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Isoproterenol inhibits resistin gene expression through a G_S-protein-coupled pathway in 3T3-L1 adipocytes

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Abstract Resistin was recently identified as a hormone secreted by adipocytes which leads to insulin resistance in vivo and in vitro and might therefore be an important link between obesity and diabetes. To clarify the regulation of resistin gene expression, 3T3-L1 adipocytes were treated with various agents known to modulate insulin sensitivity, and resistin mRNA was measured by quantitative real-time reverse transcription-polymerase chain reaction. Interestingly, isoproterenol treatment reduced the level of resistin mRNA to 20% of non-treated control cells. This effect was dose-dependent with significant inhibition occurring at concentrations as low as 10 nM isoproterenol. Moreover, pretreatment of adipocytes with the β-adrenergic antagonist propranolol almost completely reversed the inhibitory effect of isoproterenol, whereas addition of the α-adrenergic antagonist phentolamine did not have any effect. Furthermore, the effect of isoproterenol could be mimicked by activation of G_S-proteins and adenylyl cyclase. Thus, both cholera toxin and forskolin decreased resistin mRNA expression in a dose-dependent fashion by up to 90% of control levels. Taken together, these results suggest that resistin gene expression is regulated by a protein kinase A-dependent pathway in 3T3-L1 adipocytes.

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

1. Introduction

Type 2 diabetes is among the most common chronic diseases, affecting about 150 million people worldwide [1]. Type 2 diabetes is characterized by insulin resistance of peripheral tissues such as liver, muscle and fat which cannot be overcome by hypersecretion of pancreatic β-cells [2]. Insulin resistance is often associated with obesity [3]. The connection between increased adiposity and insulin resistance is still not completely clear. It has been shown that adipocytes secrete several factors such as free fatty acids (FFA), tumor necrosis factor α and angiotensinogen which cause insulin resistance in vivo and in vitro [4]. Most recently, Steppan et al. [5,6] reported the isolation of a novel adipocyte-derived signaling molecule which they named resistin. It was shown that resistin levels are significantly increased in both genetic and diet-induced obesity in vivo [5]. Furthermore, resistin administration caused impaired glucose tolerance, whereas immunoneutralization of this protein improved insulin action [5]. Most interestingly, the authors showed that resistin is downregulated by thiazolidinediones (TZDs) [5]. TZDs are a new class of anti-diabetic drugs which enhance insulin sensitivity [5]. Therefore, resistin might be both a link between obesity and diabetes and a candidate to explain the anti-diabetic effects of TZDs.

However, little is known concerning the control of resistin gene expression. Steppan et al. [5] demonstrated that resistin mRNA and protein are downregulated during fasting and upregulated upon refeeding. Furthermore, Kim et al. [7] showed a strong upregulation of resistin mRNA after streptozotocin-diabetic mice were treated with insulin. Apart from insulin, several other hormones such as catecholamines, glucocorticoids and thyroid hormones influence fat cell metabolism, as well as insulin sensitivity, and therefore, are potential regulators of resistin gene expression.

Therefore, we examined the effect of isoproterenol, dexamethasone and triiodothyronine (T3) in vitro on the expression of resistin mRNA in 3T3-L1 adipocytes. We show that isoproterenol inhibits resistin gene expression. Furthermore, we demonstrate that this inhibitory effect is mediated via a G_S-protein-coupled pathway.

2. Materials and methods

2.1. Materials

Isoproterenol, propranolol, phentolamine, cholera toxin, forskolin, T3, and dexamethasone were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Oligonucleotides were from MWG-Biotech (Ebersberg, Germany). Fetal bovine serum and cell culture reagents were purchased from Life Technologies (Grand Island, NY, USA).

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 containing 25 mM glucose, 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% fetal bovine serum (culture medium). For differentiation, confluent preadipocytes were cultured for 2 days in culture medium supplemented with 5 μM insulin, 0.5 mM isobutylmethylxanthine and 0.1 μM dexamethasone, and 2 days in culture medium with 5 μM insulin. The cells were then grown for an additional 4–8 days in culture medium after which more than 90% of the cells had accumulated fat droplets. Adipocytes were maintained for 6 h in serum-free medium before the different effectors were added for 16 h as described in the figure legends.

