RAPID COMMUNICATION

Hormonal regulation of the novel adipocytokine visfatin in 3T3-L1 adipocytes

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Abstract

Recently, visfatin was characterized as a novel adipocytokine that is upregulated in obesity and exerts insulin-mimetic effects in various tissues. To clarify expression and regulation of this adipocytokine, visfatin mRNA was measured by quantitative real-time reverse transcription-polymerase chain reaction in 3T3-L1 adipocytes during adipogenesis and after treatment with various hormones known to alter insulin sensitivity. Visfatin expression was about 6-fold higher in 3T3-L1 adipocytes *in vitro* as compared with epididymal fat *in vivo* and increased during adipogenic conversion more than 3-fold. Interestingly, 100 nM dexamethasone significantly increased visfatin mRNA by almost 1·5-fold. In contrast, 500 ng/ml growth hormone (GH), 10 ng/ml tumor necrosis factor (TNF) α, and 10 μM isoproterenol downregulated visfatin expression by 45%, 36%, and 43% respectively. Insulin did not influence synthesis of this adipocytokine. The effects of dexamethasone, GH, TNFα and isoproterenol were time- and dose-dependent. Furthermore, activation of G_s-protein-coupled pathways by forskolin and cholera toxin was sufficient to significantly downregulate visfatin mRNA. Taken together, our results show a differential regulation of visfatin mRNA by insulin resistance-inducing hormones, supporting the view that this adipocytokine might be an interesting novel candidate linking core components of the metabolic syndrome such as obesity and insulin resistance.

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Introduction

About 150 million people are currently affected by type 2 diabetes mellitus worldwide, which is characterized by insulin resistance of peripheral tissues such as liver, muscle, and fat that cannot be compensated for by increased insulin secretion from pancreatic β-cells (Matthaei *et al.* 2000). Insulin resistance and type 2 diabetes are frequently associated with obesity and a better understanding of the connection between increased adiposity and impaired insulin sensitivity has been obtained recently (Lazar 2005). Thus, various fat cell-secreted proteins, so-called adipocytokines, including tumor necrosis factor (TNF) α, interleukin (IL)-6, and resistin are upregulated in obesity and induce insulin resistance. In contrast, the adipocytokine adiponectin is downregulated in states of impaired insulin sensitivity and appears as an endogenous insulin sensitizer (Fasshauer & Paschke 2003).

Most recently, by using a differential display method, visfatin was isolated as a novel adipocytokine implicated in the development of obesity-associated insulin resistance and diabetes mellitus (Fukuhara *et al.* 2005). The authors demonstrated that visfatin, which is identical to pre-B cell colony-enhancing factor (PBEF), is preferentially expressed in visceral as compared with subcutaneous fat (Fukuhara *et al.* 2005). Furthermore, plasma visfatin concentrations correlated strongly with the amount of visceral fat in human subjects (Fukuhara *et al.* 2005). Most interestingly, visfatin had insulin-mimetic effects in various rodent models of insulin resistance and obesity *in vivo* (Fukuhara *et al.* 2005). Consistent with these effects, visfatin addition to 3T3-L1 adipocytes and L6 myocytes increased basal glucose uptake, whereas glucose release was suppressed from H4IIEC3 hepatocytes *in vitro* (Fukuhara *et al.* 2005). Further experiments indicated that visfatin directly binds to and stimulates the insulin receptor, however, the binding site appeared to be different from insulin (Fukuhara *et al.* 2005).

Thus, the data accumulated so far suggest that visfatin is a novel adipocyte-secreted factor that strongly correlates to

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visceral obesity and has insulin-mimetic effects. A number of factors have been shown to alter glucose tolerance although the underlying cellular mechanisms are not always understood. It is possible that some of these mechanisms are indirect and involve alterations in visfatin expression in adipocytes. To test this hypothesis, we examined the effect of various insulin resistance-inducing hormones on visfatin expression in 3T3-L1 adipocytes *in vitro*. We demonstrate for the first time that dexamethasone induces visfatin mRNA whereas growth hormone (GH), TNFα, and the β-adrenergic agonist isoproterenol significantly suppress synthesis of this adipocytokine. Furthermore, we present first evidence that intracellular cAMP accumulation is sufficient to downregulate visfatin mRNA.

