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Journal Title: Molecular Endocrinology -Baltimore-
Volume: Volume 20, Number 12
Publisher: Endocrine Society | 2006-12, Pages 3376-3388
Type of Work: Article | Post-print: After Peer Review
Publisher DOI: 10.1210/me.2006-0177
Permanent URL: http://pid.emory.edu/ark:/25593/fjk50

Final published version:

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Accessed December 14, 2019 1:18 AM EST
Leptin Increases Tissue Inhibitor of Metalloproteinase I (TIMP-1) Gene Expression by a Specificity Protein 1/Signal Transducer and Activator of Transcription 3 Mechanism

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Abstract

Leptin has properties of a profibrogenic cytokine. In liver, the activated hepatic stellate cell (HSC) is responsible for a net production of extracellular matrix. A key molecule synthesized is the tissue inhibitor of metalloproteinase 1 (TIMP-1), which acts to inhibit the activity of matrix metalloproteinases. The purpose of the present study was to determine how leptin, a gp130 cytokine, orchestrates the regulation of TIMP-1 gene activation and expression. Transient transfection of primary HSCs revealed that leptin significantly increased luciferase activity of a 229-bp TIMP-1 promoter construct (TIMP-1–229). An EMSA revealed that leptin enhanced specificity protein 1 (Sp1) binding. Site-directed mutagenesis for Sp1 reduced the enhancing effect of leptin on TIMP-1 transcriptional activation, and this effect was dose dependent on the number of Sp1 sites mutated. Chromatin immunoprecipitation revealed that leptin enhanced binding of Sp1; however, inhibition of signal transducer and activator of transcription (STAT) 3 phosphorylation by AG490 also blocked Sp1 phosphorylation and significantly reduced leptin-associated TIMP-1–229 promoter activity, indicating that one mechanism for leptin-increased transcriptional activity is via phosphorylation of Sp1 and subsequent promoter binding. Finally, we demonstrate that leptin also results in intranuclear pSTAT3 binding to Sp1. We propose a novel mechanism whereby leptin-mediated TIMP-1 transcription employs a Sp1/pSTAT3-dependent mechanism, one of which is a noncanonical association between Sp1 and pSTAT3. These data provide a new molecular mechanism whereby the adipocytokine leptin plays a role in complications of the metabolic syndrome.

Leptin, a 16-kDa hormone, has an array of biological effects. Recently, leptin has been shown by several groups to be critical in the development of hepatic fibrosis. In relation to tissue inhibitor of metalloproteinase 1 (TIMP-1), Cao et al. (59) recently demonstrated that leptin directly increased TIMP-1 transcriptional activation, and this effect was dose dependent on the number of Sp1 sites mutated. Their data indicate that TIMP-1 promoter activity was increased via the Janus kinase (JAK)-signal transducer and activator of transcription (STAT) 3 signaling pathway, which we have previously shown is critical to leptin-enhanced collagen production (1,2) and perpetuation of HSC proliferation and inhibition of apoptosis (3).

There are four major TIMPs (TIMP-1, TIMP-2, TIMP-3, and TIMP-4) that suppress extracellular matrix (ECM) degradation by forming an inhibitory 1:1 complex with matrix metalloproteinases.
metalloproteinases (MMPs) (4). In addition to acting as an inhibitor of most of the known MMPs, TIMP-1 has several important biological functions. TIMP-1 is a key regulator of fibrogenic events in the liver; the extent of carbon tetrachloride-induced liver fibrosis is elevated in mice that over express TIMP-1 from a liver-specific TIMP-1 transgene (5). The major cellular source of TIMP-1 in the injured liver is the activated HSC (6). HSCs are found in the normal liver in a quiescent state in which their major function appears to be the storage of vitamin A. After injury, HSCs activate or transdifferentiate into proliferating myofibroblast-like cells that produce abundant levels of fibrillar collagen and TIMP-1 (7). Secretion of abundant levels of TIMP-1 by activated HSCs leads to decreased hepatic collagenase activity and thereby promotes a net increase in ECM. TIMP-1 also promotes hepatic fibrogenesis by inhibiting apoptosis of activated HSCs, and thereby allows perpetuation of the fibrogenic process. The hypothesis that progression of liver fibrosis is associated with inhibition of matrix degradation in liver is strongly supported by studies of the relevant MMPs and TIMPs in cultured HSCs (7–9).

In chronic liver disease, and in animal models of liver fibrosis, significant increases in TIMP-1 and TIMP-2 expression have also been observed. Studies of human liver explants (obtained from patients undergoing liver transplantation) have found increased expression of TIMP-1, TIMP-2, or both in patients with primary sclerosing cholangitis, biliary atresia, primary biliary cirrhosis, and autoimmune chronic active hepatitis (8,10). These changes in mRNA for TIMP-1 were accompanied by proportional changes in TIMP-1 protein, which was increased approximately 5-fold in fibrotic vs. normal human liver as determined by ELISA.

TIMP-1 promoter activity is induced during HSC culture-activation (11). The TIMP-1 promoter is structurally conserved between rodents and humans and contains functional binding sites for activating protein-1 (AP-1) (Fos/Jun), and STATs located within a 22-bp serum-response element (SRE) and motifs for specificity protein 1 (Sp1) and BLP-1 transcription factors (12). A regulatory motif called the upstream TIMP-1 element [UTE-1] (12), is required for high level promoter activity including activated HSCs. Recent work (13) indicates that RUNX1A interacts directly with JunD, but RUNX1B failed to establish such an interaction. JunD and RUNX factors assemble at the adjacent SRE and UTE-1 sites on the TIMP-1 promoter and form functional interactions that stimulate transcription (14). Activated STAT proteins translocate to the nucleus to stimulate activation of the TIMP-1 promoter as has been demonstrated previously (13,15). Leptin signals as a gp130 cytokine; however, there is controversy related to exactly how gp130 signal transduction by IL-6 and oncostatin directs STAT binding along the TIMP-1 promoter (16) because induction of STAT binding to homologous TIMP-1 promoter sequences has not been detected. Hence, gp130 activation of TIMP-1 may likely involve cooperation with other factors such as Sp1 (17). Recently, Sp1 was reported to be critical to TIMP-1 promoter activity because mutations of the two Sp1 sites in the full-length promoter reduced TIMP-1 promoter transcription activity. In addition, the cotransfection with an antisense Sp1 oligonucleotides decreased the promoter activity, suggesting that the transcription of the TIMP-1 promoter is mediated by Sp1 (17).

