Proinflammatory adipocytokines induce TIMP-1 expression in 3T3-L1 adipocytes

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Abstract Tissue inhibitor of metalloproteinase (TIMP)-1 is an adipocyte-secreted protein upregulated in obesity which promotes adipose tissue development. Furthermore, the proinflammatory adipocytokines tumor necrosis factor \(\alpha\) (TNF\(\alpha\)) and interleukin (IL)-6 induce insulin resistance, and plasma concentrations are increased during weight gain. In the current study, the impact of TNF\(\alpha\) and IL-6 on TIMP-1 mRNA and protein expression was determined in 3T3-L1 adipocytes. Interestingly, TNF\(\alpha\) and IL-6 induced TIMP-1 protein secretion more than 3- and 2-fold, respectively. Furthermore, TIMP-1 mRNA was upregulated in a time- and dose-dependent fashion. Inhibitor experiments suggested that nuclear factor kB and p44/42 mitogen-activated protein kinase are involved in both, basal and adipocytokine-induced TIMP-1 expression. Moreover, the thiazolidinedione troglitazone partly reversed TNF\(\alpha\)- but not IL-6-induced TIMP-1 synthesis. Taken together, we demonstrate that TIMP-1 expression is selectively upregulated in fat cells by proinflammatory adipocytokines and might play a role in maintaining adipose tissue mass in obesity.

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1. Introduction

Obesity is a rapidly growing nutritional disorder characterized by excessive accumulation of adipose tissue [1]. Both, hyperplasia and hypertrophy of adipocytes can be found when weight is gained [1]. Furthermore, neovascularization of expanding adipose tissue is critical to maintain proper function [2]. These multiple processes depend on dynamic changes of cell–matrix interactions and extensive extracellular matrix (ECM) remodeling [3]. Various members of the matrix metalloproteinase (MMP) family and the tissue inhibitors of MMPs (TIMPs) appear as primary mediators of ECM remodeling [3]. MMPs consist of more than 20 neutral endopeptidases which are able to cleave ECM components and several non-ECM proteins including cytokines, adhesion molecules, and proteinase inhibitors [3]. MMP activity is inhibited through interaction with TIMPs which comprise a family of four members [3].

The potential contribution of MMPs and TIMPs to obesity-related adipose tissue enlargement has been determined recently, and TIMP-1 appears as an interesting candidate promoting fat accumulation for several reasons. First, two independent studies reported significant upregulation of adipose tissue TIMP-1 in mice with genetically based or diet-induced obesity [4,5]. Furthermore, transgenic overexpression of TIMP-1 increased the rate of adipocyte differentiation in vivo [6]. In accordance with these findings, treatment of 3T3-L1 cells with recombinant TIMP-1 dramatically accelerated lipid accumulation during differentiation in vitro [6]. Moreover, TIMP-1-deficient mice were protected from nutritionally induced obesity [7]. Thus, TIMP-1 appears as a novel fat-derived protein promoting adipose tissue development when weight is gained. However, it is unclear so far which mediators stimulate TIMP-1 expression in fat cells in obesity.

During weight gain, the proinflammatory adipocytokines tumor necrosis factor \(\alpha\) (TNF\(\alpha\)) and interleukin (IL)-6 showed increased expression in fat, and plasma concentrations correlate with the development of glucose intolerance [8]. In the current study, we, therefore, examined the effect of these cytokines on TIMP-1 expression and secretion in 3T3-L1 adipocytes in vitro. We demonstrate for the first time that both adipocytokines significantly stimulate TIMP-1 mRNA and protein synthesis in fat cells. Furthermore, we present evidence that basal and adipocytokine-induced TIMP-1 expression is mediated via nuclear factor \(\kappa\)B (NF\(\kappa\)B) and p44/42 mitogen-activated protein (MAP) kinase. Moreover, troglitazone partly reverses TNF\(\alpha\)- but not IL-6-stimulated TIMP-1.