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Abbreviations: FFA, free fatty acids; Glut, glucose transporter; G-protein, guanine nucleotide-binding protein; RT-PCR, reverse transcription-polymerase chain reaction; T3, triiodothyronine; TZD, thiazolidinedione

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2.3. Analysis of resistin gene expression

Real-time reverse transcription-polymerase chain reaction (RT-PCR) analyses were performed in a fluorescent temperature cycler (LightCycler, Roche Molecular Biochemicals, Mannheim, Germany) according to the manufacturer's instructions. Briefly, total RNA was isolated from 3T3-L1 cells using TRIzol reagent (Life Technologies) and 1 μg of total RNA was reverse transcribed using standard reagents (Life Technologies). One-tenth (2 μl) of each RT reaction served as a template 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). After initial denaturation at 94°C for 30 s, reactions were cycled 40 times using the following parameters for resistin detection: 95°C for 1 s, primer annealing at 61°C for 7 s, and primer extension at 72°C for 11 s. β-Actin cDNA was amplified as follows: 94°C for 5 s, primer annealing at 60°C for 7 s, and primer extension at 72°C for 21 s. SYBR Green I fluorescence was detected at the end of each cycle to monitor the amount of PCR product formed during that cycle. At the end of each run, melting curve profiles were produced (cooling the sample to 68°C and heating slowly to 95°C with continuous measurement of fluorescence) to confirm amplification of specific transcripts. The following oligonucleotide primers specific for mouse resistin (accession number AF323080) and β-actin (accession number X03672) were used: resistin, GTACCCACGGGATGAAGAACC (sense) and GCAGAGCCACAGGAGCAG (antisense); β-actin CCAGGGTGTGATGGTGGGAATG (sense) and CGCACGATTTCCCTCTCAGCTG (antisense). Cycle-to-cycle fluorescence emission readings were monitored and quantified using the second derivative maximum method of the LightCycler Software (Roche Molecular Biochemicals). The software determines the crossing points of individual samples by an algorithm which identifies the first turning point of the fluorescence curve. This turning point corresponds to the first maximum of the second derivative curve and correlates inversely with the log of the initial template concentration. Relative resistin mRNA levels were normalized to those of β-actin. The specificity of the amplification product 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 indicated as mean ± S.E.M. Unpaired Student's *t*-tests were used for analysis of differences between various cell treatments. $P < 0.05$ is considered significant and < 0.01 highly significant.

3. Results

3.1. Measurement of resistin mRNA levels in 3T3-L1 adipocytes

To test the reliability of the quantitative real-time RT-PCR method, increasing amounts of total cellular RNA from differentiated 3T3-L1 cells were reverse-transcribed and analyzed using specific primer pairs for resistin and β-actin (Fig. 1A,B). Linearity between total RNA used per reaction and amount of mRNA measured by the LightCycler software was obtained between 1 and 200 ng of total RNA for both mRNA products (Fig. 1A,B).

3.2. Resistin mRNA levels are decreased by isoproterenol

To identify factors modulating resistin gene expression, fully differentiated 3T3-L1 cells were treated with isoproterenol, T3, and dexamethasone for 16 h. After isolation of total RNA resistin mRNA levels normalized to β-actin mRNA were determined by real-time RT-PCR. Isoproterenol treatment reduced the levels of resistin mRNA dramatically by about 80% as compared to untreated controls ($P < 0.01$) (Fig. 2). In contrast, T3 and dexamethasone did not have a significant effect on resistin mRNA levels (Fig. 2).

To determine whether the reduction of resistin mRNA by isoproterenol is dose-dependent, differentiated 3T3-L1 cells were incubated with increasing concentrations (0–10 μM) of

