EXPERIMENTAL STUDY

Negative regulation of adipose-expressed galectin-12 by isoproterenol, tumor necrosis factor α , insulin and dexamethasone

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Abstract

Objective: Galectin-12 has recently been shown to be a predominantly adipocyte-expressed protein which is stimulated by insulin-sensitizing thiazolidinediones and possesses apoptosis-inducing activity.

Methods: To further clarify galectin-12 regulation and its potential involvement in the development of insulin resistance, 3T3-L1 adipocytes were chronically treated with various hormones known to impair insulin sensitivity, and galectin-12 mRNA was measured by quantitative real-time reverse transcription-polymerase chain reaction.

Results: Treatment of 3T3-L1 cells for 16 h with 10 μ mol/l isoproterenol, 100 nmol/l insulin, 0.6 nmol/l tumor necrosis factor α (TNF α), and 100 nmol/l dexamethasone reduced galectin-12 gene expression between 47% and 85%. These negative effects were dose-dependent with significant inhibition detectable at concentrations as low as 10 nmol/l isoproterenol, 0.06 nmol/l TNF α , and 1 nmol/l dexamethasone. Furthermore, the inhibitory effect of isoproterenol could be almost completely reversed by pretreatment with the β -adrenergic antagonist propranolol and mimicked by stimulation of G_s-proteins with cholera toxin or by activation of adenylyl cyclase with forskolin and dibutyryl-cAMP.

Conclusions: Our results suggest that galectin-12 is an adipocyte-expressed protein which is down-regulated by various insulin resistance-inducing hormones. These findings imply a role for galectin-12 in the pathogenesis of insulin resistance.

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Introduction

Type 2 diabetes affects about 150 million people worldwide and is characterized by insulin resistance of peripheral tissues such as liver, muscle and fat which cannot be overcome by hypersecretion of pancreatic β -cells (1). Insulin resistance is often associated with obesity, and various studies suggest that several adipose-expressed proteins such as tumor necrosis factor α (TNF α), resistin, and adiponectin are dysregulated in obesity leading to impaired insulin sensitivity (2). Thiazolidinediones (TZDs) are a new class of antidiabetic drugs that activate peroxisome proliferator-activated receptor (PPAR) gamma and decrease insulin resistance in target tissues (3). Several studies have shown that various candidate proteins in fat are regulated by TZDs and might mediate some of their positive effects on insulin sensitivity. Thus, Stepan *et al.* suggested resistin as a major target of TZD action (4). However, results have been controversial (5). Another

interesting candidate gene is adiponectin which is up-regulated by TZDs and improves insulin sensitivity *in vitro* and *in vivo* (6, 7). Most recently, Hotta *et al.* suggested galectin-12 as a novel adipose-expressed protein which is upregulated by TZDs (8). Interestingly, the authors demonstrated convincingly that the protein is a potent inducer of apoptosis (8). Based on their findings, they suggested galectin-12 as a TZD-responsive gene which might improve insulin sensitivity at least partly via induction of apoptosis of preferentially large insulin-resistant adipocytes (8). A PPAR element has not, so far, been described in the galectin-12 gene promoter (8).

However, potential regulation of galectin-12 gene expression by other agents modifying insulin resistance is unknown. In the current study, we therefore examined the effect of isoproterenol, insulin, TNF α , dexamethasone, angiotensin 2 (AT2), growth hormone (GH), and tri-iodothyronine (T₃) on galectin-12 gene expression in 3T3-L1 adipocytes *in vitro*. We demon-

strate for the first time that isoproterenol, insulin, $\text{TNF}\alpha$, and dexamethasone potently inhibit galectin-12 mRNA expression in 3T3-L1 cells whereas AT_2 , GH, and T_3 do not have any effect. Furthermore, we present evidence that the inhibitory effect of isoproterenol is mediated via β -adrenergic receptors, G_s -proteins and adenylyl cyclase.

Materials and methods

Materials

Cell culture reagents were purchased from Life Technologies, Inc. (Grand Island, NY, USA), and oligonucleotides were purchased from MWG-Biotech (Ebersberg, Germany). All other reagents were obtained from Sigma Chemical Co. (St Louis, MO, USA).