2. Materials and methods

2.1. Materials

Cell culture reagents were obtained from Life Technologies, Inc. (Grand Island, NY), oligonucleotides from MWG-Biotech (Ebersberg, Germany). Dexamethasone, IL-6, insulin, isobutylmethylxanthine, LY294002, parthenolide, PD98059, SB203580, TNF\(\alpha\), and troglitazone...
were purchased from Sigma Chemical Co. (St. Louis, MO). Antibodies detecting phospho- or total p44/42 MAP kinase were from Cell Signaling Technology (Beverly, MA, USA).

2.2. Culture and differentiation of 3T3-L1 cells
3T3-L1 cells (American Type Culture Collection, Rockville, MD) were differentiated as described [9]. In brief, confluent preadipocytes were cultured for three days in DMEM containing 25 mM glucose (DMEM-H), 10% fetal bovine serum, and antibiotics (culture medium) further supplemented with 1 mM insulin, 0.5 mM sodium selenite, 10 mM isoascorbate, 3 mM putrescine, and 0.1 mM dexamethasone. After this period, they were grown for three days in culture medium with 1 μM insulin and for three to six more days in culture medium. Various effectors were added to cells starved in DMEM-H only for the indicated periods of time. At the time of the stimulation experiments at least 95% of the cells had accumulated fat droplets.

2.3. Analysis of TIMP-1 and TIMP-2 mRNA
TIMP mRNA synthesis was determined by quantitative real-time RT-PCR in a fluorescent temperature cycler (ABI Prism 7000, Applied Biosystems, Darmstadt, Germany) as described previously [10]. Briefly, total RNA was isolated from 3T3-L1 adipocytes with TRizol reagent (Life Technologies, Inc., Grand Island, NY) and 1 μg RNA was reverse transcribed using standard reagents (Life Technologies, Inc., Grand Island, NY). 2 μl of each RT reaction was amplified in a 26 μl PCR. After initial denaturation at 95 °C for 10 min, 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: TIMP-1 (Accession No. NM_011593) CTATAGTGCTGCGTGTTGTG (sense) and TTCCGTCGCAAGCAGGAAGGT (antisense); TIMP-2 (Accession No. NM_011594) GGCCTCCCTCCTCTACCT (sense) and GACTTCTATATCCAGCAGACAT (antisense); 36B4 (Accession No. NM_007475) AAGCGGTCCTGGCAGTCT (sense) and CCGCAGGGCCAGCAGGTGTG (antisense). SYBR Green I fluorescence emissions were monitored after each cycle and synthesis of TIMP-1, TIMP-2, and 36B4 mRNA was quantified using the second derivative maximum method of the ABI Prism 7000 software (Applied Biosystems, Darmstadt, Germany). This method determines the crossing points of individual samples by an algorithm identifying the first turning point of the fluorescence curve. TIMP-1 and TIMP-2 expression was calculated relative to 36B4 which was used as an internal control due to its resistance to hormonal influences [11]. Specific transcripts were confirmed by melting curve profiles (cooking 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.

2.4. Analysis of TIMP-1 secretion
Quantification of TIMP-1 protein levels in 3T3-L1 cell culture supernatants was performed with a commercially available enzyme-linked immunosassay (ELISA) from RayBiotech Inc. (Norcross, GA, USA) according to the manufacturer’s instructions.

2.5. Western blotting
Western blotting was performed essentially as described [12]. Briefly, cells were harvested in lysis buffer (50 mM HEPES, 137 mM NaCl, 1 mM MgCl2, 1 mM CaCl2, 10 mM Na3PO4, 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.

2.6. 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.

3. Results
3.1. TIMP-1 secretion is stimulated by TNFα and IL-6
TIMP-1 secretion into the medium was determined in differentiated 3T3-L1 cells after TNFα (20 ng/ml) and IL-6 (30 ng/ml) treatment for 16 h. Interestingly, both effectors significantly increased TIMP-1 protein levels in the supernatants from 28.3 (basal) to 89.6 ng/ml (TNFα) and 61.7 ng/ml (IL-6), respectively (P < 0.01) (Fig. 1).