Finally, STAT3 can activate transcription without binding itself to a palindromic enhancer element (18). Leptin, functioning as a gp130 cytokine (19), has been proved by several investigators to be a critical profibrogenic molecule in hepatic fibrosis (1,20–26). The purpose of the present study, therefore, was to elucidate the transcriptional mechanisms responsible for leptin-increased TIMP-1 gene expression via examination of STAT activation and Sp1 activity. We demonstrate a dual mechanism whereby leptin mediates enhanced TIMP-1 mRNA expression: that upstream Sp1 binding is enhanced by leptin via Sp1 phosphorylation and, pSTAT3 activation assembly with Sp1, along but not directly on
the TIMP-1 promoter, is a second mechanism for leptin-enhanced TIMP-1 promoter activation and gene expression.

RESULTS

TIMP-1 mRNA Increased in Vivo from Livers of Bile Duct Ligation (BDL)-Treated Lean But Not fa/fa Rats

BDL was performed in fa/fa rats and respective lean littersmates, as was sham operation. The fatty (fa/fa, or Zucker diabetic fatty) rat is defective in leptin receptor (Ob-R) as evidenced by chromosome mapping in a region of the rat chromosome 5 (27,28). The fa mutation is a missense mutation in the extracellular domain of OB-R, which results in a glutamine<sup>269</sup> to proline<sup>269</sup> amino acid substitution (29,30). In practical terms, the homozygous genotype (fa/ fa) represents a dysfunctional leptin signal transduction apparatus because prior work demonstrates that the fa mutation results in a nearly 10-fold reduction in the cell surface localization of the OB-R (31). Hence, we examined, by real-time RT-PCR, genes known to be involved with ECM remodeling and found that fibrotic livers from lean rats subjected to BDL had a 3.5-fold increase in TIMP-1 mRNA (Fig. 1A) than did sham-operated animals or fa/ fa rats that also underwent BDL.

Dose-Response and Time-Course Studies Reveal TIMP-1 mRNA Increased in Activated HSCs in Vitro

Activated HSCs treated with leptin (Fig. 1B) also resulted in a significant increase in TIMP-1 mRNA, which was maximally sustained with 100 ng/ml leptin ($P < 0.01$). Importantly, this effect was seen beginning at 3 h and continuing for 48 h after treatment (Fig. 1C). To determine whether leptin served to stabilize TIMP-1 mRNA transcripts and was not responsible for a nascent increase in TIMP-1 mRNA, we performed an mRNA stability assay using actinomycin D (Fig. 1D). These studies failed to indicate that leptin increased the half-life of TIMP-1 mRNA transcripts. When taken together, both the in vivo and in vitro results prompted us to examine the role leptin played in the regulation of TIMP-1 promoter regulation, and to determine whether this was related to an AP-1-dependent process, which has been previously reported as a consequence of HSC activation (7,32).

Deletion Analysis of the Human TIMP-1 Promoter: cis-Regulatory Elements Associated with Increased Leptin Activity Exists between −108 and −229 bp from the Start Site of Transcription

To determine whether leptin acted via the AP-1-dependent mechanism cited previously, we constructed six deletion mutants of the TIMP-1 promoter (Fig. 2A) and transiently transfected these into freshly isolated but culture-activated HSCs. Hence, these cells by default already had basal TIMP-1 promoter activity. When compared with basal activity of the TIMP-1−229 and other deletion constructs, the luciferase activity of TIMP-1−108 decreased more than 6-fold and no significant effect of leptin treatment on TIMP-1−108 promoter activity was observed. Leptin treatment of HSCs transfected with the TIMP-1−229 or larger deletion constructs resulted in a 60−140% increase in TIMP-1 luciferase activity when compared with basal activity of the TIMP-1−229 construct (Fig. 2B), indicating that one cis-binding element critical to leptin-enhanced TIMP-1 expression would be found between −108 and −229 bp upstream from the start site of transcription.
Leptin Increased the Binding of Two Oligonucleotides Containing Sp1 Sites to HSC Nuclear Protein Extracts

To elucidate the molecular mechanism related to the effect of leptin on TIMP-1–229 promoter activation, we synthesized six oligonucleotides (probes 1–6) spanning the 229-bp promoter region constructed in the previous studies (Fig. 3A) and performed EMSA with nuclear extracts from leptin-treated HSCs (Fig. 3B). The data indicate that probe 1 and 3 bound nuclear protein from leptin-treated HSCs with higher affinity than to extracts from untreated HSCs; however, complexes formed with probe 3 (P3) were much stronger than those with probe 1 (P1) (Fig. 3B). Binding activity was also enhanced with probe 4 (P4), but this did not change in the presence of leptin. Sequence analysis revealed probes 1 and 3 (P1 and P3) contained Sp1 consensus binding sites (Fig. 3A); and leptin appeared to enhance both Sp1 and Sp3 binding to probes 1 and 3 based on supershift analysis (Fig. 3C, lanes 5 and 6). Competition analysis with excess cold oligonucleotide P3 confirmed that Sp1 and Sp3 formed specific complexes (Fig. 3C, lane 4). These data prompted further examination of potential Sp1 sites, and to determine whether leptin enhanced Sp1-TIMP-1–229 promoter binding.

Substitution Mutation of Sp1 Sites Abolished Increased Leptin Activity of the TIMP-1–229 Promoter

To demonstrate that leptin-mediated transcriptional activation of the TIMP-1–229 promoter was Sp1 dependent, we created a series of 2-bp substitution mutants in which the two Sp1 sites contained in this mutant construct were eliminated (Fig. 4A). We demonstrated that the effect of leptin on TIMP-1–229 was reduced to basal levels (Fig. 4B) when either Sp1 cis-binding region was mutated (ΔT-229MA or ΔT-229MB). Luciferase activity was reduced further by the mutation of both Sp1 sites (ΔT-229MAB). We did not observe any difference in leptin-treated or basal luciferase activity for either the TIMP-1–108 deletion mutant or the ΔT1–108MA.

Leptin Modulates Sp1 and Sp3 Phosphorylation

Western blot analysis demonstrated that leptin resulted in the phosphorylation of STAT3 at 30 min; this is in keeping with the kinetics of Jak/STAT phosphorylation (Fig. 5A). Western blot also revealed that protein levels of Sp1 or Sp3 were not changed in HSCs exposed to leptin (data not shown). Because Sp1 and Sp3 can be activated as phosphoproteins, we investigated whether leptin might modulate the phosphorylation of Sp1 and Sp3. Serum-free or leptin-treated cells were harvested, and Sp1 or Sp3 was immunoprecipitated by antibody and protein A/G-agarose beads. The immunoprecipitates were subjected to SDS-PAGE (33) and probed with antiphosphoserine antibody. Phosphorylation of Sp1 was greater at 4 h when compared with Sp3 after leptin treatment (Fig. 5B). As anticipated, this activity diminished by 24 h.