Materials and Methods

Materials

Cell culture reagents were obtained from Life Technologies Inc. (Grand Island, NY, USA), oligonucleotides from MWG-Biotech (Ebersberg, Germany). Cholera toxin, dexamethasone, forskolin, GH, insulin, isobutylmethylxanthine, isoproterenol and TNFα 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 and differentiated as described recently (Fasshauer *et al*. 2004*a*). In brief, after reaching confluence, preadipocytes were cultured for three days in Dulbecco's Modified Eagle Medium (DMEM) containing 25 mM glucose (DMEM-H), 10% fetal bovine serum and antibiotics (culture medium) further supplemented with 1 μM insulin, 0·5 mM isobutylmethylxanthine (IBMX) and 0·1 μM dexamethasone. Then, cells were kept for three more days in culture medium with 1 μM insulin (without IBMX, dexamethasone) and three to six days in culture medium (without insulin, IBMX, dexamethasone) after which about 95% of the cells had accumulated fat droplets. All stimulations were carried out after culturing the cells in DMEM-H without any additions for the time periods indicated.

Animals and tissue collection

Random-fed (n=6) 3-month-old male C57BL/6 mice (Taconic, Borup, Denmark) were sacrificed. Epididymal and subcutaneous fat pads were removed, immediately frozen in liquid nitrogen, and stored at −70 °C. Animals were housed in virus-free facilities on a 12 h light/dark cycle (07:00 h on–19:00 h off), fed a standard rodent chow and allowed water *ad libitum*. All protocols for animal use and euthanasia were in accordance with the 'Principles of laboratory animal care' (NIH Guidelines), approved by the Animal Care Committee of the University of Leipzig and followed the current version of the German Law on the Protection of Animals.

Analysis of visfatin gene expression

Visfatin mRNA synthesis was determined by quantitative real-time RT-PCR in a fluorescent temperature cycler (Taqman, Applied Biosystems, Darmstadt, Germany) as described previously (Fasshauer *et al*. 2004*a*). Briefly, total RNA was isolated from 3T3-L1 adipocytes with TRIzol (Life Technologies Inc.) and 1 μg RNA was reverse transcribed using standard reagents (Life Technologies Inc.). 2 μl of each RT reaction was amplified in a 26 μl PCR. Samples were incubated in the Taqman for an initial denaturation at 95 °C for 10 min, after which 40 PCR cycles were performed, each cycle consisting of 95 °C for 15 s, 60 °C for 1 min, and 72 °C for 1 min. The following primers specific for mouse visfatin and mouse 36B4 were used: Visfatin (accession no. NM021524) 5′–TCGGTTCTGGTGGCGCTTTGCTAC–3′ (sense) and 5′–AAGTTCCCCGCTGGTGTCCTATGT–3′ (antisense); 36B4 (accession no. NM007475) 5′–AAGCGC GTCCTGGCATTGTCT–3′ (sense) and 5′–CCGCAG GGGCAGCAGTGGT–3′ (antisense). SYBR Green I fluorescence emissions were monitored after each cycle. Synthesis of visfatin and 36B4 mRNA was quantified using the second derivative maximum method of the Taqman Software (Applied Biosystems, Darmstadt, Germany) which determines the crossing points of individual samples by an algorithm identifying the first turning point of the fluorescence curve. Visfatin synthesis was expressed relative to 36B4, which was used as an internal control due to its resistance to hormonal regulation (Laborda 1991). Amplification of specific transcripts was confirmed by melting curve profiles (cooling the sample to 68 °C and heating slowly to 95 °C with measurement of fluorescence) at the end of each PCR. The specificity of the PCR was further verified by subjecting the amplification products to agarose gel electrophoresis. Linearity between total RNA used per reaction and amount of mRNA measured by the Taqman software was obtained between 2 and 200 ng of total RNA (data not shown).

Statistical analysis

Results are shown as means ± S.E. Comparisons between groups were carried out by unpaired Student's t-test and in the case of multiple time points and treatments by one-way ANOVA. P values <0·01 are considered highly significant and <0·05 significant.