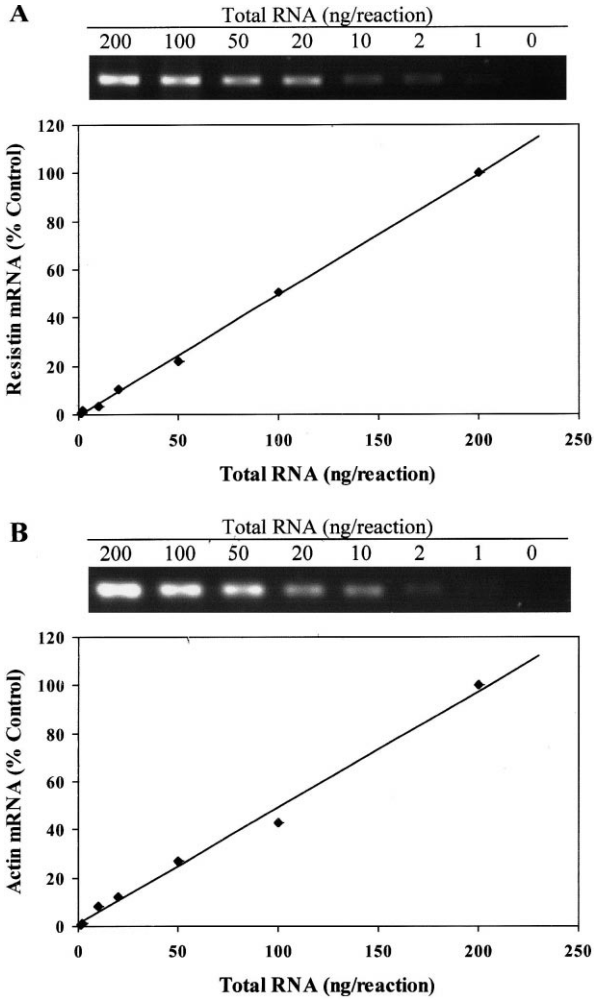

Fig. 1. Measurement of resistin mRNA levels in 3T3-L1 adipocytes. Increasing amounts of total RNA from fully differentiated 3T3-L1 cells were subjected to quantitative real-time RT-PCR with primers specific for resistin (A) and β-actin (B) as described in Section 2. Data are expressed relative to resistin and β-actin mRNA levels measured with 200 ng RNA (=100%). Top panels: agarose gel electrophoresis of the PCR products at cycles 23 (A) and 27 (B).

this compound. Isoproterenol decreased the level of resistin mRNA expression to about 20% as compared to non-treated control cells in a dose-dependent fashion (Fig. 3). At 10 nM isoproterenol resistin gene expression was reduced by about 50% ($P < 0.01$) (Fig. 3). Maximum inhibition was observed at a concentration of 10 μM isoproterenol (Fig. 3).

3.3. Pretreatment of 3T3-L1 adipocytes with propranolol reverses the inhibitory effect of isoproterenol

Isoproterenol stimulates β-adrenergic receptors. To further confirm that the reduction of resistin mRNA is mediated through this receptor, 3T3-L1 adipocytes were pretreated with selective antagonists of the α- and β-adrenergic receptors (100 μM each) for 1 h before isoproterenol (10 μM) was added for 16 h. Propranolol, a specific β-adrenergic receptor antagonist, almost completely reversed the inhibitory effect of isoproterenol on resistin gene expression ($P < 0.01$) (Fig. 4). In contrast, phentolamine, an α-adrenergic receptor antagonist, rather enhanced the inhibitory effect of isoproterenol on re-

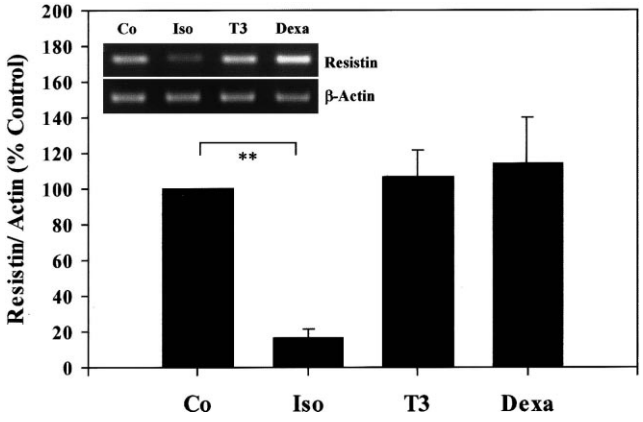

Fig. 2. Resistin gene expression is inhibited by isoproterenol. 3T3-L1 adipocytes were serum-starved for 6 h before isoproterenol (Iso, 10 μM), T3 (1 μM), or dexamethasone (Dexa, 100 nM) was added for 16 h. Total RNA was extracted and subjected to quantitative real-time RT-PCR to determine resistin mRNA levels normalized to β-actin expression as described in Section 2. Data are expressed relative to non-treated control (Co) cells (=100%). Inset: Agarose gel electrophoresis of the PCR products at cycles 23 (resistin) and 27 (β-actin). Results are the means ± S.E.M. of four independent experiments. $^{**}P<0.01$ comparing isoproterenol-treated with untreated control cells.

