Leptin stimulates tissue inhibitor of metalloproteinase-1 in human hepatic stellate cells

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Leptin is recognized as a profibrogenic hormone in the liver, but the mechanisms involved have not been clarified. The tissue inhibitor of metalloproteinase (TIMP)-1, which acts through inhibition of collagen degradation, is synthesized by activated hepatic stellate cells (HSC) in response to fibrogenic substances. The capacity of leptin to induce TIMP-1 and its signaling molecules were investigated in a human HSC cell line, LX-2. Leptin stimulated TIMP-1 protein, mRNA, and promoter activity, JAK1 and -2, as well as STAT3 and 5, were activated. After leptin, there was increased expression of tyrosine 1141-phosphorylated leptin receptor, which may contribute to STAT3 activation. AG 490, a JAK inhibitor, blocked JAK phosphorylation with concomitant inhibition of STAT activation, TIMP-1 mRNA expression, and promoter activity. Leptin also induced an oxidative stress, which was inhibited by AG 490, indicating a JAK mediation process. ERK1/2 MAPK and p38 were activated, which was prevented by catalase, indicating an H2O2-dependent mechanism. Catalase treatment resulted in total suppression of TIMP-1 mRNA expression and promoter activity. SB203580, a p38 inhibitor, prevented p38 activation and reduced TIMP-1 message half-life with down-regulation of TIMP-1 mRNA. These changes were reproduced by overexpression of the dominant negative p38 and p38β mutants. PD098059, an ERK1/2 inhibitor, opposed ERK1/2 activation and TIMP-1 promoter activity, leading to TIMP-1 mRNA down-regulation. Thus, leptin has a direct action on liver fibrogenesis by stimulating TIMP-1 production in activated HSC. This process appears to be mediated by the JAK/STAT pathway via the leptin receptor long form and the H2O2-dependent p38 and ERK1/2 pathways via activated JAK.

Leptin, a peptide hormone, is secreted mainly by adipocytes and acts on the hypothalamus to control body weight by reducing food intake and increasing energy expenditure (1). Leptin also has direct influences on hematopoiesis, immunity, reproduction, and angiogenesis (2–4) as well as the metabolism of various tissues, including the liver cells (5–7). Leptin’s actions are mediated through the leptin receptor, which belongs to the class I cytokine receptor family, and shares common features with the interleukin-6 receptor (8). In humans and rodents, two major forms of the leptin receptor (OB-R) are expressed. The long form OB-R (OB-Rb) is predominantly expressed in the hypothalamus and is present at low levels in peripheral tissues and specific cell types; it is the functional receptor isoform for leptin signaling (8–10). In contrast, the short form (OB-Rs) is found in many organs and is considered to lack signaling capability.

In liver diseases, plasma leptin levels were reported to be increased in patients with alcoholic cirrhosis (11, 12) regardless of body mass index (11), in nonalcoholic cirrhosis (13) and in nonalcoholic steatohepatitis (14), suggesting a possible involvement of leptin in the pathogenesis of liver fibrosis. Recent laboratory studies showed that hepatic fibrosis induced by chemical toxins and by Schistosoma mansoni was markedly decreased in leptin-deficient ob/ob mice (15–17), in ob/ob mice during the progression of experimental steatohepatitis (18) and in Zucker rats (fa/fa) (19), which lack a functional leptin receptor (compared with corresponding lean littersmates). These findings implicated leptin as a mediator in the development of liver fibrosis but did not elucidate the mechanisms and cell types involved. One hypothesis suggests that leptin acts on the Kupffer cells and sinusoidal endothelium (in which OB-Rs was detected) to release TGF-β1 that, in turn, stimulates fibrogenesis in activated hepatic stellate cells (HSC) (15, 19). Another view holds that leptin acts directly on HSC and triggers specific signal transduction systems, which alter collagen gene expression (16). In culture-activated rat HSC, rat HSC-T6, and human HSC-LX-1 cell lines, leptin was found to stimulate collagen I promoter activity (16), up-regulate mRNA (20, 21), and increase protein production (21). Furthermore, Tang et al. (21) reported that leptin acts to enhance expression of the TGF-β type II receptor, which sensitizes HSC to the fibrogenic actions of TGF-β1. Although leptin (15, 22) and its receptors, either the long (16) or short forms (19, 21), were detected in activated HSC and in immortalized HSC lines, the intracellular transduction molecules used by leptin signaling in HSC fibrogenesis have not been clarified.