Chromatin Immunoprecipitation (ChIP) Assay Revealed that Leptin Enhanced Only Sp1—But Not Sp3—Binding to the TIMP-1 Promoter

To determine whether leptin-enhanced TIMP-1 promoter activation was dependent on Sp1 or Sp3 we performed a ChIP assay. Sp1 and Sp3 bind TIMP-1–229, but in the presence of leptin only Sp1 binding was significantly increased (Fig. 6, A and B). These data are consistent with published reports that Sp3 may compete as an inhibitory factor to cis-binding regions associated with Sp1-enhanced transcriptional activation (34). OB-Rb signals as a gp130 cytokine, but we also failed to identify pSTAT3 binding to the TIMP-1–229 promoter (Fig. 6A). Hence, when taken together, the data indicate that leptin increased only Sp1—but not Sp3 or pSTAT3—binding to the TIMP-1–229 promoter.
Inhibition of STAT3 Phosphorylation Blocked Sp1 Phosphorylation and TIMP-1–229 Promoter Activity

The chemical inhibitor AG490 was used to block leptin-associated Jak2 phosphorylation of STAT3. AG490 abolished leptin-associated phosphorylation of both STAT3 (Fig. 7A) and Sp1 (Fig. 7B); and, AG490 also abolished leptin-enhanced luciferase activity of TIMP-1–229 (Fig. 7C). The presence of AG490 alone did not alter basal activity of the TIMP-1–229 promoter indicating that basal expression of TIMP-1 in activated HSCs is not associated with gp130 signal transduction. Overexpression of suppressor of cytokine signaling 3 (SOCS-3), a physiological inhibitor of pSTAT3 (4), blocked Sp1 phosphorylation enhanced by leptin (Fig. 7D). Hence, leptin induced STAT3 and Sp1 phosphorylation, which if abolished prohibited leptin-associated TIMP-1–229 promoter activation.

Leptin Enhanced TIMP-1–229 Promoter Activity Also Involves a Direct Interaction between pSTAT3 and Sp1

Finally, we investigated whether another molecular mechanism was responsible for leptin-induced TIMP-1–229 promoter activation. We overexpressed Sp1 and immunoprecipitated STAT3-Sp1 complexes by using Sp1 antibody and detected the coimmunoprecipitated STAT3-Sp1 complex by immunoblotting with anti-pSTAT3 antibody (Fig. 8A) or phosphoserine antibodies to detect Sp1. Only leptin-treated HSC lysates immunoprecipitated with antiphosphoserine antibody were detected with the anti-pSTAT3 antibody. In the EMSA, the addition of the anti-STAT3 antibody decreased the binding of Sp1 or Sp3 to the oligonucleotide (Fig. 8B), whereas antibodies against STAT1 did not. When taken together, these data reveal that Sp1 and pSTAT3 have a physical relationship that binds the TIMP-1 promoter to mediate leptin activation of this gene.

DISCUSSION

Hepatic fibrosis was historically thought to be a passive and irreversible process due to the collapse of the hepatic parenchyma and its substitution with collagen-rich tissue (35,36). Currently, hepatic fibrosis is considered to be a dynamic model of the wound-healing response to chronic liver injury (37). Nonalcoholic steatohepatitis (NASH) has been recognized as a major cause of liver fibrosis (38). First described by Ludwig et al. (39), it is considered a part of the spectrum of nonalcoholic fatty liver disease that ranges from steatosis to cirrhosis and can eventually lead to hepatocellular carcinoma. NASH is an important component of the metabolic syndrome, which is characterized by obesity, type 2 diabetes mellitus, and dyslipidemia, with insulin resistance as a common feature. Because the prevalence of obesity is rapidly increasing, a rise in the prevalence of NASH is anticipated. Hepatic fibrosis is the result of the wound-healing response to repeated injury and is associated with major alterations in both the quantity and composition of ECM (40). Accumulation of ECM results from both increased synthesis and decreased degradation (41). Decreased activity of ECM-removing matrix metalloproteinases (MMPs) is mainly due to an overexpression of their specific inhibitors, TIMPs—in particular TIMP-1. Strong evidence has been put forth that adipocytokines, or adipokines, clearly regulate liver fibrosis. Adipokines are cytokines primarily derived from adipose tissue. We and others (24,26) have shown that leptin is required for HSC activation, type I collagen synthesis, and perpetuation of the activated HSC phenotype—which are all associated with a net increase of hepatic ECM. Leptin-promoting events for increased ECM include increased activated HSC proliferation, impedance of HSC death by apoptosis, and increased synthesis of type I collagen (1–3). Leptin has also been associated with the fibrogenic response in kidney (42,43), fertility and blastocyst implantation in the female reproductive tract (44–46), and in wound-healing of the epidermis of leptin-deficient, ob/ob mice (47).
Our *in vivo* data from BDL-operated rats with a competent leptin signaling axis, as well as our *in vitro* data, indicate that leptin increases TIMP-1 mRNA transcripts. Such increases are not due to prolongation of the TIMP-1 mRNA half-life as a result of posttranscriptional modification (Fig. 1D). The prevailing data concerning TIMP-1 gene regulation are based on HSC activation and subsequent activation of AP-1 binding in the proximal portion of the TIMP-1 promoter as discussed previously. Long-term activated rat HSCs persistently express, among other proteins, Jun D. UTE-1 is a regulatory DNA motif essential for TIMP1 promoter activity in a variety of cell types including HSCs; and the relationship between the UTE-1 site and its adjacent upstream SRE in the promoter are critical to TIMP-1 gene regulation (14). The key regulatory sequence within the SRE is an AP-1 site that in HSC directs high level transcription via its interaction with JunD (14). Importantly, the TIMP-1 STAT element has a much lower affinity in comparison to AP-1 (13); hence, simultaneous binding of AP-1 and JunD is probably necessary to confer responsiveness after stellate cell activation, and activation of the TIMP-1 proximal promoter. To date, only one other group has examined the regulation of TIMP-1 by leptin and reports that MAPK activity increases hydrogen peroxide production as a potential mechanism for activating the TIMP-1 promoter (48). We have not found that leptin alone will up-regulate the UTE or SRE in the proximal promoter of the TIMP-1 gene. One potential reason for the lack of responsiveness of the −108 promoter in these studies is that leptin up-regulation of the TIMP-1 promoter may be transient, and these studies were conducted in the activated HSC phenotype in which activation of TIMP-1 via AP-1 has already occurred. Recent evidence also corroborates what we have found—insofar as the role of gp130 signal transduction— which exploits Sp1-TIMP-1 promoter binding to activate transcription (18). Hence, there may be several mechanisms of TIMP-1 activation in HSCs including one associated with transactivation, oxidative stress, and Sp1 phosphorylation by the Jak2/STAT 3 pathway with subsequent nuclear translocation and TIMP-1 promoter binding.

