Sp1 and Sp3 Transcription Factors Mediate Leptin-Induced Collagen α1(I) Gene Expression in Primary Culture of Male Rat Hepatic Stellate Cells

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Mechanisms by which leptin stimulates collagen α_1(I) [*Col1a(I)*] gene expression are unclear. The purposes of this study were to identify the *trans*-acting factors and *cis*-acting elements in *Col1a(I)* promoter involved in this effect as well as the pathways that are implicated. In primary cultures of rat hepatic stellate cells (HSCs), we measured the effects of leptin on *Col1a(I)* gene and protein expression and on the binding of nuclear proteins to the *Col1a(I)* promoter. We found that leptin increased *Col1a(I)* gene and protein expression in activated HSCs. Transient transfections showed that leptin exerted its effects through elements located between −220 and −112 bp of the *Col1a(I)* promoter. Gel retardation assays demonstrated that leptin induced the binding of transcription factors specific protein (Sp)-1 and Sp3 to two elements located between −161 and −110 bp of the *Col1a(I)* promoter. Leptin-induced Sp1/Sp3 phosphorylation, but this effect was suppressed by inhibiting or silencing Janus kinase-2, phosphatidylinositol-3-kinase, nonphagocytic adenine dinucleotide phosphate (NADPH) oxidase, or ERK1/2, by the use of antioxidants or catalase, or by preventing protein-aldehyde adduct formation. Leptin provoked oxidative stress, aldehyde-protein adduct formation, and increased gene expression of some components of the NADPH oxidase complex. In conclusion, in HSCs, leptin up-regulates *Col1a(I)* gene expression after activating NADPH oxidase, inducing oxidative stress, aldehyde-protein adduct formation, and ERK1/2 phosphorylation, which in turn activates Sp1/Sp3 and provokes the binding of these two factors to regulatory elements located between −161 and −110 bp of the *Col1a(I)* promoter. These findings may contribute to a better understanding of mechanisms involved in the leptin-induced liver fibrosis. **(*Endocrinology* 153: 5845–5856, 2012)**

Leptin is a 16-kDa nonglycosylated peptide and adipocyte-derived hormone that regulates food intake and energy expenditure (1). Absence of leptin is associated with a massive increase in body fat (2). Apart from its effects on energy homeostasis, leptin is a pleiotropic molecule that is also involved in many other biological effects including wound repair and extracellular matrix production (3). Fibrosis caused by liver injury is markedly diminished in animals deficient in leptin or leptin-receptors (4, 5). By contrast, leptin increases hepatic fibrosis induced by tioacetamide administration (6). On the other hand, leptin has shown to be a stimulatory factor for collagen gene expression and collagen protein production (7, 8); therefore, leptin is a profibrogenic hormone that may play a role in the development of liver fibrosis. However, intracellular

ISSN Print 0013-7227 ISSN Online 1945-7170
Printed in U.S.A.

doi: 10.1210/en.2012–1626 Received June 12, 2012. Accepted September 24, 2012.
First Published Online October 23, 2012

Abbreviations: AP2, Activator protein 2; CAT, chloramphenicol acetyltransferase; c-Krox, Krueppel-related zinc finger protein; Col1a(I), collagen α1(I); DNase, deoxyribonuclease; DPI, diphenylene-iodonium; FP1, footprinting-1 containing sequences between −103 bp and −82 bp of the *Col1a(I)* promoter; FP2, footprinting-2 containing sequences between −130 bp and −112 bp of the *Col1a(I)* promoter; FP3, footprinting-3 containing sequences between −161 bp and −133 bp of the *Col1a(I)* promoter; FP4, footprinting-4 containing sequences between −190 bp and −170 bp of the *Col1a(I)* promoter; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HSC, hepatic stellate cell; Jak2, Janus kinase-2; JNK, Jun-N-terminal kinase; NADPH, nicotinamide adenine dinucleotide phosphate; NF1, nuclear factor 1; NOX2, NADPH oxidase 2; ObRb, long form of leptin receptor; PD098059, 2′-amino-3′-methoxyflavone; pHMB, parahydroxymercuribenzoate; PI3K, phosphatidylinositol-3-kinase; P5P, pyridoxal-5-phosphate; RAC1, Rho-related C3 botulinum toxin substrate; SB203580, 4-(4-fluorophenyl)-2-(4-methylsulfonylphenyl)-5-(-4-pyridyl)1H-imidazole; siRNA, small interfering RNA; Sp, specific protein; Stat3, signal transducer and activator of transcription protein-3; TBARS, thiobarbituric acid-reacting substances; Zf9, zinc finger transcription factor 9.