3.2. TIMP-1 but not TIMP-2 mRNA is induced by TNFα and IL-6 in 3T3-L1 adipocytes
Next, we tested whether TNFα and IL-6 might influence TIMP-1 or TIMP-2 mRNA expression in 3T3-L1 adipocytes. In fact, both proinflammatory cytokines induced TIMP-1 in a time-dependent fashion (Fig. 2). Thus, significant 4.8-fold stimulation was first seen after 4 h of TNFα treatment, and activation persisted for up to 24 h (P < 0.01) (Fig. 2A). Furthermore, significant 1.6-fold upregulation of TIMP-1 was evident as early as 1 h after IL-6 addition (P < 0.05) and maximal 5.1-fold stimulation was observed after 24 of treatment (P < 0.01) (Fig. 2B). In contrast, TIMP-2 mRNA was not significantly regulated by TNFα and IL-6 (Fig. 2A and B).

TNFα and IL-6-induced TIMP-1 synthesis dose-dependently after 16 h of treatment (Fig. 3). Thus, significant stimulation was seen at concentrations as low as 3 ng/ml TNFα (P < 0.05) (Fig. 3A) and 10 ng/ml IL-6 (P < 0.05) (Fig. 3B). Furthermore, maximal effects were detectable at 100 ng/ml effector for both, TNFα (P < 0.01) (Fig. 3A) and IL-6 (P < 0.05) (Fig. 3B). Treatment of 3T3-L1 adipocytes with 20 ng/ml TNFα or 30 ng/ml IL-6 for 16 h did not affect adipocyte differentiation as assessed by oil red O staining (data not shown).

3.3. NFκB and p44/42 MAP kinase are involved in basal and adipocytokine-induced TIMP-1 expression
Various effects of proinflammatory TNFα and IL-6 are mediated via NFκB. To determine whether this signaling intermediate was involved in TIMP-1 induction, 3T3-L1 adipocytes were pretreated with parthenolide (50 μM), a specific pharmacological NFκB inhibitor for 1 h before TNFα (20 ng/ml, Fig. 4A) and IL-6 (30 ng/ml, Fig. 4B) were added for 16 h. Parthenolide significantly downregulated basal TIMP-1 expression to 43% and 61% of the levels seen in untreated control (Fig. 4A and B). Again, TNFα (Fig. 4A) and IL-6 (Fig. 4B) significantly induced TIMP-1 expression 12.2- and 3.7-fold, respectively (P < 0.01). Interestingly, this induction was significantly reversed to 86% (TNFα, P < 0.01, Fig. 4A) and 159% (IL-6, P < 0.01, Fig. 4B) of control levels. Treatment of 3T3-L1 adipocytes with both, TNFα and IL-6, did not significantly augment TIMP-1 mRNA beyond the level seen with TNFα stimulation alone (Fig. 4A).