Culture and differentiation of 3T3-L1 cells

3T3-L1 adipocytes (American Type Culture Collection, Rockville, MD, USA) were cultured as described previously (9). Briefly, preadipocytes were cultured to confluence in DMEM containing 25 mmol/l glucose, 10% fetal bovine serum, and antibiotics (culture medium). At confluence, 3T3-L1 cells were cultured for three days in culture medium further supplemented with 1 $\mu\text{mol/l}$ insulin, 0.5 mmol/l isobutylmethylxanthine and 0.1 $\mu\text{mol/l}$ dexamethasone, and for another three days in culture medium with 1 $\mu\text{mol/l}$ insulin followed by an additional three to six days in culture medium. For experiments, adipocytes were used between days 9 and 12 after induction of differentiation. At this point, at least 90% of 3T3-L1 cells showed accumulation of fat droplets.

Analysis of galectin-12 gene expression

Expression of galectin-12 was measured by quantitative real-time RT-PCR in a fluorescent temperature cycler (LightCycler, Roche Molecular Biochemicals, Mannheim, Germany) as described previously (10). Briefly, RNA was isolated from 3T3-L1 adipocytes using TRIzol (Life Technologies, Inc.). One microgram total RNA was reverse transcribed using standard reagents (Life Technologies, Inc.) and one tenth of each RT reaction was amplified in a 20 μl PCR containing 3 mmol/l MgCl_2 , 0.5 $\mu\text{mol/l}$ of each primer and 1 $\times$ LightCycler DNA Master SYBR Green I mix (Roche Molecular Biochemicals). After an initial denaturation at 94 °C for 30 s, 40 PCR cycles were performed, each cycle consisting of 94 °C for 1 s, 61 °C for 7 s, and 72 °C for 11 s. The following primer pairs were used: galectin-12 (accession no. AF244978) CTCCGCCCCCTTCCTCTTTCACC (sense) and GCCCTTGGAGCCCTTCTTAGCAGT (antisense); 36B4 (accession no. NM007475) AAGCGCGTCCTGGCATTGTCT (sense) and CCGCAGGGGCAGCAGTGGT (antisense). After each cycle SYBR Green I fluorescence

emissions were monitored. Levels of galectin-12 and 36B4 mRNA were quantified by using the second derivative maximum method of the LightCycler Software (Roche Molecular Biochemicals) which determines the crossing points of individual samples by an algorithm identifying the first turning point of the fluorescence curve. Galectin-12 expression is given relative to 36B4 which has been frequently used as an internal control due to its resistance to hormonal regulation (11).

Amplification of specific transcripts was confirmed by performing melting curve profiles (cooling the sample to 68 °C and heating slowly to 95 °C with continuous measurement of fluorescence) at the end of each PCR. Furthermore, the specificity was verified by subjecting the PCR products to agarose gel electrophoresis.

Statistical analysis

Results are shown as means $\pm$ S.E.M. Differences between various treatments were tested with unpaired Student's *t*-tests. *P* values < 0.01 are considered highly significant.

Results

Galectin-12 mRNA expression is stimulated during differentiation

First, expression of galectin-12 mRNA during differentiation was determined. Galectin-12 synthesis dramatically increased over the course of differentiation and was about 1000-fold higher on day 9 of differentiation as compared with confluent 3T3-L1 preadipocytes on day 0 (*P* < 0.01) (Fig. 1).

Figure 1 Differentiation-dependent galectin-12 gene expression. Confluent preadipocytes (day 0) were differentiated and at the indicated days of differentiation total RNA was subjected to quantitative real-time RT-PCR. Galectin-12 mRNA levels are shown relative to cells on day 0 (=100%). Results are the means $\pm$ S.E.M. of two independent experiments. ***P* < 0.01, comparing confluent cells (day 0) with differentiated adipocytes (day 6 and 9). Inset: agarose gel electrophoresis of the PCR products for galectin-12 and 36B4 respectively at cycle 30. d, day.