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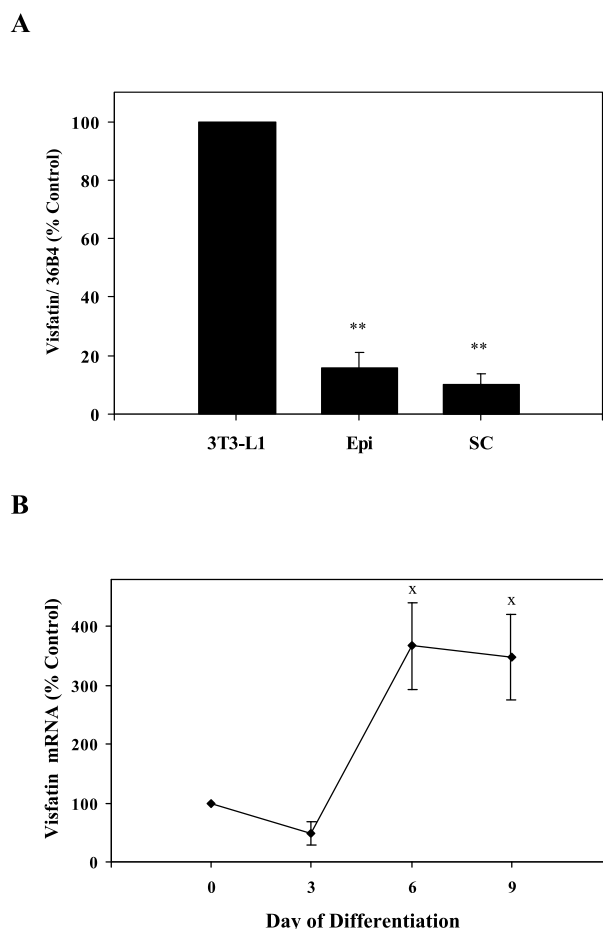

Figure 1 Expression of visfatin in 3T3-L1 adipocytes and during differentiation. (A) Expression of visfatin in fully differentiated 3T3-L1 adipocytes was compared with epididymal (Epi) and subcutaneous (SC) fat depots. (B) Confluent preadipocytes (day 0) were differentiated and at the indicated days (0, 3, 6, 9) total RNA was subjected to quantitative real-time RT-PCR. Visfatin mRNA levels are shown relative to (A) differentiated 3T3-L1 adipocytes (=100%) and (B) cells on day 0 (=100%). Results are the means ± S.E. of at least four independent experiments. **indicates highly significant downregulation ($P<0·01$) comparing 3T3-L1 adipocytes with epididymal and subcutaneous fat depots. ^{X} indicates significant upregulation ($P<0·05$) comparing confluent cells (day 0) with differentiating adipocytes (day 6 and 9).

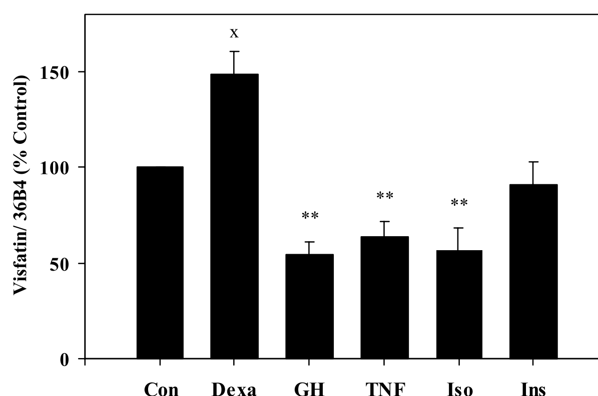

Figure 2 Visfatin mRNA expression is stimulated by dexamethasone and inhibited by GH, TNFα and isoproterenol. Fully differentiated 3T3-L1 cells were serum-deprived for 6 h before 100 nM dexamethasone (Dexa), 500 ng/ml GH, 10 ng/ml TNFα (TNF), 10 μM isoproterenol (Iso) and 100 nM insulin (Ins) were added for 16 h. Total RNA was subjected to quantitative real-time RT-PCR determining visfatin mRNA levels normalized to 36B4 relative to untreated control (Con) cells (=100%) as described in Materials and Methods. Results are the means ± S.E. of at least three independent experiments. ^{X} indicates significant upregulation ($P<0·05$), **indicates highly significant downregulation ($P<0·01$) comparing hormone-treated with non-treated cells.

