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

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Visfatin is a novel adipokine exerting insulin-mimetic effects in various insulin-sensitive tissues such as liver, muscle, and fat. In contrast, interleukin (IL)-6 is a proinflammatory adipose-secreted factor that induces insulin resistance and plasma concentrations that correlate with the development of type 2 diabetes mellitus. In the present study, the impact of IL-6 on visfatin gene expression in 3T3-L1 adipocytes was determined by quantitative real-time reverse transcription-polymerase chain reaction. Interestingly, 30 ng/ml IL-6 time-dependently downregulated visfatin synthesis with a significant 40% suppression seen after 4 h of treatment. Furthermore, the addition of IL-6 for 16 h dose-dependently suppressed visfatin mRNA with significant effects first observed at concentrations as low as 3 ng/ml and a maximal 43% reduction at 30 ng/ml effector. Moreover, inhibitor studies suggested that the negative effect of IL-6 on visfatin expression is, at least in part, mediated by p44/42 mitogen-activated protein kinase. In contrast, troglitazone did not reverse the negative effect of IL-6 on visfatin synthesis under these conditions. Taken together, our study suggests that IL-6 might influence glucose tolerance in part by regulation of the novel insulin-mimetic adipokine visfatin.

INSULIN RESISTANCE and type 2 diabetes mellitus are frequently associated with obesity (10). In recent years, the connection between increased adiposity and impaired insulin responsiveness of target tissues has been better elucidated. Thus fat cells secrete various proteins called adipokines that influence insulin sensitivity profoundly.

Among those, interleukin (IL)-6 is a proinflammatory cytokine plasma, concentrations of which correlate with the development of type 2 diabetes mellitus (6). In accordance with these findings, administration of recombinant IL-6 in rodent models and in humans in vivo leads to hyperglycemia and compensatory hyperinsulinemia (22, 23). Because ~25% of systemic IL-6 originates from subcutaneous adipocytes in vivo and omental fat cells secrete even two- to threefold more IL-6 than subcutaneous adipocytes in vitro, IL-6 is regarded as an adipokine (7, 15).

Most recently, by use of a differential display method, visfatin was isolated as a novel adipokine that is preferentially expressed in visceral compared with subcutaneous fat (8). Visfatin, which is identical to pre-B cell colony-enhancing factor (PBEF), was originally cloned as a cytokine-like growth factor, enhancing the effect of IL-7 and stem cell factor on early stage B cells (20). Fukuhara et al. (8) demonstrated convincingly that visfatin had insulin-mimetic effects in several animal models of insulin resistance and obesity in vivo. Moreover, addition of visfatin to 3T3-L1 adipocytes and L6 myocytes increased basal glucose uptake, and glucose release was suppressed from H4-II-E-C3 hepatocytes in vitro (8). Signaling studies suggested that visfatin directly stimulates the insulin receptor; however, the binding site appeared to be different from that for insulin (8). Interestingly, plasma visfatin concentrations correlated strongly with the amount of visceral fat in human subjects (8).

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 MAP 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.

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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) TCGGTGTCTTGGCGCTTTTGTAC (sense) and AAAGTCCCCGGTGTGGCGATGTTATG (antisense); and 36B4 (accession no. NM-007475) AAGCCGGCCTGGGTCATTCT (sense) and CCGCAAGGGCGACAGTTGTGT (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 MgCl$_2$, 1 mM CaCl$_2$, 10 mM Na$_3$P$_2$O$_7$, 10 mM NaN$_3$, 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 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).

Effect of IL-6 is partly mediated by p44/42 MAP kinase. We tested whether signaling proteins such as Janus kinase 2 (JAK2), p44/42 MAP kinase, p38 MAP kinase, and phosphatidylinositol (PI) 3-kinase, which have been implicated in IL-6

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 untreated 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.

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-treated cells; *$P < 0.05$ 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 adipocytokine 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 adipocytokine 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 anmniotic 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 pharmaceutical 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 (7, 16). 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 adipocytokine 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 adipocytokine 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 anmniotic 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.

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