Figure 2 Galectin-12 gene expression is inhibited by isoproterenol, insulin, TNF α , and dexamethasone. After 6 h serum deprivation, fully differentiated 3T3-L1 adipocytes were stimulated for 16 h with isoproterenol (Iso, 10 μ mol/l), insulin (Ins, 100 nmol/l), TNF α (TNF, 0.6 nmol/l), dexamethasone (Dexa, 100 nmol/l), AT2 (10 μ mol/l), T₃ (1 μ mol/l), and GH (23 nmol/l). Total RNA was subjected to quantitative real-time RT-PCR to determine galectin-12 mRNA levels normalized to 36B4 as described in Materials and methods. Expression is shown relative to untreated control (Co) cells (=100%). Results are the means $\pm$ S.E.M. of at least four independent experiments. ** $P < 0.01$, comparing hormone-treated with non-treated cells.

Isoproterenol, insulin, TNF α , and dexamethasone inhibit galectin-12 expression

As galectin-12 has recently been shown to be induced by insulin-sensitizing TZDs, we tested whether various hormones that induce insulin resistance might influence galectin-12 gene expression in 3T3-L1 adipocytes *in vitro*. Interestingly, treatment of 3T3-L1 cells with 10 μ mol/l isoproterenol, 100 nmol/l insulin, 0.6 nmol/l TNF α , and 100 nmol/l dexamethasone for 16 h decreased galectin-12 mRNA expression by 85%, 55%, 47%, and 67% respectively, as compared with untreated controls ($P < 0.01$) (Fig. 2). In contrast, AT 2 (10 μ mol/l), T₃ (1 μ mol/l) and GH (23 nmol/l) did not significantly influence expression of galectin-12 (Fig. 2).

Isoproterenol, TNF α , and dexamethasone inhibited galectin-12 mRNA synthesis in a dose-dependent fashion. Thus, a significant 42% reduction of galectin-12 mRNA expression was detectable at isoproterenol concentrations as low as 10 nmol/l ($P < 0.01$) (Fig. 3A). Moreover, significant 31% and 43% inhibition was detectable at 0.06 nmol/l TNF α (Fig. 3B) and 1 nmol/l dexamethasone respectively (Fig. 3C) ($P < 0.01$).

Inhibition of galectin-12 expression by isoproterenol is mediated via β -adrenergic receptors, G_s-proteins, and adenylyl cyclase

To verify that isoproterenol inhibits galectin-12 expression via β -adrenergic receptors, 3T3-L1 adipo-

cytes were pretreated with selective α - (phentolamine, 100 μ mol/l) and β - (propranolol, 100 μ mol/l) adrenergic antagonists for 1 h before isoproterenol (10 μ mol/l) was added for 16 h. Again, galectin-12 mRNA expression significantly decreased by 89% after addition of isoproterenol as compared with untreated control cells ($P < 0.01$) (Fig. 4). Propranolol reversed this inhibitory effect of isoproterenol on galectin-12 gene expression to 75% of the levels observed in control cells ($P < 0.01$) whereas phentolamine did not have any effect (Fig. 4). These results suggest that the reduction of galectin-12 mRNA expression by isoproterenol is mediated via β -adrenergic receptors.

As β -adrenergic receptors are typically coupled to G_s-proteins mediating the stimulation of adenylyl cyclase, the effects of cholera toxin, forskolin and dibutyryl-cAMP on galectin-12 gene expression were examined. Cholera toxin activates G_s-proteins by ADP-ribosylation, forskolin is a direct activator of adenylyl cyclase, whereas dibutyryl-cAMP is a stable cAMP analog. As shown in Fig. 5 all three effectors significantly inhibited galectin-12 gene expression between 89% and 97%. These effects were dose-dependent with significant inhibition detectable at concentrations as low as 0.12 nmol/l cholera toxin, 2 μ mol/l forskolin, and 10 mmol/l dibutyryl cAMP respectively (data not shown).

Inhibition of galectin-12 expression by TNF α is not reversed by inhibition of p44/42 MAP kinase, p38 MAP kinase, phosphatidylinositol (PI) 3-kinase, and protein kinase C (PKC)

P44/42 and p38 MAP kinase, as well as PI 3-kinase and PKC have been implicated in TNF α signaling (12). To examine a potential involvement of these proteins in TNF α -mediated downregulation of galectin-12, 3T3-L1 adipocytes were pretreated with specific pharmacological inhibitors before addition of TNF α . As shown in Fig. 6, inhibition of p44/42 MAP kinase, p38 MAP kinase, PI 3-kinase and PKC by PD98059, SB203580, LY294002 and GF109203X respectively, did not reverse but rather tended to augment inhibition of galectin-12 mRNA expression by TNF α .