The data presented here clearly indicate that leptin-mediated increase in TIMP-1 promoter activity is linked to Sp1 promoter binding. The mechanism of leptin-enhanced Sp1 TIMP1 promoter activation is the consequence of two events. Except for the TIMP-1−108 mutant, the other deletion mutant constructs revealed significant differences under leptin-treated conditions (Fig. 2B). Although we did note that Sp3 can also bind the putative Sp1 sites employed in EMSA analysis (Fig. 3C), ChIP analysis revealed that leptin enhanced only the binding of Sp1, and not Sp3, to the TIMP-1 promoter employed in these studies (Fig. 6). Importantly, leptin resulted in minimal phosphorylation activity when compared with Sp1 (Fig. 5B). Even though the principle leptin signaling pathway is via Jak2/STAT3 phosphorylation, we also failed to detect pSTAT3 binding to the promoter by ChIP assay. Conversely, AG490, which inhibits Jak2 phosphorylation of STAT3 (Fig. 7A), not only inhibited Sp1 phosphorylation (Fig. 7B) but also markedly diminished leptin-enhanced TIMP-1 promoter activity (Fig. 7C). Sp1 belongs to the family of zinc finger proteins that are ubiquitously expressed (49). In our studies, mutation of either Sp1 *cis*-binding elements resulted in a reduction in leptin-mediated TIMP-1−229 activity; importantly, the Sp1 binding site at −121 and −128 bp appear to be indispensable for leptin-stimulated TIMP-1−229 promoter activity (Fig. 4B; ΔT-229MA).

Activated STAT3 can alter transcription by dimerization, and subsequent translocation to the nucleus with binding to enhancer elements of target genes, thereby inducing transcriptional activation (50). However, we did not find this to be the case by ChIP assay for the TIMP-1−229 promoter. Instead we found that leptin resulted in phosphorylation of Sp1 (Figs. 5B and 7B) and was required for leptin-enhanced TIMP-1 promoter activation (Fig. 7C); and Sp1, but neither Sp3 nor pSTAT3, was found to enhance TIMP-1 promoter binding by ChIP assay (Fig. 6). Hence, one mechanism whereby leptin acts is via phosphorylation of Sp1 and not by direct pSTAT3 binding. Although AG490 and SOCS-3
are respective chemical and biological inhibitors of Jak2 phosphorylation and STAT3 phosphorylation, these inhibitors could affect other signaling pathways and affect the activity of other hormones such as insulin. Recently it has been shown that domain-containing tyrosine phosphatase 2 (SHP-2) and SOCS3 do not just regulate the pathways that they are known to be associated with (SHP-2 with MAPK and SOCS-3 with JAK/STAT), but also have a strong effect on the other pathway (51). Hence, future work in assessing SOCS-3 overexpression in inhibiting alternate signaling pathways and leptin-mediated gene regulation will be required.

More than a decade ago, Jackson and Tjian (52) published the first study on Sp1 phosphorylation. Because the first report many other studies on this topic have been published using various cell systems and promoters (53) and Sp1 phosphorylation has been stimulated during viral infection (54), and organ development in the lung and liver (55,56). Leptin has been shown to be a growth factor (3,44,47). To our knowledge, this is the first report indicating that leptin can potentiate transcription by phosphorylation of Sp1. In short, these data provide a direct molecular link, i.e. JAK is directly responsible for the phosphorylation of Sp1 via pSTAT3; but it does not exclude the possibility that another intermediary signaling molecule (between pSTAT3 and Sp1) is ultimately responsible for Sp1 phosphorylation. Hence, additional studies will be needed to elucidate such signaling details.

We also have provided evidence that leptin drives TIMP-1 expression via both pSTAT3 activation and Sp1 in a novel noncanonical way. This hypothesis was substantiated by the fact that STAT3, but not STAT1, antibodies blocked the leptin-induced binding of Sp1 to the Sp1 oligonucleotide sequence from the TIMP-1 promoter (Fig. 8B); and, by overexpression of Sp1, we have convincingly demonstrated that only leptin-treated lysates immunoprecipitated with anti-Sp1 antibodies reveal pSTAT3 when subjected to immunoblot with antibodies to pSTAT3 or antiphosphoserine antibodies to detect Sp1 (Fig. 8A). There is evidence that transcription factors can activate transcription without DNA binding. STAT3 and Sp1 cooperatively activate the CAAT-enhancer-binding protein δ promoter (57), albeit through adjacent Sp1 and STAT3 binding sites. However, the TIMP-1 promoter region examined in the EMSA here contained only Sp1 binding sites, but no STAT3 binding elements (SBEs). We describe here a novel mechanism (Fig. 9) for the transcriptional regulation of TIMP-1 by which leptin not only activates Sp1 phosphorylation via STAT3 activation but also facilitates STAT3 association with Sp1 bound to the SBE-less TIMP-1 promoter. The characterization of this mechanism may be relevant for the design of novel treatment strategies, particularly for liver fibrosis related to NASH.

**MATERIALS AND METHODS**

**Experimental Animals**

The BDL protocol was approved by the Institutional Animal Care and Use Committee of Emory University, and all animals received human care and were euthanized in accord with Institutional and Ethical Guidelines of the American Veterinary Association.

**Animal Model for in Vivo Studies**

Four-week-old fa/fa rats and their lean littermates were purchased from Charles River Laboratories (Wilmington, MA). Animals were housed for four additional weeks in a temperature-controlled environment (20–22 C) with a 12-h light, 12-h dark cycle, and fed ad libitum with Laboratory Chow (Ralston Purina, St. Louis, MO) and water. Rats were assigned to one of four groups (n = 10): BDL (lean, or LLB), BDL (fa/fa, or FFB), sham-operated (lean, LLN) and sham-operated (fa/fa, or FFN). BDL, or sham operation was
performed as described elsewhere (58). Twenty-one days after surgery, total heptectomy was performed, and the animals were exsanguinated. Livers harvested were snap-frozen in liquid nitrogen.