Results

Visfatin mRNA expression is high in 3T3-L1 adipocytes and stimulated during adipogenesis

We compared expression of visfatin in 3T3-L1 adipocytes *in vitro* to visceral and subcutaneous fat depots of mice *in vivo*. As shown in Fig. 1A, visfatin expression was about 6-fold higher in 3T3-L1 adipocytes as compared with visceral (=epididymal) fat ($P<0·01$). Moreover, expression of this adipocytokine was further decreased by 42% in subcutaneous as compared with visceral adipose tissue (Fig. 1A). Next, visfatin synthesis during differentiation of 3T3-L1 cells was determined. As compared with undifferentiated preadipocytes (day 0), visfatin mRNA increased significantly more than 3-fold on day 6 and day 9 of differentiation ($P<0·05$) (Fig. 1B).

Dexamethasone increases visfatin mRNA whereas TNFα, GH and isoproterenol decrease expression of this adipocytokine

Various hormones which have been shown to induce insulin resistance were tested for their ability to regulate visfatin expression in 3T3-L1 fat cells. Treatment with 100 nM dexamethasone for 16 h significantly increased visfatin mRNA almost 1·5-fold ($P<0·05$) (Fig. 2). However, dexamethasone treatment did not influence expression of adipogenic markers including peroxisome proliferator activated receptor γ (PPARγ), CCAAT/enhancer binding protein-α (C/EBPα) and the glucose transporter 4 (Glut4) (data not shown). In contrast, addition of 500 ng/ml GH, 10 ng/ml TNFα and 10 μM isoproterenol for 16 h decreased visfatin expression by 45% ($P<0·01$), 36% ($P<0·01$) and 43% ($P<0·01$) respectively (Fig. 2). Insulin did not significantly influence visfatin mRNA synthesis (Fig. 2). Furthermore, dexamethasone, GH, TNFα, isoproterenol, insulin and forskolin did not significantly influence cell viability as determined by trypan blue staining (data not shown).

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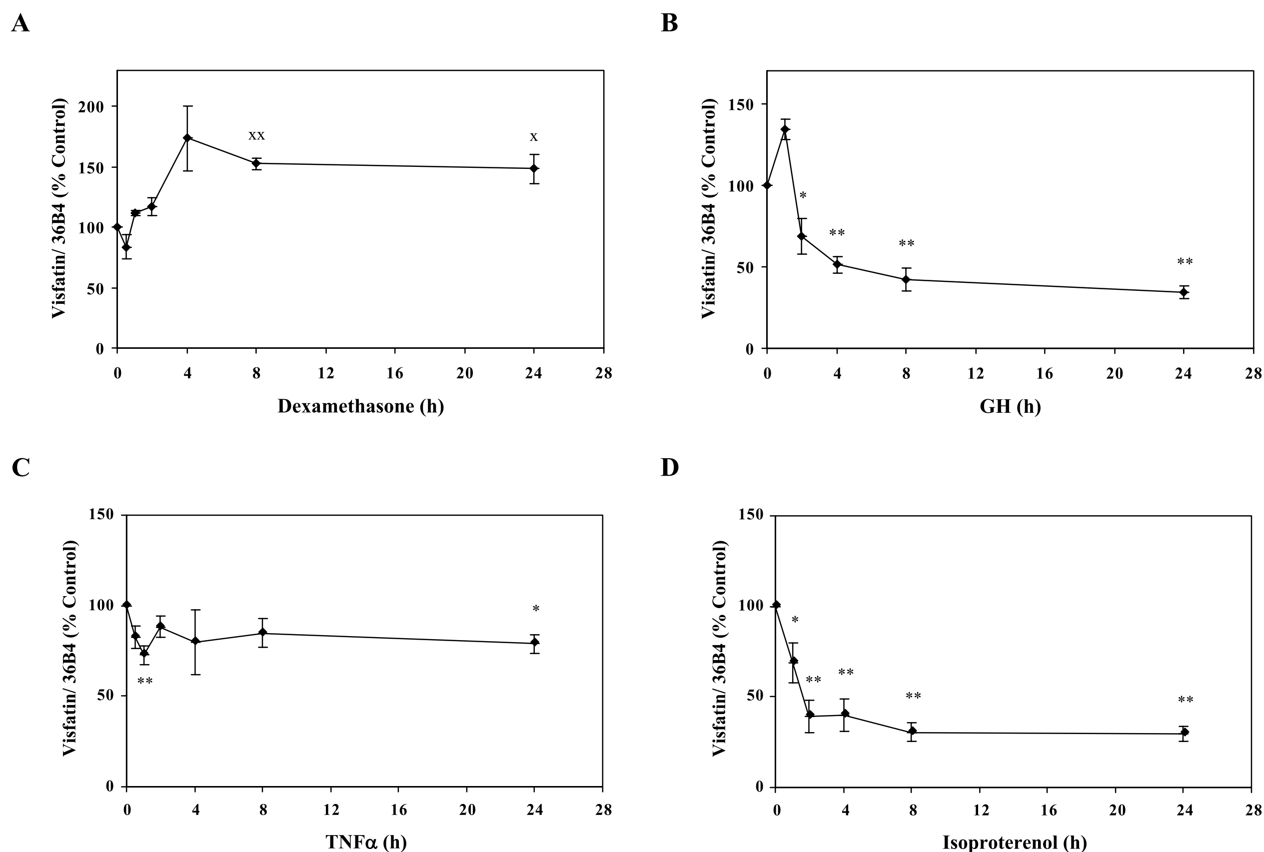