We next tested whether signaling proteins such as p44/42 MAP kinase, p38 MAP kinase, and phosphatidylinositol (PI) 3-kinase might play a role in stimulation of TIMP-1 synthesis. For this purpose, 3T3-L1 adipocytes were pretreated with specific pharmacological inhibitors for 1 h before TNFα (20 ng/ml, Fig. 5A) and IL-6 (30 ng/ml, Fig. 5B) were added for 16 h. Interestingly, the p44/42 MAP kinase inhibitor PD98059 (50 μM) and the PI 3-kinase inhibitor LY294002 (10 μM) alone significantly downregulated basal TIMP-1 synthesis to 43% and 61% of the levels seen in untreated control
cells, respectively (P < 0.05) (Fig. 5A and B). In contrast, inhibition of p38 MAP kinase with SB203580 (20 µM) did not significantly influence basal TIMP-1 mRNA (Fig. 5A and B). TIMP-1 mRNA was stimulated 6.5-fold after 16 h of TNFα treatment (P < 0.01) (Fig. 5A). Interestingly, inhibition of p44/42 MAP kinase by PD98059 significantly reversed this activation by 76–154% of the expression seen in untreated adipocytes (P < 0.05) (Fig. 5A). In contrast, inhibition of p38 MAP kinase and PI 3-kinase did not significantly influence stimulation of TIMP-1 synthesis by TNFα (Fig. 5A). IL-6 upregulated TIMP-1 mRNA 2.2-fold as compared to controls (P < 0.01) (Fig. 5B). Again, PD98059-pretreatment significantly reversed this activation by more than 50–109% of control expression (P < 0.05) (Fig. 5B). In contrast, LY294002 and SB203580 did not significantly influence stimulation of TIMP-1 by IL-6 (Fig. 5B). Furthermore, we determined whether TNFα directly stimulates p44/42 MAP kinase phosphorylation. Treatment of 3T3-L1 adipocytes with 20 ng/ml TNFα time-dependently increased phosphorylation of p44/42 MAP kinase with maximal effects detectable 15 min after effector addition (Fig. 5C). Furthermore, TNFα-induced p44/42 MAP kinase phosphorylation could be completely inhibited by pretreatment with 50 µM PD98059 for 17 h (Fig. 5D).

3.4. Troglitazone partly reverses TNFα- but not IL-6-induced TIMP-1 expression

Insulin-sensitizing thiazolidinediones have been shown to prevent certain actions of proinflammatory TNFα and IL-6...
in 3T3-L1 adipocytes [22,23]. Therefore, we determined whether the thiazolidinedione troglitazone might affect stimulation of TIMP-1 by TNFα and IL-6. Treatment of 3T3-L1 cells with 10 μM troglitazone significantly reduced basal TIMP-1 expression to 70% of controls (P < 0.01) (Fig. 6). Again, TNFα and IL-6 upregulated TIMP-1 synthesis 7.3- and 5.2-fold, respectively (P < 0.01) (Fig. 6). Interestingly, TNFα-induced TIMP-1 expression was significantly reversed by about 50% by troglitazone pretreatment (P < 0.05) (Fig. 6). In contrast, troglitazone did not influence IL-6-induced TIMP-1 expression (Fig. 6).

4. Discussion

In the current study, we show for the first time that TIMP-1 but not TIMP-2 expression is induced in adipocytes by TNFα and IL-6 in vitro. Since TNFα and IL-6 are increased during
TIMP-1 mRNA synthesis normalized to 36B4 is expressed relative to untreated control (Con) cells (= 100%). Results are the means ± SEM of three independent experiments. ** denotes P < 0.01 comparing untreated with TNFα or IL-6 in 3T3-L1 adipocytes [22,23]. Thus, troglitazone or pioglitazone reverse the reduction in insulin receptor (IR) or IR substrate-1 phosphorylation caused by TNFα [22]. Furthermore, pioglitazone and IL-6 do not have synergistic effects on TIMP-1 expression.

Insulin-sensitizing thiazolidinediones activate peroxisome proliferator-activated receptor (PPAR) γ and prevent certain actions of TNFα and IL-6 in 3T3-L1 adipocytes [22,23]. Thus, troglitazone or pioglitazone reverse the reduction in insulin receptor (IR) or IR substrate-1 phosphorylation caused by TNFα [22]. Furthermore, pioglitazone and IL-6 do not have synergistic effects on TIMP-1 expression. In the current study, we have not determined direct effects of TIMP-1 on 3T3-L1 adipocytes. However, a study by Alexander et al. [6] has demonstrated that treatment of 3T3-L1 cells with recombinant TIMP-1 dramatically accelerates lipid accumulation during differentiation in vitro. Taken together, we demonstrate for the first time that TIMP-1 expression and secretion are selectively regulated in adipocytes by proinflammatory adipocytokines and might play a role in maintaining adipose tissue mass in obesity. Further studies are needed to more clearly define the mechanisms by which TIMP-1 promotes fat accumulation.

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References