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Collagen I accumulation in liver fibrosis results, in part, from inhibition of its degradation by interstitial collagenase due to increased activity of the tissue inhibitor of metalloproteinase (TIMP)-1 (23). The latter is synthesized and secreted by activated HSC in response to fibrogenic cytokines in particular TGF-β1 (24, 25). Furthermore, serum TIMP-1 is increased in alcoholic and with early fibrosis and can serve as a marker of precirrhotic and cirrhotic states (26). The regulation of TIMP-1 gene transcription has been extensively studied in HSC (27, 28), but much less is known about ligand-induced intracellular signaling molecules that modulate its production. Leptin was found to increase TIMP-1 expression in human endothelial and vascular smooth muscle cells, although the pathways were not characterized (29). Thus far there is no report on the effects of leptin on TIMP-1 production and the associated signaling pathways in HSC.

Leptin signals from the OB-Rb through the JAK/STAT pathway in the hypothalamic nuclei (30) and in various cell types as well. But leptin can also use other signaling cascades via JAK activation. Among these are p38 (31), extracellular signal-regulated kinase (ERK1/2) (2, 31–36), and c-Jun terminal/stress-activated protein kinases (37) of the mitogen-activated protein kinase (MAPK) family members. Furthermore, leptin was found to generate increased amounts of H2O2 in isolated vascular endothelial cells in association with atherogenic processes (37, 38). H2O2 was shown to stimulate p38 in culture-activated rat HSC, resulting in α(I) procollagen mRNA up-regulation (39), and to phosphorylate ERK1/2 in human fibrosarcoma cells, leading to increased matrix metalloproteinase-1 transcription (40). In addition, Sohara et al. (41) reported that ERK1/2 mediates TIMP-1 production in liver myofibroblasts in response to oncostatin M, which is, like leptin, a member of the interleukin-6 cytokine family that signals through class I cytokine receptors. These findings raise the possibility that leptin may stimulate TIMP-1 production, using the H2O2-dependent p38 and ERK1/2 signal transduction pathways via JAK activation.

Specifically, our aims were to assess whether the fibrogenic actions of leptin involve the induction of TIMP-1 in the LX-2 cell line derived human HSC, the principal cells that mediate hepatic fibrogenesis (42, 43). The respective roles of the JAK/Stat and H2O2-dependent p38 and ERK1/2 MAPK signal transduction pathways in this process were evaluated. We also determined the expression of the leptin receptor and its signaling capabilities in LX-2 cells in response to leptin.

**EXPERIMENTAL PROCEDURES**

**Materials**— Dulbecco’s modified Eagle’s medium (DMEM) was purchased from Invitrogen (Rockville, MD). Human TIMP-1 and β-actin cDNA probes were obtained from American Type Culture Collection (Manassas, VA). [32P]dCTP and random priming DNA labeling kit from Amersham Biosciences (Arlington, IL). Human TIMP-1 ELISA kits was purchased from R&D System (Minneapolis, MN) and proteinase inhibitor mixture from ICN (Irvine, CA). Leptin, catalase, 2 mM l-glutamine, 100 IU penicillin, and 100 μg/ml streptomycin and incubated at 37 °C in a 5% CO2-air humified atmosphere. The medium was changed 48 h after plating. At subconfluence, cells were washed in serum-free DMEM, and leptin with or without inhibitors was added. Leptin was used at concentrations of 25–100 ng/ml. The inhibitors and their concentrations were: 50 μM JAK inhibitor AG 490 (45–47); 1000 units/ml catalase (49); 20 μM p38 inhibitor SB203580 (39, 48); 20 μM SB202474 (an inactive analog of SB203580) (52); and 30 μM ERK1/2 inhibitor PD98059 (39, 50). MeSO was used as vehicle control for the inhibitors. In these experiments, LX-2 cells were used from passages 20 to 30.

**Rat Hepatic Stellate Cells**—HSCs were isolated from male Sprague-Dawley rats and cultured in DMEM supplemented with 10% FCS and antibiotics until subconfluence as previously described (39). Cells were trypsinized, subcultured, and used 3 days later (as passage 1). Animal experimental procedures followed the National Research Council’s recommendations for animal care and were approved by the Institutional Animal Care and Use Committee.

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Hydrothidine is oxidized by O$_2^*$ produced by the cells. Hence, the loss of fluorescence is proportional to the amount of superoxide generated. Hydroethidine fluorescence was measured at 352 nm for excitation and 434 nm for emission.

**Lipid Peroxidation**—This was assessed by the addition of cis-parinaric acid to LX-2 culture at a final concentration of 5 μM. Subsequent to peroxidative stress, cis-parinaric acid is degraded, resulting in decreased intensity. The loss of fluorescence is proportional to lipid peroxidation. The cis-parinaric acid fluorescence was measured at 325 nm for excitation and 413 nm for emission.

