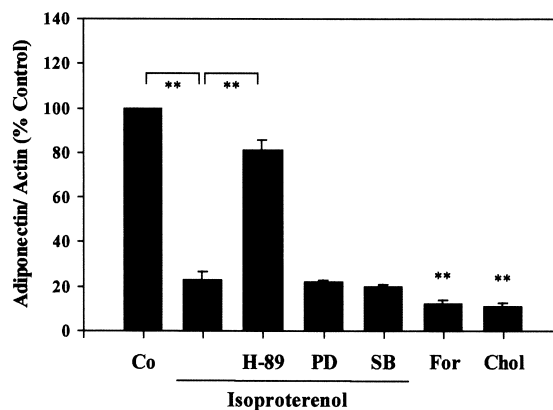

Fig. 4. Inhibition of adiponectin gene expression by isoproterenol is mediated via G_S-proteins, adenylyl cyclase, and PKA. After 5 h serum-starvation, 3T3-L1 adipocytes were cultured in the presence or absence of H-89 (10 μM), PD 98059 (PD, 50 μM), and SB 203580 (SB, 20 μM) for 1 h before isoproterenol (10 μM), forskolin (For, 200 μM), and cholera toxin (Chol, 1000 ng/ml) were added for 16 h. Total RNA was extracted and quantitative real-time RT-PCR was performed as described in Section 2. Adiponectin mRNA levels are normalized to β-actin and expressed relative to non-treated control (Co) cells (= 100%). Results are the means ± S.E.M. of four independent experiments. **$P < 0.01$ comparing non-treated with isoproterenol-treated, forskolin-treated and cholera toxin-treated adipocytes or comparing isoproterenol-treated and H-89-pretreated cells.

cyclase and PKA. To confirm that the inhibitory effect of isoproterenol on adiponectin gene expression is mediated via PKA, 3T3-L1 adipocytes were pre-incubated with H-89 (10 μM), a selective antagonist of PKA, for 1 h before isoproterenol (10 μM) was added for 16 h. In fact, H-89 almost completely reversed the inhibitory effect of isoproterenol on adiponectin gene expression ($P < 0.01$) (Fig. 4). Moreover, the effects of cholera toxin, an activator of G_S-proteins, and forskolin, a direct stimulator of adenylyl cyclase, on adiponectin gene expression were examined. As shown in Fig. 4, both effectors significantly inhibited adiponectin gene expression by about 90%. This effect was dose-dependent with significant inhibition detectable at concentrations as low as 10 ng/ml cholera toxin and 2 μM forskolin, respectively (data not shown). By contrast, specific inhibition of p44/42 and p38 MAP kinases by PD 98059 (50 μM) and SB 203580 (20 μM), respectively, did not rescue isoproterenol-induced suppression of adiponectin gene expression (Fig. 4).

3.4. The inhibitory effect of isoproterenol on adiponectin gene expression is reversible

To confirm that the reduction of adiponectin mRNA by β-adrenergic stimulation was not due to a 'dedifferentiation' effect or toxic effect of isoproterenol, fully differentiated adipocytes were treated with isoproterenol (10 μM) for 16 h and the medium was then replaced by DMEM containing 25 mM glucose for an additional 24 h. Incubation of 3T3-L1 adipocytes with isoproterenol again decreased adiponectin gene expression to about 25% of untreated control cells (Fig. 5, columns 1 and 2, $P < 0.01$). However, removal of isoproterenol from the medium increased adiponectin mRNA to control levels (Fig. 5, columns 3 and 4). Interestingly, serum-starvation for an additional 24 h appeared to inhibit adiponectin gene expression by almost 40% (Fig. 5, columns 1 and 3).
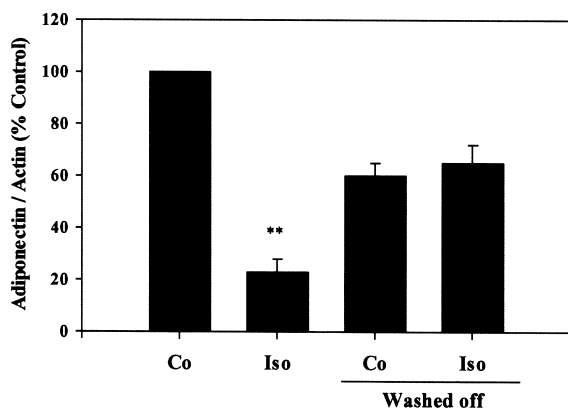
Fig. 5. Inhibition of adiponectin gene expression by isoproterenol is reversible. Fully differentiated 3T3-L1 cells were serum-starved for 6 h before isoproterenol (Iso) was added for 16 h (columns 1, 2). After this period cells were rinsed once with DMEM and cultured in DMEM containing 25 mM glucose for an additional 24 h (columns 3, 4). Extraction of total RNA and quantitative real-time RT-PCR were performed as described in Section 2. Adiponectin gene expression normalized to β-actin mRNA levels is expressed relative to untreated control (Co) cells (=100%). Results are the means ± S.E.M. of two independent experiments. **$P < 0.01$ comparing isoproterenol-treated with non-treated cells.

