Interleukin-6 is a negative regulator of visfatin gene expression in 3T3-L1 adipocytes

Susan Kralisch,1 Johannes Klein,2 Ulrike Lossner,1 Matthias Bluher,1 Ralf Paschke,1 Michael Stumvoll,1 and Mathias Fasshauer1

1Department of Internal Medicine III, University of Leipzig, Leipzig; 2 Department of Internal Medicine I, University of Lübeck, Lübeck, Germany

Submitted 1 March 2005; accepted in final form 11 May 2005


Thus the data accumulated so far suggest that visfatin is a novel adipocyte-secreted factor that strongly correlates to visceral obesity and has insulin-mimetic effects. However, it is not known whether proinflammatory IL-6 might impair glucose tolerance, at least in part, by downregulation of visfatin. In the present study, therefore, we examined the effect of IL-6 on visfatin gene expression in 3T3-L1 adipocytes in vitro. We demonstrate for the first time that IL-6 suppresses visfatin mRNA synthesis. Furthermore, we present evidence that this inhibitory effect is partially mediated via p44/42 mitogen-activated protein kinase but is not reversible by troglitazone pretreatment under the conditions studied.

MATERIALS AND METHODS

Materials. Cell culture reagents were purchased from Life Technologies (Grand Island, NY). Oligonucleotides were purchased from MWG-Biotech (Ebersberg, Germany). Dexamethasone, IL-6, insulin, isobutylmethylxanthine, and troglitazone were purchased from Sigma Chemical (St. Louis, MO). Antibodies detecting phospho- or total p44/42 MAP kinase were purchased from Cell Signaling Technology (Beverly, MA).

Culture and differentiation of 3T3-L1 cells. 3T3-L1 cells (American Type Culture Collection, Rockville, MD) were differentiated as described (3). Briefly, confluent preadipocytes were cultured for 3 days in 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, and 0.1 μM dexamethasone and 3 days in culture medium with 1 μM insulin. After this period, 3T3-L1 cells were grown for 3–6 more days in culture medium, after which at least 95% of the cells had accumulated fat droplets. IL-6 treatment was performed in DMEM-H without any additions.
Analysis of visfatin mRNA. Visfatin mRNA expression was determined by quantitative real-time RT-PCR in a fluorescent temperature cycler (ABI Prism 7000; Applied Biosystems, Darmstadt, Germany), as described previously (1). In brief, total RNA was isolated from 3T3-L1 adipocytes with TRIzol reagent (Life Technologies), RNA (1 μg) was reverse transcribed using standard reagents (Life Technologies), and 2 μl of each RT reaction was amplified in a 26-μl PCR. Samples were incubated in the ABI Prism 7000 for an initial denaturation at 95°C for 10 min. Then, 40 PCR cycles were performed using the following conditions: 95°C for 15 s, 60°C for 1 min, and 72°C for 1 min. The following primer pairs were used: visfatin (accession no. NM-021524) TCCTGCCTGTCGAGCGCTTAC (sense) and AAGTCCCGCGTCGTTCATATG (antisense); and 36B4 (accession no. NM-007475) AAGCCGCTCCTGACATTGCT (sense) and CCGCAGGGCGACAGTTGCT (antisense). SYBR Green I fluorescence emissions were determined after each cycle, and synthesis of visfatin and 36B4 mRNA was quantified using the second derivative maximum method of the ABI Prism 7000 software (Applied Biosystems). This method determines the crossing points of individual samples by an algorithm identifying the first turning point of the fluorescence curve. Visfatin synthesis was calculated relative to 36B4, which was used as an internal control due to its resistance to hormonal regulation (13). Specific transcripts were 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, and the specificity of the PCR was further verified by subjecting the amplification products to agarose gel electrophoresis.

Western blotting. Western blotting was performed essentially as described (11). Briefly, cells were harvested in lysis buffer (50 mM HEPES, 137 mM NaCl, 1 mM MgCl2, 1 mM CaCl2, 10 mM NaN3, 10 mM NaF, 2 mM EDTA, 10% glycerol, 1% Igepal CA-630, 2 mM vanadate, 2 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml aprotinin, pH 7.4) and lysates were clarified. Equal amounts of protein were resolved by SDS-PAGE, transferred to nitrocellulose membranes, blocked for 1 h, and immunoblotted with p44/42 MAP kinase antibodies for 2 h. Specifically bound primary antibodies were detected with peroxidase-coupled secondary antibody and enhanced chemiluminescence.

Statistical analysis. Results are shown as means ± SE. Differences between various treatments were analyzed by unpaired Student’s t-tests with P values <0.01 considered highly significant and <0.05 considered significant.