Figure 3 Visfatin mRNA expression is stimulated by dexamethasone and inhibited by GH, TNFα and isoproterenol in a time-dependent manner. Fully differentiated 3T3-L1 cells were serum-deprived overnight before (A) 100 nM dexamethasone, (B) 500 ng/ml GH, (C) 10 ng/ml TNFα, and (D) 10 μM isoproterenol were added for the indicated periods of time. Quantitative real-time RT-PCR was performed determining visfatin mRNA levels normalized to 36B4 and relative to untreated control cells (=100%) as described in Materials and Methods. Results are the means ± S.E. of at least three independent experiments. ^{XX} indicates highly significant ($P<0·01$), ^{X} significant ($P<0·05$) upregulation, **indicates highly significant ($P<0·01$), *significant ($P<0·05$) downregulation comparing hormone-treated with non-treated adipocytes.

The effect of dexamethasone, GH, TNFα and isoproterenol on visfatin expression is time- and dose-dependent

Next, we determined time- and dose dependent effects of dexamethasone, GH, TNFα, and isoproterenol on visfatin mRNA. Significant 1·5-fold stimulation of visfatin expression by 100 nM dexamethasone was first seen at 8 h of treatment and persisted for up to 24 h ($P<0·05$) (Fig. 3A). In contrast, 500 ng/ml GH significantly decreased visfatin synthesis by more than 30% after 2 h of treatment ($P<0·05$), with maximally 65% suppression after 24 h ($P<0·01$) (Fig. 3B). 10 ng/ml TNFα significantly suppressed visfatin expression at 1 h and 24 h of treatment with a maximal decrease of 27% at 1 h ($P<0·01$) (Fig. 3C). Isoproterenol at 10 μM time-dependently inhibited visfatin mRNA synthesis with significant effects first seen after 1 h ($P<0·05$) and maximally 71% suppression observed after 24 h of treatment ($P<0·01$) (Fig. 3D).

Incubation of 3T3-L1 adipocytes with dexamethasone for 16 h stimulated visfatin mRNA synthesis dose-dependently with significant 1·5-fold upregulation first seen at 100 nM and maximal effects at 1000 nM effector ($P<0·05$) (Fig. 4A). GH-treatment for 16 h inhibited visfatin mRNA expression with a significant 40% reduction seen at concentrations as low as 50 ng/ml ($P<0·05$) (Fig. 4B). TNFα significantly suppressed visfatin synthesis by almost 30% at 3 ng/ml ($P<0·01$) (Fig. 4C). Significant downregulation of visfatin by isoproterenol was first seen at 100 nM (data not shown).

Forskolin and cholera toxin downregulate visfatin expression

We determined whether a cellular increase in cAMP concentrations would be sufficient to downregulate visfatin mRNA. Cholera toxin activates G_S-proteins by