4. Discussion

Several lines of evidence suggest that the fat-secreted factor adiponectin is a new insulin-sensitizing adipocytokine and might represent a novel treatment strategy for insulin resistance and type 2 diabetes [7,8].

It appears possible that various hormones mediate their insulin sensitivity-modulating effect at least partly via up- or downregulation of adiponectin. Growing evidence suggests that catecholamines impair insulin sensitivity and that increased activity of the sympathetic nervous system contributes to insulin resistance [18–21]. In accordance with this view, we have previously demonstrated molecular interactions on several levels between adrenergic and insulin signaling cascades [25,26]. In a clinical context, our group recently demonstrated that patients with pheochromocytoma are insulin-resistant due to increased serum levels of catecholamines [27]. Furthermore, insulin resistance could be reduced by surgical removal of the tumors in most cases [27]. The mechanisms by which catecholamines induce insulin resistance are still not completely understood. In the present study, we demonstrate for the first time that β-adrenergic stimulation decreases adiponectin gene expression in 3T3-L1 adipocytes. Thus, β-adrenergic downregulation of this insulin-sensitizing adipocytokine may be an important new mechanism of inducing insulin resistance.

Presently, the mechanisms by which adiponectin improves insulin sensitivity are largely unknown. Yamauchi et al. reported increased fatty acid transport, combustion and energy dissipation in muscle after adiponectin treatment [7]. These processes lead to reduced muscle triglyceride content and serum FFA levels [7]. As a result of these changes, insulin signaling in muscle was improved [7]. These data suggest that decreased levels of adiponectin may increase FFA serum levels and impair insulin signaling in insulin-sensitive tissues. Interestingly, both effects can be observed in catecholamine-induced insulin resistance. Thus, we and others have shown

that β-adrenergic stimulation leads to increased serum concentrations of FFA and inhibits insulin signaling molecules essential for insulin action such as insulin receptor substrates [25,28–30]. It appears possible that reduced expression of adiponectin contributes to increased FFA levels and impaired insulin signaling after chronic catecholamine treatment. However, direct effects of β-adrenergic activation on lipolysis and insulin signaling molecules most likely also contribute [23–25,30].

In the present study, we show evidence that the inhibitory effect of isoproterenol on adiponectin expression is mediated via activation of PKA. These results are in accordance with the classical view of β-adrenergic receptors being coupled to PKA via G_S-proteins and adenylyl cyclase [31]. Furthermore, we exclude the possibility that suppression of adiponectin gene expression is simply due to a toxic or 'dedifferentiation' effect of catecholamines. The signaling pathway of isoproterenol downstream of PKA inhibiting adiponectin gene expression remains to be elucidated. Activation of PKA by β-adrenergic stimulation has been shown to stimulate p44/42 and p38 MAP kinases in adipocytes [26,32–34]. However, pretreatment of 3T3-L1 cells with selective inhibitors of either molecule does not rescue isoproterenol-induced suppression of adiponectin gene expression. Thus, both molecules are unlikely candidates mediating the inhibitory effect of isoproterenol downstream of PKA. As another possibility, lipolysis in response to a rise in intracellular cAMP levels may play a role in the regulation of adiponectin mRNA expression. Interestingly, we show that starvation of 3T3-L1 adipocytes for an additional 24 h, a condition accompanied by increased lipolysis and elevated concentrations of intracellular FFA, inhibits adiponectin gene expression by about 40%.

In summary, we demonstrate for the first time that β-adrenergic stimulation reduces adiponectin gene expression. Furthermore, we present evidence that this effect is mediated via G_S-proteins, adenylyl cyclase and PKA. This mechanism may play an important role in the pathogenesis of insulin resistance and the insulin resistance syndrome. More work is needed to clearly define the signaling pathways by which adiponectin exerts its effects on insulin sensitivity and to further characterize the complex interplay between insulin, adrenergic and adiponectin signaling.

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