**Reduced Glutathione Measurement**—GSH levels in LX-2 cells were determined, using the Cayman’s GSH assay kit according to the manufacturer’s instruction.

**p38 and ERK1/2 MAPK Phosphorylation Assays**—These were performed by Western blots, using the components provided in the PhosphoPlus p38 MAPK and ERK/2 MAPK antibody kits as previously described (39, 51). p38 activation was assayed using rabbit polyclonal phospho-p38 (Thr-180/Tyr-182) antibody. ERK1/2 phosphorylation was detected using the rabbit polyclonal phosho-ERK1/2 (Thr-202/Tyr-204) antibody. The antibodies were used at 1:1000 dilution. Horseradish peroxidase-conjugated anti-rabbit IgG (1:2000) was used as the secondary antibody. Equal protein loading was controlled by immunoblotting of corresponding nonphosphorylated p38 or ERK1/2 proteins. Immunoreactive proteins on the blots were visualized using the LumiGLO chemiluminescent reagents and then exposed to x-ray film. Signal intensities were quantified with the Evaluating Image Analysis System MCID.

**p38 Kinase Activity Assay**—This was performed by the detection of phosphorylation of activating transcription factor (ATF)-2, a substrate of p38, using the p38 MAP Kinase Assay kit as described previously (39). Whole cell lysates (200 μg) were immunoprecipitated with a 20-μl aliquot of monoclonal phospho-p38 (Thr-180/Tyr-182) antibody immobilized to agarose beads. The immunoprecipitated pellet was incubated with 2 μg of ATP-2 fusion protein in the presence of 100 μM ATP and 50 μl of kinase buffer for 30 min at 30 °C. A 20-μl sample was resolved on a 12% SDS-PAGE gel, transblotted to nitrocellulose membrane, probed with phospho-ATF-2 (Thr-71) antibody (1:1000) as the primary antibody and horseradish peroxidase-conjugated anti-rabbit IgG (1:2000) as the secondary antibody. Immunoreactive proteins were visualized and quantified as described above. Equal protein loading was controlled by immunoblotting of the corresponding nonphosphorylated ATF-2.

**Transfection of TIMP-1 Promoter and Chloramphenicol Acetyltransferase Assay**—The TIMP-1 promoter function was studied using the CAT reporter plasmid (pBLCA T3) containing nucleotides 102 to 96 (minimal promoter) of the human TIMP-1 gene (27). For transient transfection, LX-2 cells were grown on 24-well plates (3 × 10⁵ cells/well) to 90% confluence in DMEM supplemented with 5% FCS. The cell medium was changed, and transfection was performed using the LipofectAMIN™ kit per the manufacturer’s protocol. 1 μl of LP2000 reagent was diluted to 50 μl with DMEM and incubated for 5 min at room temperature. To this was added 320 ng of the reporter plasmid DNA or the promotorless pBLCT3 suspended in 50 μl of DMEM. The mixture was incubated at room temperature for 20 min to allow the formation of DNA/LF2000 complex. Finally, the mixture (100 μl) was added to the culture, mixed gently, and incubated with the cells at 37 °C for 24 h. Thereafter, the cells were treated with leptin in the absence or presence of AG 490, catalase, SB203580, or PD98059 (30 μM) was added. After 2, 8, and 12 h of incubation, total RNA from LX-2 cells was isolated for Northern blot analysis of TIMP-1 mRNA levels, and the decay time course in the absence or presence of the inhibitors was analyzed. Similar experiments were performed with LX-2 cells transiently transfected with p38dn and p38Δdn.

**TIMP-1 mRNA Stability Determination**—To assess whether p38 and ERK1/2 regulate TIMP-1 gene expression at the post-transcriptional level, LX-2 cells were treated with leptin (75 ng/ml) for 24 h to induce TIMP-1 mRNA. This was followed by actinomycin D (10 μg/ml) treatment for 20 min to block the transcription (53). The cell medium was changed, and fresh medium containing SB203580 (20 μM) or PD98059 (30 μM) was added. After 2, 8, and 12 h of incubation, total RNA from LX-2 cells was isolated for Northern blot analysis of TIMP-1 mRNA levels, and the decay time course in the absence or presence of the inhibitors was analyzed. Similar experiments were performed with LX-2 cells transiently transfected with p38dn and p38Δdn.