**Isolation and Culture of Primary Hepatic Stellate Cells**

Quiescent stellate cells were isolated as described in detail elsewhere. HSCs were isolated from Sprague Dawley rats (Charles River). All rats received humane care, and the Institutional Animal Care and Use Committee of Emory University approved the HSC isolation protocol. In brief, *in situ* perfusion of the liver was performed with 20 mg/dl Pronase (Boehringer Mannheim, Indianapolis, IN), followed with collagenase (Crescent Chemical, Hauppauge, NY). Dispersed cell suspensions were layered on a discontinuous density gradient of 8.2% and 15.6% Accudenz (Accurate Chemical and Scientific, Westbury, NY). The resulting upper layer consisted of more than 95% HSCs. Cells were plated in modified medium 199 OR containing 20% fetal bovine serum (FBS) (Flow Laboratories, Naperville, IL). The purity of cells was assessed by immunolocalization of smooth muscle α actin in the monolayer as well as by intrinsic autofluorescence. The viability of all cells was verified by phase-contrast microscopy as well as the ability to exclude propidium iodide. Cell viability of cultures used for experiments was greater than 95%. Subconfluent activated cells in culture (75%) 7–10 d after isolation were washed twice with PBS and serum-starved for 16 h with 0.1% FBS and 1% penicillin-streptomycin in DMEM. The concentration of leptin was determined previously and its use is described elsewhere (1).

**Real-Time Quantitative RT-PCRs (RT-qPCR) from Whole Liver Post-Bile Duct Ligation and Cultured HSCs to Detect Matrix Gene Expression**

Total RNA from either whole liver from BDL-injured rats or HSCs was obtained using Trizol Reagent (Invitrogen, Carlsbad, CA). Total RNA (1µg) was reverse-transcribed in a 20-µl reaction containing random primers and iScript reverse Transcriptase (Bio-Rad, Hercules, CA). Real-time PCR was performed with iCycler IQ Real-time detection system (Bio-Rad) using IQ SYBR Green super mix (Bio-Rad). The primers used for the amplification of the rat TIMP-1 (accession no. NM_053819) were: forward 5′- CCAGCCATGGAGAGCCTCTG-3′, and reverse 5′-TCAGATTATGCCAGGGAACC-3′. All samples were run in triplicate. The relative fold increase of specific RNA was calculated by the comparative cycle of threshold detection method and values were normalized to 18 S rRNA. Final results, expressed as n-fold differences of mRNA between different tissues relative to the 18 S rRNA, were calculated with the following formula that is based on two replications in each cycle: n = fold = 2^[(Ct - Ct)/2], where C represents cycle threshold (Ct) for target gene or 18 S rRNA gene detection in control tissues; t represents Ct for target gene or 18 S rRNA gene detection in other tissues.

**TIMP-1 mRNA Stability Assay**

Primary HSCs are grown in DMEM with 0.1% FBS for 16 h, treated with leptin (0 or 100 ng/ml) for 4 h and subsequently treated with actinomycin D (5 µg/ml) in 100-mm culture dishes. HSCs were harvested for RNA at 0, 3, 8, 24, and 48 h after the addition of actinomycin D. The leptin concentrations used in these studies has been previously used (3,22,48,59). Total RNA was prepared using Trizol (Invitrogen) and isolated according to the manufacturer’s protocol. TIMP-1 mRNA and 18 S rRNA were quantitated for each sample relative to the sample at 0 h using RT-qPCR. The quantity of TIMP-1 mRNA was normalized to the amount of 18 S rRNA and the results were expressed as fold change. The entire experiment was repeated three separate times. The mRNA stability of TIMP-1 mRNA was evaluated by drawing the best-fit linear curve on a linear plot of the TIMP-1 mRNA fold change vs. time.
TIMP-1 Promoter Constructs

Human genomic DNA was extracted from the human MCF-7 cell line by using the QIAamp DNA blood kit (QIAGEN, Valencia, CA). TIMP-1 promoters were amplified from human genomic DNA using the PCR. Forward primers, which anneal at −1482, −989, −582, −369, −229, and −108 bp upstream from the transcription start site, were used with a 21-bp reverse primer ending at position +88 bp of the human TIMP-1 promoter. XhoI and HindIII restriction sites were introduced for subsequent cloning into the XhoI/HindIII-digested pGL3 basic luciferase reporter vector (Promega, Madison, WI). Primer sequences were: forward primers 5′-CTCGAGTAGATTAGAGGGTACAGCTGCTG-3′, 5′-CTCGAGTGGGGTGAGGATTAGTGGTTC-3′, 5′-TCGAGCCATGTATTGACTCTGTGATCC-3′, 5′-CTCGAGGAAGGGCTGAACTAATTTGGG-3′, 5′-CTCGAGAGGCGGCTTGGAAGGAATAG-3′, 5′-CTCGAGGTGGGTGGATGAGTAATGCA-3′ and reverse primer 5′-AAGCTTCAGCTCCGGTCCCTG-3′. One nanogram of human genomic DNA and 1.25 pmol of each primer were used for the amplification of the TIMP-1 promoter. Annealing of primers was performed at 60°C for 1 min followed by 72°C in 30 1-min cycles and elongation at 72°C for 10 min using a High Fidelity PCR kit (Invitrogen). The PCR was analyzed by agarose gel electrophoresis and the expected products were extracted and purified using the QIA-quick gel extraction (QIAGEN). The purified product was used for ligation into the TOPO TA vector (Invitrogen). After XhoI/HindIII digestion of the TIMP-1, inserts were ligated into the TOPO TA vector and extracted by using the Gel extraction kit from QIAGEN. Vectors were recloned in the pGL3 basic vector to yield TIMP-1–1482, TIMP-1–989, TIMP-1–582, TIMP-1–369, TIMP-1–229, and TIMP-1–108. The correct size and orientation of the cloned insert were analyzed by sequencing the plasmid DNA following standard plasmid preparation procedure by alkaline lysis.

Site-Directed Mutagenesis

The Sp1 mutant constructs were generated using the QuikChange site directed mutagenesis kit (Stratagene, La Jolla, CA) using TIMP-1–229 and TIMP-1–108 constructs as templates. Substitution mutation at both Sp1 sites, i.e. −34 and −35 bp, and −124 and −125 bp upstream from the start site of transcription were created with the following sequences: Sp1 mutant A sense, 5′-CCCGTGCACCCCCATCCCATGCGTGGAC-3′, Sp1 mutant A antisense, 5′-GTCGACCGCTAGGATGGGGTGACGC GG-3′, Sp1 mutant B sense, 5′-CTTTCGTCGGCCATCCCCTGCGTGGAC-3′, Sp1 mutant B antisense, 5′-GCAGAAGCCAAAGGGATGGC CGACGAA AGGG-3′. The mutations were confirmed by DNA sequencing.