Inhibition of galectin-12 expression by insulin is not mediated via p44/42 MAP kinase, PI 3-kinase, and p70S6 kinase

The effect of inhibition of p44/42 MAP kinase, PI 3-kinase and p70S6 kinase by PD98059, LY294002 and rapamycin respectively, on insulin-mediated suppression of galectin-12 expression was assessed. Interestingly, pretreatment with the three inhibitors did not significantly alter the insulin-induced decrease of galectin-12 mRNA (Fig. 6).

Figure 3 Dose-dependent inhibition of galectin-12 gene expression by isoproterenol, $\text{TNF}\alpha$, and dexamethasone. 3T3-L1 cells were serum-starved for 6 h before the indicated concentrations of isoproterenol (Iso, A), $\text{TNF}\alpha$ (TNF, B), and dexamethasone (C) were added for 16 h. After extraction, total RNA was subjected to quantitative real-time RT-PCR to determine galectin-12 mRNA levels normalized to 36B4 as described in Materials and methods. Data are expressed relative to untreated control cells (=100%). Results are the means $\pm$ S.E.M. of at least two independent experiments. ** $P < 0.01$, comparing effector-treated with non-treated cells.

Discussion

In this study, we demonstrate a potent negative regulation of galectin-12 by insulin resistance-inducing hormones. Until now, 12 members of the galectin family have been isolated from various tissues. All members

share a highly conserved carbohydrate-recognition domain which recognizes the carbohydrate moieties of cell surface proteins (13). Galectins have been shown to contribute to cell adhesion, migration, and growth regulation (14). Furthermore, several members induce cellular apoptosis including galectin-12 which

Figure 4 Negative regulation of galectin-12 gene expression by isoproterenol is mediated via β -adrenergic receptors. After serum starvation for 5 h, adipocytes were cultured in the presence or absence of phentolamine (Phen, 100 μ mol/l) or propranolol (Prop, 100 μ mol/l) for 1 h before isoproterenol (10 μ mol/l) was added for 16 h. Total RNA was extracted and subjected to quantitative real-time RT-PCR as described in Materials and methods. Galectin-12 mRNA levels normalized to 36B4 expression are shown relative to non-treated control (Co) cells (=100%). Results are the means $\pm$ S.E.M. of four independent experiments. ** $P < 0.01$, comparing isoproterenol-treated with non-treated and propranolol-pre-treated adipocytes.

was cloned as a galectin preferentially expressed in fat tissue (8). Synthesis of galectin-12 increased in adipose tissue after treatment with troglitazone and significantly correlated with the percentage of apoptotic nuclei in fat (8). Interestingly, induction of apoptosis of preferentially large adipocytes has been suggested as one mechanism by which TZDs increase insulin sensitivity (15). Based on these findings it was suggested that galectin-12 mediates some of the insulin-sensitizing effects of TZDs via induction of apoptosis in fat cells. Moreover, it is possible that various insulin resistance-inducing hormones influence insulin sensitivity at least partly via downregulation of galectin-12. To test this hypothesis, 3T3-L1 adipocytes were used as an *in vitro* model as they show high expression of galectin-12

and are responsive to treatment by various hormones (8, 16).

There is growing evidence that increased activity of the sympathetic nervous system contributes to insulin resistance and that catecholamines impair insulin sensitivity (17–21). In a clinical context, our group recently demonstrated that patients with pheochromocytoma are insulin-resistant due to increased serum levels of catecholamines (22). Moreover, insulin sensitivity could be improved by surgical removal of the tumors in most cases (22). The mechanisms by which β -adrenergic activation impairs insulin sensitivity are not completely understood. In the present study, we demonstrate for the first time that isoproterenol decreases galectin-12 gene expression in 3T3-L1

Figure 5 Inhibition of galectin-12 gene expression by isoproterenol is mediated via G_s -proteins and adenyllyl cyclase. After 3T3-L1 adipocytes were serum-starved for 6 h, isoproterenol (Iso, 10 μ mol/l), cholera toxin (Cholera, 12 nmol/l), forskolin (For, 200 μ mol/l), and dibutyryl-cAMP (cAMP, 100 mmol/l) were added for 16 h. Total RNA was extracted and quantitative real-time RT-PCR determining galectin-12 mRNA levels normalized to 36B4 was performed as described in Materials and methods. Data are expressed relative to non-treated control (Con) cells (=100%). Results are the means $\pm$ S.E.M. of four independent experiments. ** $P < 0.01$, comparing non-treated with isoproterenol-, cholera toxin-, forskolin-, and dibutyryl-cAMP-treated cells.