mechanisms and collagen gene elements by which leptin induces fibrosis are partially unknown. Leptin exerts its actions through signaling pathways upon its binding and activation of the long form of leptin receptor (ObRb), which in turn activates Janus kinase-2 (Jak2)/signal transducer and activator of transcription protein-3 (Stat3) pathway, translocates phosphorylated Stat3 into the nucleus and regulates activity of target gene promoter (9). The specific transcriptional factors required for leptin-induced activation of the collagen promoter and the specific sites on this promoter that are critical for this activation in humans remain to be elucidated. In fact, collagen α1(I) [*Col1a(I)*] promoter lacks Stat3 consensus binding sites. Some studies have suggested that oxidative stress may mediate these effects of leptin. Thus, in a number of cell types, leptin produces reactive oxygen species, likely with the contribution of the nonphagocytic adenine dinucleotide phosphate (NADPH) oxidase (10). In previous studies, we have shown that oxidative stress induces *Col1a(I)* gene expression by the mediation of aldehyde-protein adducts and transcription factors specific protein (Sp)-1 and Sp3 acting on *Col1a(I)* promoter (11, 12).

The aims of this study were the following: 1) to determine the effect of leptin on collagen production in primary culture of hepatic stellate cells (HSCs), 2) to recognize the *trans*-acting factors and *cis*-acting elements in *Col1a(I)* promoter involved in this effect, and 3) to identify the intracellular pathways used by leptin to induce *Col1a(I)* gene expression.

Materials and Methods

HSC isolation and culture

HSCs were isolated from adult male Sprague Dawley rats as described by Rippe *et al.* (13). All procedures were carried out in accordance with the Spanish Guidelines for the Care and Use of Laboratory Animals. The purity of HSC cultures was tested by flow cytometry at d 2 and 12. This study showed that at d 2 most isolated cells were reelin positive or glial fibrillary acidic protein positive, and only a minority of them were smooth muscle actin positive, fibulin-2 positive (14). Cells were cultured as described elsewhere (12). At d 12 of culture, 98% of cells were reelin and smooth muscle actin positive, and less than 2% were ectodermal dysplasia 1 and 2 positive, two macrophage markers (BioNova Científica SL, Madrid, Spain). After an activation period of 12–14 d in culture, the effect of leptin (recombinant rat leptin; MBL International Corp., Woburn, MA) was examined by the addition of this peptide to cells cultured in serum-free medium overnight.

Quantitative RT-PCR

Gene expression of *Col1a(I)*, *TGFβ1*, *Sp1*, NADPH oxidase components [$p47^{phox}$, $p22^{phox}$, NADPH oxidase 2 (NOX2), and Rho-related C3 botulinum toxin substrate (RAC1)], *ERK1*, and *ERK2* were measured by quantitative RT-PCR as described elsewhere (15). Sequences of primers used in these experiments are shown in Supplemental Table 1, published on The Endocrine Society's Journals Online web site at http://endo.endojournals.org.

Northern blots

On some occasions, *Col1a(I)* gene expression was also evaluated by Northern blot as previously described (14).