Transient Transfections and Reporter Assays

Activated HSCs, after no more than two passages, were grown in DMEM containing 10% FBS, penicillin (100 U/ml), and streptomycin (100 µg/ml), until cells were 60–80% confluent. Transient transfection assays were performed using Lipofectamine Plus reagent (Invitrogen,) with 1 µg of the TIMP-1 reporter plasmid plus 200 ng of Renilla luciferase plasmid (Promega) as an internal control for transfection efficiency. Four hours after transfection, the cells were supplemented with complete media. HSCs were serum starved overnight before stimulation with leptin (100 ng/ml). Luciferase activity in cell extracts was measured using a Dynatech luminometer (Dynatech Laboratories, Chantilly, VA). Firefly luciferase activity was normalized to Renilla luciferase activity.
Nuclear Extract Preparation

Nuclear extracts were prepared as described previously. Briefly, HSCs were washed and scraped in ice-cold PBS and transferred to centrifuge tubes. Samples were spun at 4000 × g for 3 min at 4°C. The pellet was resuspended in buffer containing 10 mM HEPES (pH 7.0); 1.5 mM MgCl₂; and 10 mM KCl on ice for 10 min. The samples were vortexed for 10 sec and spun at 4000 × g for 3 min. The resulting pellet was resuspended in buffer containing 20 mM HEPES (pH 7.9), 420 mM NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, and 25% glycerol, and incubated on ice for 20 min. These samples were spun at 16,800 × g for 2 min and the supernatant was transferred to Eppendorf tubes (Eppendorf International, Hamburg, Germany) and stored at −80°C. Protein concentration was determined by the Bradford assay (Bio-Rad) (60). All buffers contained protease inhibitor cocktail (Roche, Nutley, NJ) and 1 mM Na₃VO₄, 20 mM NaF, and 1 mM Na₄P₂O₇.

EMSA

Oligonucleotides were annealed and labeled with T4 polynucleotide kinase (New England Biolabs, Beverly, MA) and [γ-³²P] deoxy (d)-ATP (DuPont, Wilmington, DE; 3000 Ci/mmol). Labeled oligonucleotides were used as probes or remained unlabeled as competitors. A total of 5 µg protein was incubated with 35 fmol of [γ-³²P]dATP-labeled probe in binding buffer [10 mM HEPES (pH 7.9), 1 mM EDTA, 5 mM MgCl₂, 10% glycerol, 5 mM dithiothreitol, protease inhibitor cocktail and 1 µg of poly(deoxyinosine-deoxycytosine)] for 10 min on ice. The [γ-³²P]dATP-labeled oligonucleotides were added and the mixture was incubated on ice for 60 min. DNA-protein complexes were separated from free DNA by electrophoresis across a 4% nondenaturing polyacrylamide gel. All gels were electrophoresed in 0.5× Tris-borate-EDTA buffer at 200 V for 1–2 h. Gels were dried under vacuum and exposed to film (Eastman Kodak, Rochester, NY). For competition experiments, a 50-fold molar excess of unlabeled oligonucleotide was added to the binding reaction. For supershift experiments, 5 µg of nuclear protein extract were incubated 60 min on ice, each with 1 µg of the appropriate antibody in a maximum volume of 40 µl before the binding reaction. Antibodies used were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

ChIP Assay

ChIP analysis was performed using a commercially available kit (Upstate, Charlottsville, VA) on HSCs treated with or without leptin for 24 h as detailed elsewhere (61). Proteins bound to DNA were cross-linked using formaldehyde at a final concentration of 1% for 20 min at room temperature. Protein-DNA complexes were immunoprecipitated using primary antibodies for Sp1, Sp3, and STAT3 (Santa Cruz Biotechnology). Sp1- and Sp3-TIMP-1 promoter complexes were measured by real-time PCR. The primers used for the amplification of the rat TIMP-1 promoter (accession no. NM_053819) region between −62~−230 bp were: forward 5′-AGGGGCTTAAGCGGTCTTTA-3′, and reverse 5′-CACCCACTGCTGAAGTCAA-3′. The samples were electrophoresed using a 2% agarose gel, and visualized by ethidium bromide staining.

Overexpression of SOCS-3 and Sp1

The mammalian expression vectors harboring SOCS 3 (pEFFLAG-1/mSOCS-3) was a kind gift of Dr. D. Hilton (Victoria, Australia) (62). The human Sp1 expression vector (pN3-Sp1FL-complete), was kindly provided by Dr. Guntram Suske (Universität Marburg, Marburg, Germany) (49). Subconfluent (90%) activated HSCs were transfected with FLAG-SOCS-3 expression vector or corresponding empty vector (PEGFP-N1) using Lipofectamine Plus reagent (Invitrogen). The cells were serum starved for 16 h, and then cultured with leptin (100 ng/ml), IL-6 (10 ng/ml) or vehicle for 24 h before cellular proteins were
harvested for immunoprecipitation study. For the overexpression of Sp1, 90% confluent HSCs in were transfected with 24 µg of Sp1 expression vector, serum starved for 16 h and treated with or without leptin for 24 h. Nuclear protein was extracted as described above and 200 µg of protein were used for immunoprecipitation experiments.

**Coimmunoprecipitation, Immunoprecipitation, and Western Blot Analysis**

For coimmunoprecipitation, HSCs were lysed, and nuclear proteins were extracted and immunoprecipitated with 5 µl of antiserum per 200 µg of nuclear proteins. For immunoprecipitation, HSCs were lysed, and lysates were immunoprecipitated with 5 µl of antiserum per 0.5 ml of cell lysate as described (24). The immunocomplexes were collected by adding protein A/G-agarose beads. For Western blots, leptin-treated or -untreated HSCs were washed with PBS, lysed in SDS-PAGE (33) loading buffer (200 µl/60³-mm dish), boiled 10 min, and clarified by centrifugation (8 min, full speed, Eppendorf microcentrifuge). Protein extracts were fractionated across 10% acrylamide SDS-PAGE gels. Proteins were transblotted onto polyvinylidene difluoride membranes. Blots were probed with anti-pSTAT3, anti-Sp1 and anti-Sp3 antibodies or antiphosphoserine antibody (Sigma, St. Louis, MO) to investigate the phosphorylation status of Sp1 or Sp3 (63). Immunoreactive proteins were blotted with a secondary antirabbit horseradish peroxidase (Santa Cruz Biotechnology) and visualized using chemiluminescence reagents (Pierce, Rockford, IL) and exposed to x-ray film. Signal intensities were analyzed using the Fluorchem 800 Advanced Fluorescence Image Analysis System (Alpha Innotech, San Leandro, CA).