**Western Blot Analysis of Leptin Receptor (OB-R)—**Protein lysates of LX-2 cells treated or not with leptin (75 ng/ml) for 24 h were analyzed for OB-R expression by Western blot using 12% SDS-PAGE. The primary antibody was rabbit anti-human OB-R (H300) antibody. For the detection of Tyr-1141-phosphorylated OB-R, a goat anti-human phospho-OB-R (Tyr-1141) antibody was used. Mouse monoclonal antibody against GADPH was used as the control for equal protein loading. Immunoreactive proteins were visualized with Immun-Star Enhancer and Immun-Star Substrate (1:100) and then exposed to x-ray film. Signal intensities were analyzed using the Evaluating Image Analysis System MCID.

**Immunocytochemical Staining**—LX-2 cells grown on 4-chambered glass slides were incubated with monoclonal anti-vimentin, anti-α-SMA, or anti-desmin antibodies, followed by detection with the Super Sensitive MultiLink kit. The immunoreaction was visualized by using the chromogen diaminobenzidine.

**Protein Determination**—Cell lysate protein content was determined using the BCA protein assay kit from Pierce, Rockford, IL.
Statistics—Data are reported as means ± S.E. Statistical analysis was performed by analysis of variance followed by Student-Newman Keuls tests for multiple comparisons between treatment groups using Instat (3.01) and Sigma Stat (2.0) software (Jandel Scientific Software, San Rafael, CA). \( p < 0.05 \) was considered to be significant.

RESULTS

Leptin Increases TIMP-1 mRNA and Protein: Dose-dependent Effect—Human LX-2 HSC expressed TIMP-1 mRNA of 0.9 kb, as analyzed by Northern blot (Fig. 1). Leptin treatment for 24 h elicited a dose-dependent increase in TIMP-1 mRNA, reaching a maximal level of 3.6-fold at 75 ng/ml leptin. This effect was accompanied by a 3.4-fold increase in TIMP-1 protein in the culture media. The induction of TIMP-1 by leptin in LX-2 cells was reproduced in culture-activated rat HSC (data not shown).

Phosphorylation of JAK and STAT after Leptin: Time-dependent Effect—A significant increased phosphorylation was first detected at 10 min for both JAK1 and JAK2 in LX-2 cells and reached a maximum (about 4.5-fold, respectively) between 30 and 60 min after 75 ng/ml leptin (Fig. 2A). The values

![Image](https://example.com/image1.png)

**Fig. 2. Time course of JAK and STAT phosphorylation by leptin.** LX-2 cells were treated with leptin (75 ng/ml) for the time indicated, and immunoprecipitates of p-JAK1 and p-JAK2 (A) and p-STAT3 and p-STAT5 (B) were analyzed by Western blot, using the respective phosphospecific antibodies. The band intensities of p-JAKs and p-STATs were normalized to that of the corresponding nonphosphorylated JAKs and STATs, and values are expressed as -fold change relative to the control (0 min), which was assigned a value of 1. Lower panels are the corresponding histograms of data of three separate Western blot analyses. *, \( p < 0.05 \); **, \( p < 0.01 \); and ***, \( p < 0.001 \) compared with the values at 0 min.
returned to control levels at 240 min. There was an associated increase in phosphorylation of STAT3 (3.4-fold) and STAT5 (2.5-fold) at 30 min which remained maximal until 60 min (Fig. 2B). A significant increase in p-STAT3, but not p-STAT5, was still evident at 360 min. The total JAK and STAT protein contents did not change after leptin. No phosphorylation of STAT1 by leptin was detected (data not shown).

**JAK Inhibitor AG-490 Inhibits JAK Phosphorylation and Its Effects on STAT Phosphorylation and TIMP-1 mRNA**—Treatment of LX-2 cells with AG 490 for 30 min completely blocked the phosphorylation of JAK1 and JAK2 induced by leptin (75 ng/ml) (Fig. 3A). This treatment also resulted in total inhibition of STAT3 and STAT5 phosphorylation (Fig. 3B), as well as TIMP-1 mRNA expression (Fig. 3C). Me$_2$SO, in a concentration (2.1 mM) equivalent to that present in AG 490, had no effect on TIMP-1 mRNA expression, whether induced by leptin or not.