Reverse transcription-polymerase chain reaction

Gene expression of the long forms of leptin receptor (ObRb) was assessed by RT-PCR. cDNA synthesis and predenaturation were at 42 C for 60 min followed by 94 C for 10 min. PCR amplification sequence was performed for 35 cycles at 94 C for 30 sec, 57 C for 30 sec, and 72 C for 1 min. After amplification, each sample was applied to 1.2% agarose/ethidium bromide gel. The resolved PCR products were photographed under UV illumination.

Immunoprecipitation

The immunoprecipitation assays were performed as previously described (16). Cellular proteins were precipitated with polyclonal antibodies against Sp1 or Sp3 (Santa Cruz Biotechnology, Santa Cruz, CA). Immune complexes were recognized by Western blot using specific antibodies against Sp1/Sp3 and antiphosphoserine (Santa Cruz Biotechnology).

Western blot analysis

Cell culture serum-free media were collected and proteins were extracted from the media as described elsewhere (17). The filters containing proteins were incubated with appropriated polyclonal antibodies [Col1a(I), Sp1, Sp3, ERK1/2, phosphoserine, phosphor-Sp1, β-actin, glyceraldehyde-3-phosphate dehydrogenase (GAPDH)] (Santa Fe Biotechnology, Santa Fe, CA).

Plasmids

The p(−3700)ColCAT, p(−905)ColCAT, and p(−220)ColCAT (18) constructs contain the *Xba*I-*Xba*I (nucleotides −3700 to +116), the *Pvu*2-*Xba*I (nucleotides −905 to +116), or the *Bgl*II-*Xba*I (nucleotides −220 to +116) fragment, respectively, and the 5′ untranslated region of the first exon of the *Col1a(I)* gene fused to the bacterial chloramphenicol acetyltransferase (*CAT*) gene. The vector plasmid pUCCAT, also containing the *CAT* gene, was obtained by digesting p(−3700)ColCAT with *Xba*I, removing the *Xba*I-*Xba*I fragment and ligating both ends.

Transient transfection and chloramphenicol acetyl transferase (CAT) assay

In general, 5×10^6 HSCs were transiently transfected before confluence by the LipofectAMINE technique (Invitrogen, Carlsbad, CA) (17). Cell lysates were prepared, and CAT activity was determined as described elsewhere (19). Luciferase activity was determined using the enhanced luciferase assay kit according to the manufacturer's protocol (Analytical Luminiscence, San Diego, CA). Cell lysates were prepared in 125 μl of cell lysis buffer. Luciferase activity was determined using 50 μl aliquots, and protein concentrations determined with 5-μg aliquots using the Bradford protein assay (Bio-Rad Laboratories, Hercules, CA) (20). All reporter genes were normalized for transfection effi-

ciency by cotransfecting a constant amount of the *pRSVβ-gal* reporter gene and determining β-galactosidase activity (21).

Preparation of nuclear extracts and gel retardation assays

Extraction of nuclear proteins from untreated and treated HSCs, measurement of protein concentration, and gel retardation assays were performed as described elsewhere (17). The oligonucleotides used in these assays are shown in Supplemental Table 1. For competition experiments, 200-fold appropriated, unlabeled, annealed oligonucleotides were added to binding reactions. Supershift assays were performed as a standard mobility shift assay, except that 3 μl (200 μg per 0.1 ml) specific polyclonal antisera raised against Sp1, Sp3, nuclear factor 1 (NF1), activator protein 2 (AP2), Krueppel-related zinc finger protein (c-Krox), and Zinc finger transcription factor-9 (Zf9) was added to the binding reaction for 1 h at 4 C. Gel retardation assays were also performed using cells with silenced NOX2, RAC1, ERK1, or ERK2.

Determination of lipid peroxides in cells

Lipid peroxidation was determined by measuring thiobarbituric acid-reacting substances (TBARS) in cells as described previously (11). The TBARS were measured by spectrophotometry (absorbance, 532 nm) using tetramethoxypropane standards.

Measurement of cellular glutathione

We determined total cellular glutathione using the modification of Eady *et al.* (22) of Tietze's assay as described elsewhere (11).