**Abbreviations**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>AP-1</td>
<td>Activating protein-1</td>
</tr>
<tr>
<td>BDL</td>
<td>bile duct ligation</td>
</tr>
<tr>
<td>ChIP</td>
<td>chromatin immunoprecipitation assay</td>
</tr>
<tr>
<td>ECM</td>
<td>extracellular matrix</td>
</tr>
<tr>
<td>HSC</td>
<td>hepatic stellate cell</td>
</tr>
<tr>
<td>Jak</td>
<td>Janus kinase</td>
</tr>
<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
</tr>
<tr>
<td>FFB</td>
<td>ZDF bile-duct ligated</td>
</tr>
<tr>
<td>FFN</td>
<td>ZDF-sham operated</td>
</tr>
<tr>
<td>GAPDH</td>
<td>glyceraldehyde phosphate dehydrogenase</td>
</tr>
<tr>
<td>LLB</td>
<td>lean bile-duct ligated</td>
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<tr>
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<tr>
<td>MMP</td>
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<td>leptin receptor</td>
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<td>Ob-Ra</td>
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</tr>
<tr>
<td>PI-3K</td>
<td>phosphatidylinositol-3 kinase</td>
</tr>
<tr>
<td>RT-qPCR</td>
<td>real-time quantitative RT-PCR</td>
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</table>
SBE Stat-binding element
SHP-2 domain containing tyrosine phosphatase 2
SF serum-free
SOCS3 suppressor of cytokine signaling 3
Sp1 specificity protein 1
SRE serum-response element
STAT3 signal transducer and activator of transcription
TIMP-1 tissue inhibitor of metalloproteinase 1
UTE-1 upstream TIMP-1 element 1

Acknowledgments

This work was supported by National Institutes of Health Grants DK062092 and DK064399.

REFERENCES


Fig. 1. TIMP-1 Gene Expression Is Elevated by Leptin Both in Vivo and in Vitro

A, Total RNA extracted from whole livers from the four treatment groups was used to determine mRNA expression of TIMP-1 as described in Materials and Methods by RT-qPCR. Level of mRNA expression, observed in sham-operated lean littermates was set at 100% of control. Results are means ± SE and represent 12 reactions for each treatment condition. *, P < 0.01 compared with lean sham-operated values.

B, Subconfluent activated rat HSCs were serum starved for 16 h with 0.1% FBS in DMEM and then exposed to increasing concentrations of leptin for 24 h and analyzed for TIMP-1 mRNA expression by RT-qPCR. Values are means ± SE, expressed as fold change relative to the control (no leptin). *, P < 0.01. The results are representative of three independent experiments.

C, Activated rat
HSCs were treated with leptin (100 ng/ml) for the times indicated and analyzed for TIMP-1 mRNA expression by RT-qPCR. The results, representative of three independent experiments, are compared with respect to control (no leptin) at the respective time points. *, $P < 0.01$. D. Activated HSCs were grown in DMEM with 0.1% FBS for 16 h pretreated with leptin (0 or 100 ng/ml) for 4 h and subsequently treated with actinomycin D [5 µg/ml]. Cells were harvested for total RNA at time points indicated after addition of actinomycin D. TIMP-1 mRNA was quantitated using RT-qPCR and normalized to 18 S rRNA. Results are expressed as fold change relative to the samples at time zero. The entire experiment was repeated three separate times in triplicate. TIMP-1 mRNA stability was evaluated by computing the best fit linear curve on a linear plot of the mRNA fold change vs. time for leptin and nonleptin treatment groups. SF, Serum free.
Fig. 2. Localization of a Human TIMP-1 Promoter Region (−1482 to +88) Mediating the Effect of Leptin on TIMP-1 Transcription in Activated Rat HSCs

A. Various lengths of human TIMP-1 promoter sequences were cloned upstream from luciferase reporter gene as described in Materials and Methods. The schematic diagram represents six deletion mutants: TIMP-1–1482, TIMP-1–989, TIMP-1–582, TIMP-1–369, TIMP-1–229, and TIMP-1–108 used for promoter analysis. 

B. Transient transfection of activated HSCs with respective promoter activities by firefly luciferase assay. The assay was repeated three times in triplicate and represent the mean ± se; *, P < 0.01 with respect to TIMP-1–229. SF, Serum free.
Fig. 3. The Effect of Leptin on the Binding of HSC Nuclear Extracts to Oligonucleotides Spanning the 229-bp TIMP-1 Promoter

A, Schematic diagram of six oligonucleotides spanning the 229 bp TIMP-1 promoter were synthesized, annealed and radiolabeled as probes for EMSA. B, Nuclear extracts (5 µg) were prepared from activated primary rat HSCs, serum starved for 16 h, and exposed to leptin (100 ng/ml) for 24 h. Leptin enhanced binding of nuclear extracts prepared from treated HSCs to probes 1 and 3 when compared with binding activities of untreated extracts. C, To identify complexes formed for probes 1 and 3, competition and antisera reactions were performed as described in Materials and Methods. FP, Free probe; SF, serum-free, untreated control; L, leptin treatment; Cold 50×, competition assay from leptin-treated nuclear extracts in the presence of 50-fold excess unlabeled probe 3 (P3). Sp1 and Sp3 antisera were added to reaction mixtures for supershift assay in lanes 5 and 6, respectively, and supershift complexes were formed (long arrow). The assay is representative of three independent experiments.
Fig. 4. Mutation of the Sp1 Sites Abolishes Effect of Leptin on TIMP-1 Promoter Activity
A. Four substitution mutation constructs for the TIMP-1–229 deletion mutant were synthesized as described in Materials and Methods. B. Subconfluent primary activated HSCs were transiently transfected with these constructs to determine the role of Sp1 in mediating leptin-enhanced TIMP-1 promoter activity as assessed by firefly luciferase activity. Relative promoter activity from three independent experiments performed in triplicate expressed as mean ± SE. *, $P < 0.01$ with respect to basal TIMP-1–229 promoter activity. SF, Serum free.
Fig. 5. Leptin Increases Phosphorylation, But Not Protein Level, of Sp1 and Sp3
A, Subconfluent primary activated HSCs were serum starved for 16 h, and then cultured in the presence of leptin (100 ng/ml) or vehicle for the times indicated before cellular proteins were harvested. The Western blots were performed as described in Materials and Methods in triplicate using antibody to pSTAT3. B, Primary activated HSCs were grown and starved as described above, and then cultured in the presence of leptin (100 ng/ml) or vehicle for the times indicated. Sp1 and Sp3 proteins were immunoprecipitated (IP) and the immunoprecipitates were subjected to SDS-PAGE. The membranes were probed with antiphosphoserine antibody to evaluate the phosphorylation status of Sp1 and Sp3. The membranes were also probed with anti-Sp1 and -Sp3 antibody to monitor the quantity of Sp1 or Sp3. The gels shown were representative of three independent experiments. Maximal phosphorylation occurs for Sp1 at 4 h with minimal phosphorylation of Sp3. IB, Immunoblot.
Fig. 6. ChIP Assay Reveals that Leptin Enhances the Binding of Sp1, But Not Sp3 or pSTAT3, to the Rat TIMP-1 Promoter