**Generation of Oxidative Stress by Leptin and Its Inhibition by JAK Inhibitor AG 490**—The leptin (75 ng/ml)-induced oxidative stress was time-dependent (Fig. 4A). Peak levels of H$_2$O$_2$ and superoxide formation and lipid peroxidation occurred at...
1 h after leptin (6.5-fold, 3.3-fold, and 2.6-fold versus controls at 0 time, respectively). At 24 h, the H$_2$O$_2$ level was still 2.9 times higher than in control, whereas superoxide and lipid peroxidation values returned to control levels. Cellular GSH fell in a time-dependent manner after leptin treatment, with a 39% decrease after 1 h and a further 59% decrease at 24 h. Fig. 4 shows that AG 490 treatment of LX-2 cells for 1 h prevented H$_2$O$_2$ and superoxide formation, lipid peroxidation, and restored the decreased cellular GSH level seen after leptin, suggesting a mediation by activated JAK1 and JAK2 of the leptin-induced oxidative stress.

**p38 MAPK Activation by Leptin and Its Inhibition by JAK Inhibitor AG 490, Catalase, p38 Inhibitor SB203580, and p38 Dominant Negative Mutants**—Because leptin induced oxidative stress and because the latter stimulated p38 MAPK in activated rat HSC (39), we evaluated whether leptin induces the activation of p38 through H$_2$O$_2$ in LX-2 cells. Fig. 5A shows phosphorylation of p38 by leptin in a dose- and time-dependent manner. A maximal stimulation was elicited by leptin at 75 ng/ml, and, at this concentration, a significant increase was first observed at 1 h (2.5-fold), and it peaked (about 3.6-fold) from 2 to 24 h. Treatment of LX-2 cells with AG 490 or catalase for 2 h prevented p38 phosphorylation (Fig. 5B), demonstrating involvement of p-JAK1 and p-JAK2 in the process; p38 phosphorylation was also inhibited by the p38 inhibitor SB203580 but not by its inactive analog SB202474 or...
the ERK1/2 inhibitor PD098599. Transfection of p38dn and p38dn into LX-2 cells resulted in a total suppression of leptin-induced p38 phosphorylation. Leptin also increased the kinase activity of p38 (Fig. 5C), and the rise was abolished by AG-490, catalase, and SB203580 as well as by overexpressed p38dn and p38dn in LX-2 cells, but not by SB202474 or PD098059, further substantiating the participation of JAK and H$_2$O$_2$ in the activation of p38 induced by leptin.

**ERK1/2 MAPK Phosphorylation by Leptin and Its Inhibition by JAK Inhibitor AG 490, Catalase, and ERK1/2 Inhibitor PD098599**—Because leptin has been shown to activate ERK1/2 in a variety of cell types (32–36), we assessed phosphorylation of ERK1/2 by leptin in LX-2 cells and the involvement of JAKs and H$_2$O$_2$ in this process. Fig. 6A shows a dose-dependent stimulation of ERK1/2 phosphorylation by leptin, with a maximal effect (5-fold) at 75 ng/ml. At this leptin concentration, increased phosphorylation of ERK1/2 was observed at 1 h (3-fold) and was maximal (4.3-fold) at 2 h (Fig. 6A). A significant effect was still evident at 24 h. Treatment with AG 490 or catalase for 2 h abolished the leptin stimulation of ERK1/2 phosphorylation (Fig. 6B), demonstrating an involvement of p-JAK1 and -2 and H$_2$O$_2$ in the process. PD098059 prevented the rise in ERK1/2 activation induced by leptin. SB203580, SB202474, or the p38 negative mutants had no effect on ERK1/2 phosphorylation.

**Inhibition of Leptin-induced TIMP-1 mRNA by Catalase, p38 Inhibitor SB203580, Dominant Negative p38 Mutants, and ERK1/2 Inhibitor PD098599**—To determine whether H$_2$O$_2$-dependent MAPK signaling participates in the up-regulation of TIMP-1 mRNA by leptin, LX-2 cells were treated with leptin (75 ng/ml) in the presence or absence of the respective inhibitors, and TIMP mRNA was analyzed after a 24-h incubation. As shown in Fig. 7A, catalase prevented the 4-fold rise in TIMP-1 mRNA induced by leptin. SB203580, but not its inactive analog SB202474, and overexpressed p38dn and p38dn halved the level of TIMP-1 mRNA after leptin (Fig. 7B), implicating a role for p38 in the induction of TIMP-1 mRNA by leptin. PD098059, like the p38 inhibitor, reduced the rise in TIMP-1 mRNA level to one-half (Fig. 7C). These results suggest that the up-regulation of TIMP-1 mRNA by leptin is mediated, at least in part, by H$_2$O$_2$ through the p38 and ERK1/2 MAPK signaling pathways.