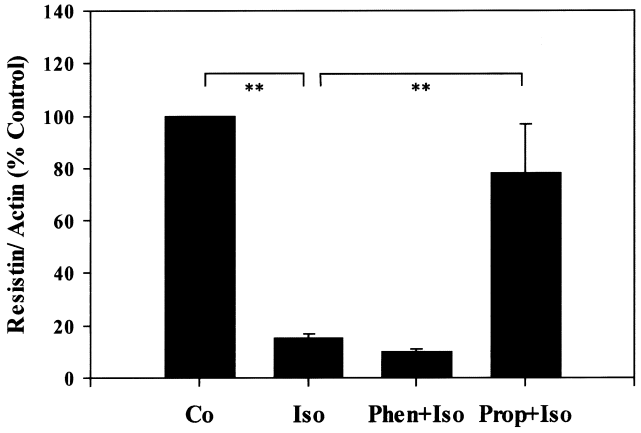

Fig. 4. Inhibitory effect of isoproterenol on resistin gene expression is mediated via β-adrenergic receptors. After 5 h serum starvation, 3T3-L1 adipocytes were cultured in the presence or absence of phentolamine (Phen, 100 μM) or propranolol (Prop, 100 μM) for 1 h before isoproterenol (Iso, 10 μM) was added for 16 h. Total RNA was extracted and quantitative real-time RT-PCR was performed as described in Section 2. Resistin 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 isoproterenol-treated with non-treated or propranolol-pretreated adipocytes.

sistin mRNA (Fig. 4). These results suggest that the reduction of resistin gene expression by isoproterenol is mediated via β-adrenergic receptors.

3.4. The effect of isoproterenol on resistin gene expression can be mimicked by cholera toxin and forskolin

β-Adrenergic receptors are typically coupled through G_S-proteins to the stimulation of adenylyl cyclase and protein kinase A. To confirm that the inhibitory effect of isoproterenol is mediated via a G_S-protein pathway, the effects of cholera toxin and forskolin on resistin gene expression were examined. Cholera toxin activates G_S-proteins by ADP-ribosylation, whereas forskolin is a direct activator of adenylyl cyclase. 3T3-L1 adipocytes were treated with different concentrations of cholera toxin (10, 100, 1000 ng/ml) and forskolin (2, 20, 200 μM) for 16 h and resistin mRNA levels were determined by real-time RT-PCR. As shown in Fig. 5, both effectors significantly inhibited resistin gene expression in a dose-dependent manner. At most 90% reduction of resistin mRNA was observed at concentrations of 1000 ng/ml cholera toxin and 200 μM forskolin (Fig. 5).

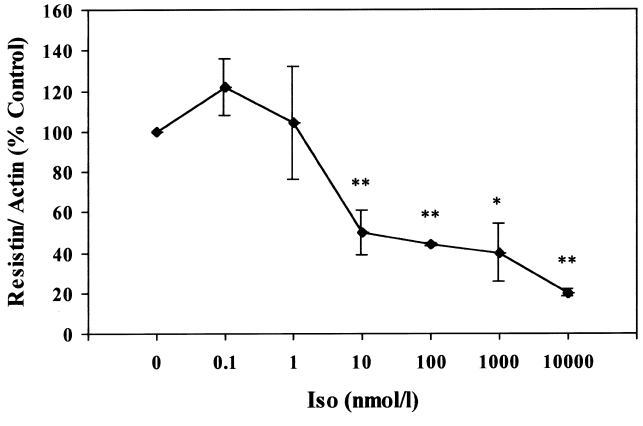

Fig. 3. Inhibition of resistin gene expression by isoproterenol is dose-dependent. Fully differentiated 3T3-L1 cells were serum-starved for 6 h before various concentrations of isoproterenol (Iso) were added for 16 h. Extraction of total RNA and quantitative real-time RT-PCR were performed as described in Section 2. Resistin gene expression normalized to β-actin mRNA levels is expressed relative to untreated control cells (=100%). Results are the means ± S.E.M. of two independent experiments. $^{*}P<0.05$, $^{**}P<0.01$ comparing isoproterenol-treated with non-treated cells.

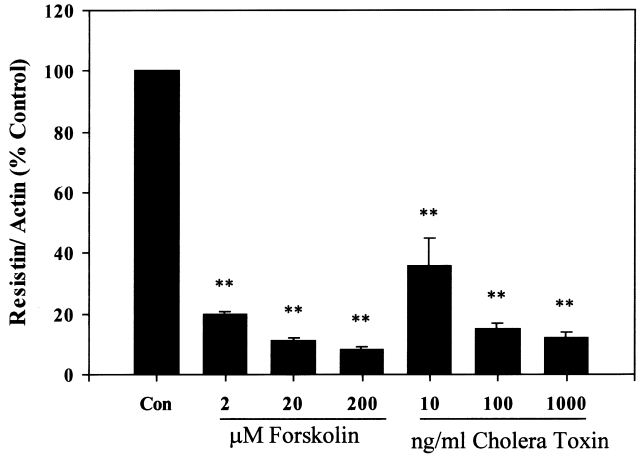

Fig. 5. Inhibition of resistin expression by isoproterenol can be mimicked by cholera toxin and forskolin. 3T3-L1 adipocytes were serum-starved for 6 h before various concentrations of forskolin and cholera toxin were added for 16 h as indicated. After extraction of total RNA, quantitative real-time RT-PCR was performed as described in Section 2. Resistin 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 four independent experiments. $^{**}P<0.01$ comparing untreated with forskolin- or cholera toxin-treated cells.