Determination of aldehyde-protein adducts was done using the OxiSelect 4-hydroxynonenal adduct ELISA kit (Cell Biolabs, Inc., San Diego, CA) according to the manufacturer's protocol.

RNA interference

Silencing RNAs for NOX2, RAC1, $p47^{phox}$, ERK1, ERK2, and nonspecific control RNA used as negative control were purchased from Santa Cruz Biotechnology. For the transfection experiments, we followed the procedure described elsewhere (14).

Statistical analysis

All experiments were repeated at least three times, and RT-PCR and luciferase analysis as well as TBARS and glutathione determinations were done by quintuplicate. Histograms are expressed as mean ± SD. A Student's *t* test was used to evaluate the difference of means between groups, accepting that a $P < 0.05$ was considered to be significant.

Results

Leptin induces *Col1a(I)* gene expression in primary culture of HSCs

Because the long-form of the leptin receptor (ObRb) is essential for signal transduction, we determined whether the primary culture of HSCs we used in our experiments express then *ObRb* gene and how this expression progresses during the activation of these cells. As Fig. 1A shows, ObRb cDNA is present in trace quantities in resting

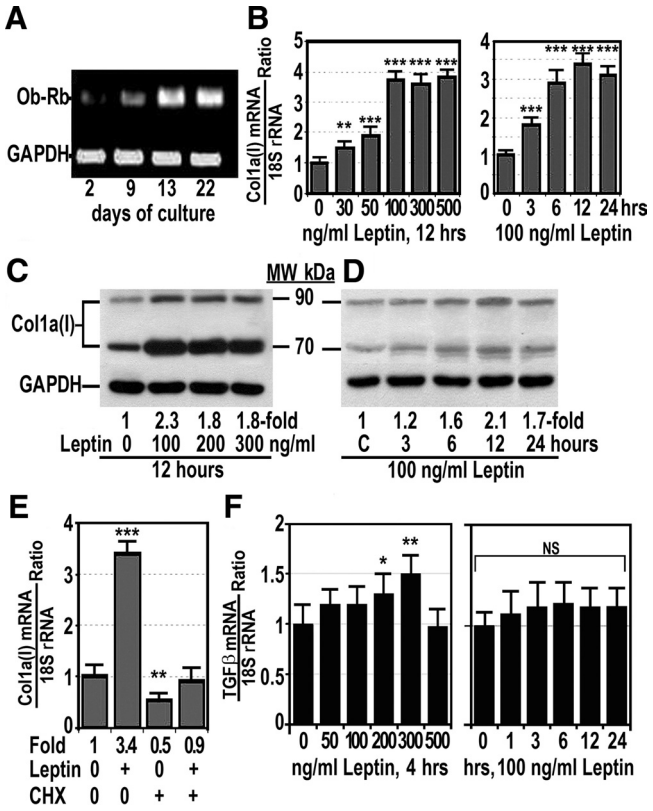

FIG. 1. Leptin increases collagen gene (B) and protein (C and D) expression in primary cultures of rat HSCs. A, RT-PCR showing that leptin receptor *ObRb* gene expression increases during activation of HSCs in primary cultures. B, Dose- and time-response effects of leptin on collagen α1(I) gene expression [Col1a(I) mRNA to 18S rRNA ratio] (quantitative RT-PCR). C and D, Dose- (C) and time-response (D) effects of increasing dosage of leptin on collagen α1(I) [Col1a(I)] protein production normalized to GAPDH content (Western blots). E, Pretreatment of cells with 0.1 mM cycloheximide (CHX) for 1 h abrogates the effect of leptin on *Col1a(I)* gene expression (RT-PCR). F, Time- and dose-response effects of leptin on *TGFβ1* gene expression (RT-PCR). Results of RT-PCR studies are representative of three separated experiments that were done by quintuplicate. Data are shown as mean ± SD. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

HSCs, but its amount increases markedly as the cells become active between d 9 and 22 of culture.