**Leptin Stimulates TIMP-1 Promoter Activity and Its Inhibition by JAK Inhibitor AG 490, Catalase, and ERK1/2 Inhibitor PD098059**—To determine whether leptin-induced TIMP-1 analyzed by Western blot, using a phospho-p38 (Thr-180/Tyr-182) antibody as the primary antibody. The intensity of bands on the blots was normalized to that of total p38, detected by a nonphosphorylated p38 antibody, and values are presented as -fold change relative to controls (without leptin or at 0 h), assigned a value of 1. The time-dependent effect of leptin on p38 phosphorylation was studied using 75 ng/ml leptin, because this concentration elicited a maximal level of p38 phosphorylation. Upper panels are typical Western blots, and lower panels are histograms of data of three separate analyses. *, * p < 0.05 and **, p < 0.01 versus control. B, inhibition of leptin-induced p38 phosphorylation. LX-2 cells were treated for 2 h with leptin (75 ng/ml) alone or with AG 490 (50 µM), catalase (1000 units/ml), SB203580 (20 µM), SB202474 (20 µM), or PD098059 (30 µM). LX-2 cells transfected with p38dn and p38dn were likewise incubated with the same concentration of leptin. The phosphorylated and total p38 protein contents were analyzed by Western blot as in A. The numbers above the immunoblots refer to the mean values of three separate analyses. C, inhibition of leptin-induced p38 kinase activity. Conditions of treatment of LX-2 cells with leptin and inhibitors were the same as in B. p38 kinase activity was assayed by immunoprecipitation and Western blot analysis of phosphorylated ATF-2, using phospho-ATF-2 (Thr-71) antibody as described under “Materials and Methods.” The values of phospho-ATF-2 were normalized to that of nonphosphorylated ATF-2 and are presented as -fold change relative to the control, assigned a value of 1. The numbers above the immunoblots refer to the mean values of three separate analyses.

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**Fig. 5.** Increased p38 MAPK activation by leptin and its inhibition by AG 490, catalase, p38 inhibitor SB203580, and by overexpression of dominant negative p38 mutants. A, dose and time course of p38 phosphorylation by leptin. LX-2 cells were incubated with leptin at 25–100 ng/ml for 2 h. Phosphorylated p38 protein content was
gene expression is regulated at the transcriptional level in LX-2 cells, the activity of TIMP-1 promoter was assayed in cells with transfections of the human TIMP-1 minimal promoter (nucleotides -102 to 96). Leptin (75 ng/ml) treatment raised the promoter activity 3-fold (Fig. 8). The rise was prevented equally by AG 490, catalase, and PD098059, but not SB203580 or overexpressed p38\(^{\text{dn}}\) and p38\(^{\text{dn}}\). Transfection with the minimal promoter in the absence of leptin treatment caused a slight increase in the promoter activity compared with transfection with the promoterless empty pBLCAT vector, but the change was not affected by any of the inhibitors tested. These results implicate that leptin-induced TIMP-1 gene expression in LX-2 cells is mediated by activated JAK1 and JAK2, which, in turn, activate the ERK1/2 signaling pathway via H\(_2\)O\(_2\) formation and suggest that p38 is not involved in the transcriptional regulation of the TIMP-1 gene.

\(\text{p38 Inhibitor SB203580, but Not ERK1/2 Inhibitor PD098059, Inhibits Leptin-induced TIMP-1 Message Stability—}\)

Because p38 activation does not appear to regulate TIMP-1 mRNA expression at the transcriptional level in LX-2 cells, we tested whether p38 acts to stabilize TIMP-1 message induced by leptin. Treatment of LX-2 cells with SB203580 resulted in an increased decay of TIMP-1 mRNA with an approximately half-life (\(t_{1/2}\)) of 4 h compared with a \(t_{1/2}\) of 10 h without SB203580 treatment, suggesting a stabilization of TIMP-1 message by p38 (data not shown). This effect of p38 was reproduced by overexpression of p38\(^{\text{dn}}\) and p38\(^{\text{dn}}\) in LX-2 cells. By contrast, in LX-2 cells treated with PD098059, no change in TIMP-1 mRNA decay was observed. These results demonstrate that the leptin-induced TIMP-1 mRNA expression via the p38 pathway involves a stabilization of the TIMP-1 message.