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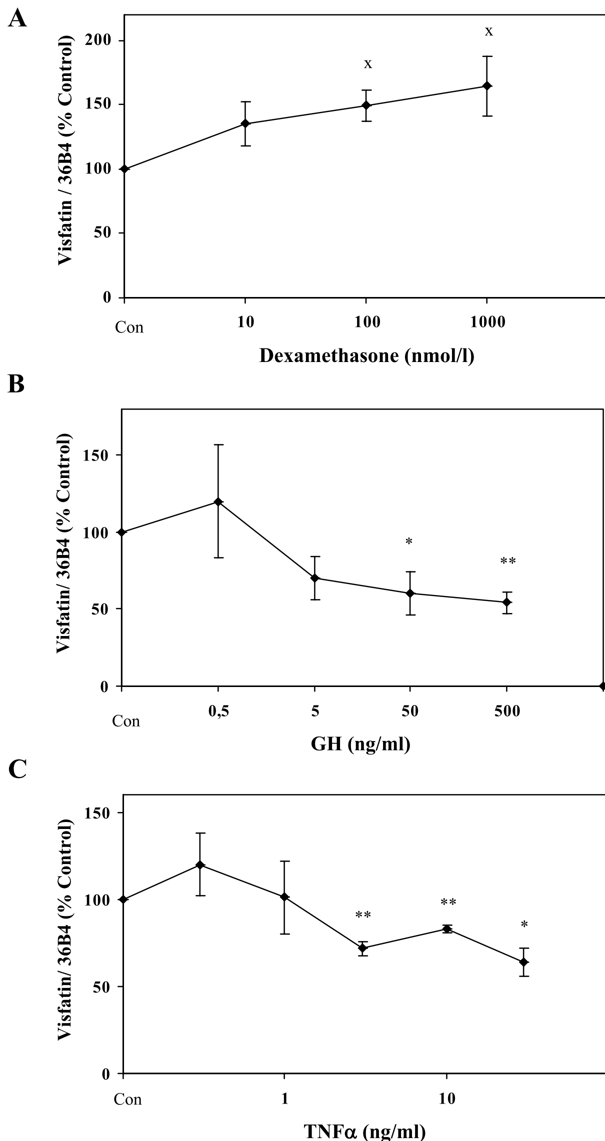

Figure 4 Dose-dependent regulation of visfatin by dexamethasone, GH, and TNFα. 3T3-L1 cells were serum-starved for 6 h before various concentrations of (A) dexamethasone, (B) GH, and (C) TNFα were added for 16 h. Total RNA was extracted and subjected to quantitative real-time RT-PCR to determine visfatin mRNA levels normalized to 36B4 expression as described in Materials and Methods. Data are expressed relative to untreated control (Con) cells (=100%). Results are the means ± S.E. of at least three independent experiments. [x] indicates significant ($P<0{\cdot}05$) upregulation, **indicates highly significant ($P<0{\cdot}01$), *significant ($P<0{\cdot}05$) downregulation comparing hormone-treated with non-treated cells.

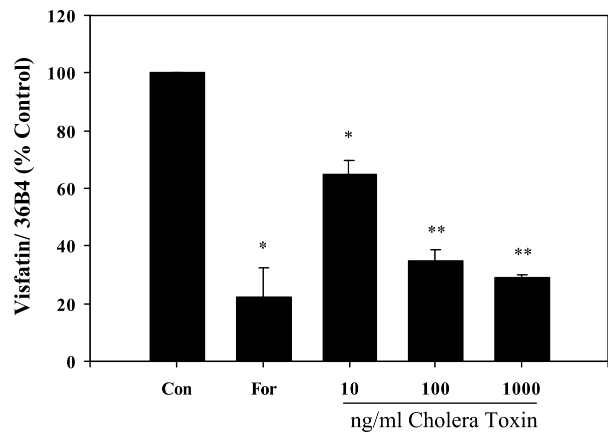

Figure 5 Forskolin and cholera toxin downregulate visfatin mRNA. After serum-starvation for 6 h, 3T3-L1 cells were cultured in the presence or absence of 200 μM forskolin (For) and the indicated concentrations of cholera toxin for 16 h. Total RNA was extracted and subjected to quantitative real-time RT-PCR to determine visfatin normalized to 36B4 expression as described in Materials and Methods. Data are expressed relative to non-treated control (Con) cells (=100%). Results are the means ± S.E. of two independent experiments. **denotes $P<0{\cdot}01$, *denotes $P<0{\cdot}05$ comparing effector-treated with non-treated fat cells.

ADP-ribosylation whereas forskolin is a direct activator of adenylyl cyclase. 3T3-L1 adipocytes were treated with 200 μM forskolin and different concentrations of cholera toxin for 16 h and visfatin mRNA levels were determined by real-time RT-PCR. As shown in Fig. 5, both effectors significantly inhibited visfatin expression. A maximal 78% ($P<0{\cdot}05$) and 72% ($P<0{\cdot}01$) reduction of visfatin mRNA was observed at concentrations of 200 μmol/l forskolin and 1000 ng/ml cholera toxin respectively (Fig. 5).