4. Discussion

Resistin was recently identified as an adipocyte-secreted factor which is overexpressed in obesity and causes insulin resistance [5]. In the present study, we demonstrate for the first time that β-adrenergic stimulation decreases resistin gene expression in 3T3-L1 adipocytes in a dose-dependent fashion.

There is growing evidence that increased activity of the sympathetic nervous system contributes to insulin resistance [8]. Our group recently demonstrated that patients with pheochromocytoma are insulin-resistant due to increased serum levels of catecholamines [9]. In most cases insulin resistance could be reduced by surgical removal of the tumors [9]. At the molecular level β-adrenergic stimulation is a strong activator of lipolysis, which leads to increased serum concentrations of FFA. FFA have been shown to cause insulin resistance at least partly by inhibiting insulin signaling [10]. Moreover, serum levels of FFA are already increased before glucose tolerance is impaired [11]. Furthermore, we and others have shown that β-adrenergic activation itself inhibits insulin signaling molecules such as insulin receptor substrates, which have been shown to be essential for insulin action [12–14]. However, in the present study we clearly demonstrate that resistin mRNA levels are decreased upon isoproterenol treatment. Thus, it appears likely that β-adrenergic stimulation has a dual effect on insulin sensitivity. On one hand, activation of β-adrenergic receptors induces insulin resistance, e.g. by negative interaction with insulin signaling molecules, on the other hand, insulin sensitivity might be improved by inhibition of resistin gene expression. In vivo, insulin resistance-inducing effects might be more prominent as compared to insulin resistance-reducing pathways. Interestingly, β-adrenoreceptor antagonists, e.g. propranolol, have been shown to impair insulin sensitivity in vivo [15]. Based on our studies, this effect might be explained at least partly by increased resistin gene expression after antagonist treatment. In vivo experiments determining the effects of various β-adrenergic agonists and antagonists on insulin sensitivity and resistin gene expression will help to better define the physiological significance of our in vitro findings.

In this study, we present evidence that the inhibitory effect of isoproterenol on resistin gene expression is mediated via increased intracellular levels of cAMP. These results are in accordance with the classical view of β-adrenergic receptors being coupled to G_S-proteins, leading to activation of adenylyl cyclase and protein kinase A [16]. The signaling pathway of isoproterenol downstream of protein kinase A decreasing resistin mRNA is presently unclear. One possibility is that lipolysis in response to a rise in intracellular cAMP levels may play a role in the regulation of resistin gene expression. Interestingly, prior in vivo studies have shown that resistin mRNA expression is inhibited by starvation, a condition accompanied by increased lipolysis and elevated concentrations of intracellular FFA [5]. Similarly, a reduction of cytosolic FFA induced by the antilipolytic action of insulin was shown to increase resistin mRNA levels [7]. Clearly, more work is needed to elucidate the exact mechanism by which increased cAMP levels lead to reduced resistin gene expression.

Glucocorticoids and thyroid hormones have profound effects on insulin sensitivity and fat cell metabolism. Thus, it has been demonstrated that glucocorticoid excess causes insulin resistance [17]. At the molecular level, it has been shown that dexamethasone treatment decreases expression of glucose transporter (Glut) 1 and translocation of Glut 4 [18]. Increased serum levels of thyroid hormones can also impair glucose tolerance [19]. This effect might be at least partly due to impaired expression and translocation of Glut 4 [20]. In our experimental system glucocorticoids and T3 do not affect resistin gene expression. Therefore, our data do not support a major role for resistin in insulin resistance caused by these two hormones. However, in vivo data e.g. of patients affected by Cushing's syndrome or Graves' disease will be necessary to clearly define the role of resistin in insulin resistance under conditions of glucocorticoid or thyroid hormone excess.

In summary, we demonstrate for the first time that β-adrenergic stimulation by isoproterenol reduces the level of resistin mRNA. Furthermore, we show that this inhibitory effect is mediated via activation of G_S-proteins and adenylyl cyclase. More work will be needed to clarify the signaling mechanisms linking increased cAMP levels to reduced resistin expression and to clearly define the physiological significance of the inhibitory effect observed.

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