**Expression of Leptin Receptors (OB-R) in LX-2 Cells—**With the OB-R H300 antibody, which reacts with an amino acid sequence 541–840 of the extracellular and transmembrane domains that are common to both the long and short forms of OB-R (3, 8), multiple immunoreactive bands were revealed by Western blot near 102 kDa, representing the short isoforms of OB-R, and a single band between 122 and 204 kDa, representing the long form of OB-R (7, 16), but their intensities were not increased after leptin treatment (Fig. 9A). In contrast, when probed with the anti-human p-OB-R (Tyr-1141) antibody directed at the Tyr-1141-phosphorylated cytoplasmic domain at the C terminus of the long form OB-R, an immunoreactive band between 122 and 204 kDa was detected, and there was a striking
**FIG. 7.** Inhibition of leptin-induced TIMP-1 mRNA by catalase, p38 inhibitor PD098059, dominant negative p38 mutants, and ERK1/2 inhibitor PD098059. LX-2 cells were incubated with leptin (75 ng/ml) alone or with the addition of catalase (1000 units/ml), SB203580 (20 μM), SB202474 (20 μM), or PD098059 (30 μM) for 24 h. LX-2 cells with transfection of p38dn and p38βdn were likewise incubated with leptin. The intensity of TIMP-1 mRNA bands was normalized with β-actin, and values are presented as fold change relative to the control (no leptin and inhibitor) assigned a value of 1. Upper panels are typical Northern blots, and the corresponding histograms summarizing results of three separate analyses are depicted in the lower panels. *p < 0.05 and ***p < 0.001 versus control; **p < 0.01 and ###p < 0.001 versus leptin treatment alone.

**DISCUSSION**

This study demonstrates that leptin has the capacity to stimulate TIMP-1 gene expression and to increase TIMP-1 fibrils. By electron microscopy, LX-2 cells contained modest amounts of rough and smooth endoplasmic reticulum, free ribosomes, Golgi apparatus, mitochondria, and a fair number of lipid droplets. Filamentous structures were not prominent, in agreement with the images by phase contrast microscopy.

Increase in its immunoreactivity after leptin treatment (Fig. 9B), suggesting up-regulation of Tyr-1141 phosphorylation of OB-Rβ.

**Cytochemical Staining and Ultrastructure of LX-2 Cells**—LX-2 cells stained positively for vimentin and α-SMA, but negatively for desmin. Staining for glial fibrillary acidic protein, synaptophysin, calponin, and myosin was also observed. Viewed under the phase contrast microscope and by comparison to activated rat or human HSC in culture, the majority of LX-2 cells displayed a lesser stellate shape with only a modest amount of visible stress fibrils. By electron microscopy, LX-2 cells contained modest amounts of rough and smooth endoplasmic reticulum, free ribosomes, Golgi apparatus, mitochondria, and a fair number of lipid droplets. Filamentous structures were not prominent, in agreement with the images by phase contrast microscopy.
protein production in activated human LX-2 HSC. This effect is associated with increased phosphorylation of JAK1 and JAK2 as well as of STAT3 and STAT5, accompanied by increased expression Tyr-1141-phosphorylated OB-RL. Leptin also activates the H$_2$O$_2$-dependent ERK1/2 and p38 MAPK pathways via activated JAK. ERK1/2 appears to act at the transcriptional level through stimulation of the TIMP-1 promoter activity, whereas p38 acts at the post-transcriptional level through stabilization of the TIMP-1 mRNA. These signaling pathways are schematically illustrated in Fig. 10.

LX-2 is an immortalized cell line derived from human HSC and has the features of activated HSC (44). Our cytochemical staining showed that LX-2 cells express α-SMA, a marker for activated HSC. Vimentin staining of LX-2 cells reveals their mesenchymal origin and the lack of desmin reflects their origin from human HSC (54). Glial fibrillary acidic protein and synaptophysin were observed in human HSC (55). The presence of calponin and myosin, which are smooth muscle-associated contractile proteins (56), suggests the acquisition by LX-2 of a contractile property, a characteristic of HSC.

All isoforms of the leptin receptor contain an identical extracellular domain of about 816 amino acids and a transmembrane domain of 23 amino acids but differ in their intracellular domains (3, 8). OB-R$_s$, the major short form of the receptor, has...
a cytoplasmic region of about 34 amino acids, whereas OB-RL
has a long 303-amino acid cytoplasmic domain, which contains
box 1 and box 2 motifs for interaction with the receptor-asso-
ciated JAK and a box 3 motif for STAT3 activation. Leptin
binding to OB-RL triggers activation of JAK2 (and possibly
JAK1), which then phosphorylates Tyr-1141 (homologous to
murine Tyr-1138) at the extreme C terminus of the cytoplasmic
domain of the receptor (see Fig. 10). The phosphorylated Tyr-
1141 binds STAT3 from the cytosol, which becomes activated
by JAK2 (32, 33, 57, 58). The activated STAT protein translo-
cates to the nucleus to bind to the TIMP-1 promoter (67, 70).