RESULTS

Measurement of visfatin mRNA levels in 3T3-L1 adipocytes. First, the reliability of the quantitative real-time RT-PCR method was tested. For this purpose, increasing amounts of reverse-transcribed total cellular RNA were quantified with specific primer pairs for visfatin (Fig. 1). Linearity between total RNA used per reaction and amount of mRNA measured by the ABI Prism 7000 software was obtained between 2 and 200 ng of total RNA (Fig. 1).

Visfatin mRNA expression is downregulated by IL-6. We tested whether the adipocytokine IL-6 might influence visfatin synthesis in 3T3-L1 adipocytes in vitro. In fact, IL-6 treatment for 16 h significantly decreased visfatin mRNA in a dose-dependent manner (Fig. 2). A significant 31% downregulation was first seen at concentrations as low as 3 ng/ml IL-6 and
signaling, might play a role in downregulation of visfatin mRNA. For this purpose, 3T3-L1 adipocytes were pretreated with specific pharmacological inhibitors for 1 h before IL-6 (30 ng/ml) was added for 16 h. The PI 3-kinase inhibitor LY-294002 (10 μM) alone significantly downregulated basal visfatin mRNA expression to 45% of the levels seen in untreated control cells (P < 0.01; Fig. 4A). In contrast, inhibition of JAK2, p44/42 MAP kinase, and p38 MAP kinase with AG-490 (10 μM), PD-98059 (50 μM), and SB-203580 (20 μM), respectively, did not significantly influence basal visfatin mRNA synthesis (Fig. 4A). Again, visfatin mRNA was downregulated to 47% of control levels after 16 h of IL-6 treatment (P < 0.01; Fig. 4A). Interestingly, inhibition of p44/42 MAP kinase by PD-98059 significantly reversed this inhibition to 75% of the expression seen in untreated adipocytes (P < 0.05; Fig. 4A). In contrast, inhibition of JAK2, p38 MAP kinase, and PI 3-kinase did not significantly influence suppression of visfatin mRNA by IL-6 (Fig. 4A). Furthermore, we determined whether IL-6 directly stimulates p44/42 MAP kinase phosphorylation. Treatment of 3T3-L1 adipocytes with 30 ng/ml IL-6 time-dependently increased phosphorylation of p44/42 MAP kinase with maximal effects that were detectable 15 min after effector addition (Fig. 4B). Furthermore, IL-6-induced p44/42 MAP kinase phosphorylation could be completely inhibited by pretreatment with 50 μM PD-98059 (Fig. 4C).

Troglitazone does not affect downregulation of visfatin by IL-6 and TNF-α. We determined whether the thiazolidinedione troglitazone might affect downregulation of visfatin by IL-6. Treatment of 3T3-L1 cells with 10 μM troglitazone reduced basal visfatin expression to 79% of controls; however, this effect did not reach statistical significance (P > 0.05; Fig. 5). Again, IL-6 downregulated visfatin expression to 69% of controls (P < 0.01; Fig. 5). Interestingly, this decrease was not significantly reversed by troglitazone pretreatment under these conditions (Fig. 5). Furthermore, we have recently

**Fig. 4.** Inhibition of visfatin expression by IL-6 is mediated in part via p44/42 MAP kinase. A: after 5 h of serum starvation, differentiated 3T3-L1 adipocytes were cultured in the presence or absence of AG-490 (AG, 10 μM), PD-98059 (PD, 50 μM), SB-203580 (SB, 20 μM), or LY-294002 (LY, 10 μM) for 1 h before IL-6 (30 ng/ml) was added for additional 16 h. After total RNA extraction, quantitative real-time RT-PCR was performed as described in MATERIALS AND METHODS. Visfatin mRNA expression normalized to 36B4 is expressed relative to untreated Con cells (=100%). Results are means ± SE of 3 independent experiments, **P < 0.01 comparing untreated with IL-6- and LY-294002-treated cells; *P < 0.05 comparing IL-6-treated with PD-98059-pretreated adipocytes. IL-6 (30 ng/ml) was added for the indicated time periods (B) or for 30 min (C) in the presence or absence of PD-98059 pretreatment (50 μM, 17 h). Western blotting was performed as described in MATERIALS AND METHODS. Representative blots from ≥2 independent experiments are shown.

maximal 43% suppression of visfatin mRNA at 30 ng/ml effector (P < 0.01; Fig. 2). Moreover, the negative effect of IL-6 was time dependent (Fig. 3). Thus significant 40% downregulation of visfatin mRNA was first seen after 4 h of treatment, and suppression persisted for ≤24 h (P < 0.01; Fig. 3).