Discussion

Visfatin expression in differentiated 3T3-L1 adipocytes is about 6-fold higher as compared with visceral fat, supporting the notion that 3T3-L1 adipocytes constitute an appropriate model to determine hormonal regulation of this adipocytokine. Furthermore, in agreement with the original study (Fukuhara *et al.* 2005), visfatin mRNA synthesis is lower in subcutaneous as compared with visceral fat and is induced during adipogenesis.

In the current study, we demonstrate for the first time that the glucocorticoid dexamethasone significantly induces visfatin mRNA expression in 3T3-L1 adipocytes. It has long been known that glucocorticoids cause insulin resistance *in vitro* and *in vivo* (Ward *et al.* 2003). Thus, in 3T3-L1 fat cells, insulin-induced glucose uptake is significantly impaired after chronic treatment with dexamethasone (Sakoda *et al.* 2000, Bazuine *et al.* 2004). However, it is not clear to what extent increased glucocorticoid levels contribute to impaired insulin sensitivity seen in obesity. Thus, cortisol serum concentrations are not consistently increased in overweight persons (Wajchenberg 2000).

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On the other hand, transgenic overexpression of 11β-hydroxysteroid dehydrogenase type 1 (11β-HSD-1) in fat, which results in increased local levels of corticosterone, is accompanied by various symptoms of the metabolic syndrome including insulin resistance and obesity (Masuzaki *et al.* 2001). Furthermore, studies in humans confirm that local overexpression of 11β-HSD-1 in adipocytes might contribute to insulin resistance found in obesity (Bahr *et al.* 2002). Since dexamethasone stimulates visfatin expression in 3T3-L1 adipocytes *in vitro*, increased local glucocorticoid levels in fat might contribute to upregulation of visfatin found in visceral obesity *in vivo* (Fukuhara *et al.* 2005). However, our data also point to the fact that fat-secreted visfatin is probably not a mediator of glucocorticoid-induced glucose intolerance. Here, other mechanisms including downregulation of insulin-sensitizing adiponectin and inhibition of insulin signaling molecules probably contribute (Sakoda *et al.* 2000, Fasshauer & Paschke 2003). Furthermore, since visfatin is not exclusively expressed in fat, visfatin serum levels in patients with hypercortisolism need to be determined to further define its role in states of increased glucocorticoid levels.

GH, which is secreted primarily by the anterior pituitary gland as a 22 kDa polypeptide, significantly downregulates visfatin mRNA in 3T3-L1 adipocytes. It has long been known that GH potently antagonizes insulin action on insulin sensitive tissues such as muscle, fat and liver *in vivo* and *in vitro* (Frank 2001). Thus, Takano and co-workers showed significant suppression of insulin-induced glucose uptake in 3T3-L1 adipocytes at 500 ng/ml effector which was added for 16 h comparable to the highest concentration used in our study (Takano *et al.* 2001). Furthermore, GH receptor is expressed in 3T3-L1 adipocytes and upregulated during adipogenic conversion (Zou *et al.* 1997, Iida *et al.* 2003). Clinically, acromegalic patients are insulin resistant (Hansen *et al.* 1986) and nocturnal GH secretion appears to contribute to nocturnal hyperglycemia in diabetic patients (Campbell *et al.* 1985). Our current findings suggest that downregulation of visfatin in adipocytes may contribute to impaired glucose tolerance caused by increased GH levels. However, it has to be pointed out that stimulation of suppressor of cytokine signaling (SOCS)-1 (Fasshauer *et al.* 2004*b*) and -3 (Fasshauer *et al.* 2002), activation of MCP-1 (Fasshauer *et al.* 2004*a*) and IL-6 (Fasshauer & Paschke 2003), as well as altered insulin signaling downstream of phosphatidylinositol 3-kinase (Takano *et al.* 2001), are additional mechanisms by which GH affects glucose metabolism.