Figure 6 Suppression of galectin-12 mRNA by $\text{TNF}\alpha$ is not mediated via p44/42 MAP kinase, p38 MAP kinase, PI 3-kinase, and PKC, and suppression by insulin is not mediated via p44/42 MAP kinase, PI 3-kinase, and p70S6 kinase. After serum-starvation for 5 h, 3T3-L1 adipocytes were pre-treated in the presence or absence of PD98059 (PD, 50 $\mu\text{mol/l}$), SB203580 (SB, 20 $\mu\text{mol/l}$), LY294002 (LY, 10 $\mu\text{mol/l}$), GF109203X (GFX, 10 $\mu\text{mol/l}$), and rapamycin (Rapa, 1 $\mu\text{mol/l}$) before addition of $\text{TNF}\alpha$ (0.6 nmol/l) or insulin (100 nmol/l) for 16 h as indicated. After total RNA extraction, quantitative real-time RT-PCR determining galectin-12 mRNA levels normalized to 36B4 was performed as described in Materials and methods. Data are expressed relative to non-treated control (Con) cells (=100%). Results are the means $\pm$ S.E.M. of two to six independent experiments.

adipocytes which might contribute to insulin resistance induced by catecholamines. Thus, based on the original findings (8), it appears possible that downregulation of galectin-12 impairs reduction of large insulin-resistant fat cells via apoptosis which, in turn, contributes to insulin resistance. However, as 3T3-L1 adipocytes show low basal levels of apoptosis (23, 24), the influence of various hormones on apoptotic rate was not directly assessed in the current study. Furthermore, we demonstrate for the first time that insulin, $\text{TNF}\alpha$, and dexamethasone are potent inhibitors of galectin-12 gene expression. These data suggest that suppression of galectin-12 may be a novel mechanism by which hyperinsulinemia, $\text{TNF}\alpha$, and glucocorticoids impair glucose tolerance and lend support to the hypothesis that this could represent a general biological mechanism for the induction of insulin resistance. In concert with previously demonstrated mechanisms such as downregulation of insulin signaling molecules (1), molecular interactions between adrenergic and insulin signaling cascades (25, 26), as well as regulation of other adipocytokines such as adiponectin (27), galectin-12 may be another intriguing player in the complex pathophysiological scenario causing the insulin resistance syndrome.

Furthermore, we show evidence that isoproterenol inhibits galectin-12 gene expression via activation of G_s -proteins and adenylyl cyclase. These results are in accordance with the classical view of β -adrenergic receptors being coupled to protein kinase A via G_s -proteins (28). Finally, we demonstrate that inhibition of various well-defined $\text{TNF}\alpha$ and insulin signaling molecules such as p44/42 MAP kinase and PI 3-kinase does not reverse suppression of galectin-12 mRNA induced by either hormone. Thus, further studies are

needed to clarify which alternative pathways mediate the inhibitory effect of both insulin and $\text{TNF}\alpha$.

Increased serum levels of thyroid hormones, AT2 or GH have also been shown to impair glucose tolerance profoundly (29–31). In our *in vitro* system, none of these hormones affect galectin-12 gene expression. Therefore, our data do not suggest a major role for galectin-12 in insulin resistance induced by these hormones.

In summary, we demonstrate for the first time that isoproterenol, insulin, $\text{TNF}\alpha$, and glucocorticoids significantly inhibit galectin-12 expression in 3T3-L1 cells *in vitro*. As galectin-12 is a T2D-responsive factor that induces apoptosis, regulation of this protein may constitute an important element in the pathogenesis of insulin resistance and the insulin resistance syndrome.

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