**Fig. 5.** Troglitazone does not reverse the negative effect of IL-6 and TNF-α on visfatin expression. After 5 h of serum starvation, differentiated 3T3-L1 adipocytes were cultured in the presence or absence of troglitazone (Tro, 10 μM) for 1 h before IL-6 (30 ng/ml) or TNF-α (TNF, 20 ng/ml) were added for an additional 16 h. Total RNA was extracted and subjected to quantitative real-time RT-PCR as described in MATERIALS AND METHODS. Visfatin mRNA expression normalized to 36B4 is expressed relative to untreated Con cells (=100%). Results are means ± SE of 3 independent experiments, **P < 0.01 comparing untreated with IL-6- or TNF-α-treated adipocytes; *P < 0.05 comparing untreated with TNF-α-treated adipocytes.
shown that TNF-α downregulates visfatin expression (12). In accord with these results, treatment of 3T3-L1 cells with 20 ng/ml TNF-α reduced visfatin expression by 25% (P < 0.05; Fig. 5). Again, troglitazone pretreatment did not influence this downregulation, similar to the data obtained with IL-6 (Fig. 5).

DISCUSSION

In the present study, we show for the first time that visfatin expression is inhibited by IL-6 in fat cells in vitro. Several mechanisms by which IL-6 influences glucose tolerance have been suggested in recent years. Thus IL-6, similar to TNFα, impairs insulin signaling in fat cells and hepatocytes, a mechanism that might be mediated by suppressor of cytokine signaling proteins (5, 19, 21). Furthermore, we have recently demonstrated paracrine downregulation of insulin-sensitizing adiponectin (4), as well as stimulation of insulin resistance-inducing monocyte chemoattractant protein-1 (2), in fat cells by IL-6. Our present findings suggest that IL-6-mediated downregulation of the insulin-mimetic visfatin in fat may be a novel mechanism by which this adipokine impairs glucose tolerance. Furthermore, because IL-6 plasma levels increase with body weight, its effect on adipose cells probably does not contribute to increased visfatin levels found in obesity (8). However, it has to be pointed out that visfatin is not exclusively expressed in fat and that regulation of this adipokine by IL-6 might be different in other tissues. In fact, Ognjanovic et al. (17) have demonstrated significant induction of visfatin (=PEBF) mRNA expression after IL-6 treatment in amniotic epithelial cells. Clearly, more work is needed to determine the expression and regulation of visfatin in other insulin-sensitive tissues, including liver and muscle, to better understand its role in glucose metabolism.

The major steps in IL-6 signaling have been elucidated in more detail in recent years. IL-6 induces gp130 homodimerization at the plasma membrane, and gp130-associated kinases such as JAK1, JAK2, and tyrosine kinase 2 become activated and phosphorylate the cytoplasmic tail of gp130 (9, 16). In the present study, we show that pharmacological inhibition of JAK2 by AG-490 does not reverse inhibition of visfatin mRNA synthesis by IL-6. These data suggest that kinases apart from JAK2, such as JAK1, might mediate the negative effect of IL-6 on visfatin mRNA. Downstream signaling proteins such as p44/42 MAP kinase, p38 MAP kinase, and PI 3-kinase are activated by signal transducer and activator of transcription-1 and -3, as well as Src homology 2 (SH2)-domain-containing tyrosine phosphatase-2, which bind to the tyrosine-phosphorylated gp130 (9). In the present study, we confirm that IL-6 potently induces p44/42 MAP kinase phosphorylation. Because effective pharmacological inhibition of p44/42 MAP kinase by PD-98059 partly reverses inhibition of visfatin expression, this molecule appears to mediate some of the negative effect of IL-6. Moreover, PI 3-kinase appears as a positive regulator of basal visfatin synthesis in fat cells because inhibition of this signaling protein leads to significantly lower mRNA levels of this adipocytokine. In contrast, p38 MAP kinase is probably not involved in visfatin regulation.

Insulin-sensitizing thiazolidinediones that activate peroxisome proliferator-activated receptor (PPAR)γ prevent certain actions of IL-6- and TNF-α related to insulin resistance in 3T3-L1 adipocytes (14, 18). Thus troglitazone or pioglitazone pretreatment reverses the reduction in insulin receptor or insulin receptor substrate-1 phosphorylation caused by TNF-α (18). Furthermore, rosiglitazone reestablishes insulin-induced lipogenesis impaired by IL-6 (14). In the current study, PPARγ activation by troglitazone is not sufficient to reverse IL-6- and TNF-α-induced visfatin downregulation, implying that this nuclear hormone receptor is not primarily involved in regulation of visfatin by IL-6 and TNF-α under the conditions studied.

Taken together, our results demonstrate for the first time significant suppression of visfatin gene expression in 3T3-L1 adipocytes by IL-6. Furthermore, we present evidence that this effect is mediated in part via p44/42 MAP kinase and that PI 3-kinase may have a role in maintaining basal visfatin expression. Because visfatin has been suggested as a novel insulin-mimetic adipokine, downregulation of this factor by IL-6 in fat might play a role in the pathogenesis of the insulin resistance syndrome.

GRANTS

This work was supported by a grant of the Deutsche Forschungsgemeinschaft (FA-376/3-1) and the Deutsche Diabetes Gesellschaft to M. Fasshauer.

REFERENCES