TNFα, originally identified in 1985, is a cytokine primarily produced by macrophages and adipocytes (Hotamisligil *et al.* 1993). Several studies have shown that TNFα potently induces insulin resistance and that serum levels of this cytokine are increased in human and murine obesity (Spiegelman & Flier 2001). In 3T3-L1 adipocytes, TNFα addition for 24 h at 85 ng/ml suppressed insulin-induced glucose uptake (Stephens & Pekala 1991). In the present study, we demonstrate that TNFα suppresses visfatin gene expression in a dose- and time-dependent fashion, which possibly contributes to impaired glucose tolerance seen in states of increased TNFα levels. However, direct inhibition of insulin signaling molecules by TNFα (Hotamisligil 1999), suppression of adiponectin (Fasshauer & Paschke 2003), as well as upregulation of MCP-1 (Fasshauer *et al.* 2004*a*) and IL-6 (Fasshauer & Paschke 2003), probably represent additional mechanisms for TNFα-induced glucose intolerance.

There is growing evidence suggesting that catecholamines impair insulin sensitivity and that increased activity of the sympathetic nervous system contributes to insulin resistance and diabetes mellitus (Facchini *et al.* 1996, Reaven *et al.* 1996, Hoieggen *et al.* 2000, Maison *et al.* 2000). In a clinical context, our group recently demonstrated that patients with pheochromocytoma are insulin resistant due to increased serum levels of catecholamines and insulin resistance improved after removal of the tumors in most cases (Bluher *et al.* 2000). In 3T3-L1 adipocytes significant impairment of insulin-stimulated glucose uptake is seen after treatment with 4 μM isoproterenol for 16 h (Kaestner *et al.* 1991). Several mechanisms by which catecholamines induce insulin resistance have been suggested, including molecular interactions on several levels between adrenergic and insulin signaling cascades (Klein *et al.* 1999, Klein *et al.* 2000), upregulation of IL-6 (Fasshauer & Paschke 2003) and SOCS-3 (Fasshauer *et al.* 2002), as well as suppression of adiponectin (Fasshauer & Paschke 2003). Based on the findings in our current study, downregulation of the insulin-mimetic visfatin may also contribute to catecholamine-induced glucose intolerance. Furthermore, we present evidence that the inhibitory effect of isoproterenol can be mimicked by forskolin and cholera toxin, which increase 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 (PKA) (Collins & Surmit 2001). Interestingly, TNFα has also been shown to activate PKA by downregulating cyclic-nucleotide phosphodiesterase 3B in 3T3-L1 adipocytes (Rahn Landstrom *et al.* 2000). Thus, it appears possible that both, isoproterenol and TNFα, suppress visfatin synthesis via the same signaling pathways.

It has to be pointed out that in most experiments significant regulation of visfatin by dexamethasone, GH, TNFα and isoproterenol was only seen at supraphysiological concentrations. However, various studies in 3T3-L1 adipocytes determining effects of these hormones on insulin sensitivity have also been performed with supraphysiological effector concentrations similar or higher as compared with our study. Thus, dexamethasone-induced insulin resistance was studied in detail in 3T3-L1

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adipocytes at 1 μM (Sakoda *et al.* 2000) and 100 nM (Bazuine *et al.* 2004) effector respectively. Furthermore, insulin resistance was induced by 500 ng/ml GH (Takano *et al.* 2001), 85 ng/ml TNFα (Stephens & Pekala 1991), or 4 μM isoproterenol (Kaestner *et al.* 1991). Taking these studies into account, it appears likely that supraphysiological concentrations of dexamethasone, GH, TNFα and isoproterenol are commonly needed in 3T3-L1 adipocytes *in vitro* to show relevant physiological effects. To our knowledge, the reasons for the need to use high effector concentrations have not been studied systematically. It could be postulated that receptor and/or signaling protein expression might be lower in adipocytes differentiated *in vitro* as compared with mature adipocytes *in vivo*.

Hyperinsulinemia caused by peripheral insulin resistance is an integral part in the development of type 2 diabetes (Gerich 1998). In our experimental system, insulin does not affect visfatin gene expression. Thus, any correlational association between high visfatin and hyperinsulinemia cannot be explained by insulin inducing visfatin gene expression in fat cells.

Taken together, we demonstrate for the first time that dexamethasone stimulates whereas GH, TNFα, and isoproterenol inhibit visfatin synthesis in 3T3-L1 adipocytes *in vitro*. These data indicate that expression of visfatin in fat is a selectively regulated mechanism that might constitute an important element in the pathogenesis of insulin resistance, obesity, and associated metabolic and cardiovascular disorders.

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