**Fig. 10. Schematic representation of the respective roles of the JAK-STAT and JAK-mediated H₂O₂-dependent MAPK pathways in TIMP-1 induction.**

Leptin triggers OB-RL signaling, leading to JAK1 and -2 phosphorylation. Activated JAK2 and (possibly JAK1) phosphorylate Tyr-1141 of the OB-RL. The phosphorylated Tyr-1141 recruits STAT3 that, in turn, becomes phosphorylated by JAK2. STAT3 translocates to the nucleus to bind to the TIMP-1 promoter (67, 70). Activated JAK1 and -2 also induce H₂O₂ formation that, in turn, activates ERK1/2 and p38. ERK1/2 stimulates TIMP-1 promoter activity with enhanced TIMP-1 mRNA expression. p38 acts through a mechanism involving a stabilization of the TIMP-1 message with up-regulation of TIMP-1 expression.

Because its expression in LX-2 cells was not increased by
It was reported that leptin induces H2O2 formation through brogenesis in HSC (39). The present data revealed that leptin signal transduction pathways and are potent mediators of fibrosis. Activation was mediated by JAK1 and JAK2 through H2O2. It is known that the TIMP-1 promoter contains a binding site for STAT3 (59, 62). It is expected that STAT-DNA binding activity at this site could contribute to the enhanced transcription of TIMP-1 gene observed after leptin. Such a mechanism has been described in human renal mesangial cells in response to thrombin (60) and in hepatoma cells after oncostatin M stimulation (59). Because this study did not examine STAT3 nuclear translocation and its binding to the TIMP-1 promoter in leptin-treated LX-2 cells, a direct role for the STAT pathway in TIMP-1 gene activation has yet to be established.

STAT5 was also activated by leptin, although at a lower level than STAT3. The role of STAT5 in leptin signaling is not known, but it was found to be activated together with STAT3 in COS and hepatoma cell lines cotransfected with OB-Rb and STAT cDNAs (6, 9, 10), and in porcine medullary cells after leptin treatment (35). Functionally, STAT5 regulates expression of milk proteins in the response of mammary tissue to prolactin (63).

Leptin could act through signaling cascades other than the JAK-STAT pathway (8, 63). Reactive oxygen species, in particular H2O2, act as second messengers that mediate diverse cellular activities, such as that which occurs in some organ systems (30, 58). Nevertheless, its coexpression with H2O2 may have a role in modulating the activities of OB-Rb, such as which occurs in some organ systems (30, 58).

Our finding of increased STAT3 phosphorylation after leptin in LX-2 cells is consistent with the observation by Saxena et al. (16) in primary cultured rat HSC. We found, in addition, that the leptin-induced STAT3 phosphorylation was preceded by JAK1 and JAK2 activation and was inhibited by AG 490, indicating mediation of STAT3 activation by JAK. AG 490 treatment also resulted in a total suppression of TIMP-1 promoter activity (Fig. 8) as well as TIMP-1 mRNA expression (Fig. 3). It is known that the TIMP-1 promoter contains a binding site for STAT3 (59, 62). It is expected that STAT-DNA binding activity at this site could contribute to the enhanced transcription of TIMP-1 gene observed after leptin. Such a mechanism has been described in human renal mesangial cells in response to thrombin (60) and in hepatoma cells after oncostatin M stimulation (59). Because this study did not examine STAT3 nuclear translocation and its binding to the TIMP-1 promoter in leptin-treated LX-2 cells, a direct role for the STAT pathway in TIMP-1 gene activation has yet to be established.

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In conclusion, the capacity of leptin to stimulate TIMP-1 gene activation and to increase its protein production in human HSC implicates that the hormone has a direct action on the stellate cells and could explain its fibrogenic role in the liver. Key future studies are needed to determine the binding of STAT3 to the TIMP-3 promoter and to evaluate possible crosstalk between the STAT pathway and the MAPK pathways (45, 64). It is important to note that the signaling molecules and pathways observed in cell lines could differ considerably from those actually activated in vivo (8, 30). Nevertheless, the present study illustrates that the human LX-2 HSC line is a useful tool for the elucidation of molecular mechanisms of hepatic fibrogenesis that could be potential targets for therapeutic intervention with fibrogenic agents.

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Leptin Stimulates Tissue Inhibitor of Metalloproteinase-1 in Human Hepatic Stellate Cells: RESPECTIVE ROLES OF THE JAK/STAT AND JAK-MEDIATED H2O2-DEPENDENT MAPK PATHWAYS

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