A. Subconfluent primary activated HSCs were serum starved for 16 h, and then cultured in the presence of leptin (100 ng/ml) or vehicle for 24 h before the cells were lysed and sonicated. Formaldehyde cross-linked chromatin from HSCs was incubated with Sp1, Sp3, pSTAT3, or control antisera. Immunoprecipitated DNA was analyzed by PCR with primer to proximal promoter of rat TIMP-1 gene or glyceraldehyde phosphate dehydrogenase (GAPDH) gene. Total input DNA at a 1:100 dilution was used as a positive control of the PCRs. DNA prepared from chromatin that was immunoprecipitated with nonimmune rabbit sera (+IgG) was used as a negative control for assays. The lanes are designated either

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according to the antisera used for the ChIP assay or by “Input,” which indicates the preimmunoprecipitated chromatin used in the PCR. The gel shown is representative of three independent experiments. B, Protein-DNA complexes immunoprecipitated were measured by real-time PCR. Results, expressed as fold change differences of DNA (amplified promoter) between leptin and vehicle samples were calculated relative to GAPDH as described in Materials and Methods. *, P < 0.01, leptin treatment vs. untreated control. Results are means ± se and represent three independent studies. SF, Serum free.
Fig. 7. AG490 Inhibited Leptin-Stimulated STAT3 and Sp1 Phosphorylation and Abolished the Leptin-Enhanced TIMP-1 Promoter Activity

A, Subconfluent primary activated HSCs were serum starved for 16 h, and then cultured with leptin (100 ng/ml) or vehicle with or without AG490 (10 µM) for 4 h before cellular proteins were harvested. IL6 (10 ng/ml) was used as positive control for STAT3 activation. pSTAT3 and STAT3 were assayed by immunoblot (IB) analysis as shown. Equal loading of proteins in each lane was confirmed by probing membranes for β-actin. B, Identical experimental design to A was conducted to probe to determine whether AG490 blocked phosphorylation of Sp1 after immunoprecipitation and probed with anti-phosphoserine antibody to evaluate the phosphorylation status of Sp1. The membranes were also probed with anti-Sp1 to monitor the quantity of Sp1. The present study is representative of two independent experiments. C, Subconfluent primary activated HSCs were transfected with TIMP-1–229, serum starved for 16 h, and then cultured as described above for 24 h before cell lysates were harvested for measurement of luciferase activity. Relative promoter activity from three independent experiments performed in triplicate are expressed as means ± SE; *, P < 0.01. D, 90% confluent primary activated HSCs were transfected with FLAG-SOCS-3 expression vector or corresponding empty vector as described elsewhere. The cells were serum starved for 16 h, and then cultured with leptin (100 ng/ml), IL-6 (10 ng/ml) or vehicle for 4 h before cellular proteins were harvested. The lysates were immunoprecipitated using Sp1 antibody or control anti-sera. Total input protein was assessed to show the successful transfection of FLAG-SOCS-3 expression vector using flag antibody.
Fig. 8. Sp1 and STAT3 Complex Was Detected and STAT3 Antibodies Disrupt the Leptin-Induced Binding of Sp1 to the TIMP-1 Promoter

A, Primary activated HSCs were transfected with 24 µg of Sp1 expression vector (pN3-Sp1FL-complete), serum starved for 16 h, and treated with or without leptin for 24 h. Nuclear protein was extracted and 200 µg of protein were subjected to immunoprecipitation (IP) using an antibody against Sp1 or antirabbit IgG (nonimmune sera). The immunoprecipitated proteins were separated and subjected to immunoblot analysis with either anti-pSTAT3, or anti-phosphoserine. Twenty micrograms of nuclear protein were input for detecting Sp1 expression. Immunoblot (IB) with either anti-pSTAT3 or antiphosphoserine antibodies of leptin-treated primary activated HSC nuclear protein lysate immunoprecipitated with anti-Sp1 antibody revealed pSTAT3-Sp1 complexes. COIP, Coimmunoprecipitation. B, To identify a physical mechanism whereby STAT3 would complex with Sp1, EMSA was carried out using 5 µg nuclear extracts prepared from leptin-treated HSCs for all lanes. Lane 1, Free probe; lane 2, serum free (SF) exposed HSC nuclear extracts; lane 3, leptin-treated primary activated HSC nuclear extract with probe 3 (cf. Fig. 3) only (Leptin); lane 4, supershift assay with nonimmune serum IgG; lane 5, antibody to Sp1 resulting in supershift of Sp1-oligonucleotide complex (Sp1); lane 6, STAT3 antisera disrupted Sp1 or Sp3 binding to the oligonucleotide, but STAT1 antisera (lane 7) did not...
result in nuclear protein-oligonucleotide disruption. This EMSA is representative of three independent experiments.
Fig. 9. Potential Mechanism for Leptin Activation of STAT3 and Sp1 in the Regulation of the TIMP-1 Promoter
Based upon the data presented leptin activates STAT3 phosphorylation via Jak2. This process also results in the phosphorylation of Sp1 as well as nuclear translocation and binding of phosphorylated Sp1 to the TIMP-1 promoter. Based on ChIP analysis (Fig. 5) and coimmunoprecipitation and EMSAs (Fig. 8), we propose that STAT3 nuclear translocation results in the physical association of pSTAT3 and phosphorylated Sp1.