Treatment of cells with various concentrations of leptin led to a significant dose- and time-dependent increase in the level of Col1a(I) mRNA without change in the 18S rRNA. This effect was maximal by treating cells with 100 ng/ml leptin for 12 h (Fig. 1B). Similar results were obtained when Col1a(I) protein production was measured by Western blotting in culture media of leptin-treated HSCs (Fig. 1, C and D). The effect of leptin on collagen gene expression was prevented by blocking protein synthesis with 0.1 mM cycloheximide for 1 h before the addition of leptin (Fig. 1E). Because the effects of leptin on collagen gene expression have been suggested to be mediated by TGFβ1 (23), we determined the effect of leptin on the *TGFβ1* gene expression in primary culture of HSCs. As

shown in Fig. 1F, *TGFβ1* gene expression did not increase significantly in cells treated with 100 ng/ml leptin.

Treatment of HSCs with leptin activates *Col1a(I)* gene expression acting through an element located between −220 bp and −111 bp of the *Col1a(I)* promoter

Addition of 100 ng/ml leptin to confluent HSCs transiently transfected with a reporter CAT plasmid driven by the whole *Col1a(I)* promoter [p(−3700)ColCAT] resulted in a 2.9 ± 0.15-fold increase in the CAT activity at 12 h (Fig. 2A). This effect was maximal at the dose of 100 ng/ml leptin (Fig. 2B). Deletion of promoter sequences upstream of −220 bp relative to the transcription start site did not abrogate the stimulatory effect of leptin on *Col1a(I)* gene expression (Fig. 2C). By contrast, the effect of leptin did not occur in cells transfected with the construct p(−111)ColCAT, which lacks sequences upstream of −110 bp (Fig. 2D), suggesting that the leptin-responsive element is located in sequences between −220 and −111 bp relative to the transcription start site of the *Col1a(I)* promoter.

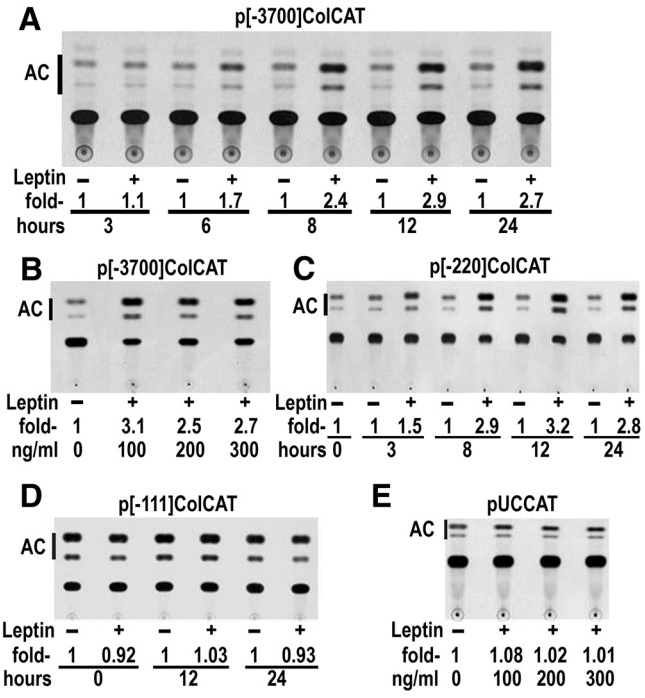

FIG. 2. Leptin stimulates *Col1a(I)* gene expression acting on elements located between −220 and −111 bp relative to the transcription start site. HSCs were transiently cotransfected by the LipofectAMINE technique (Invitrogen) with pRSVβ-gal plasmid and ColCAT constructs obtained by removing progressively more 5′-flanking sequences of the *Col1a(I)* promoter. Leptin (100 ng/ml) was added to confluent cells after 12 h of starvation, and this treatment was maintained for another 24 h. Cell lysates were assayed for CAT and β-galactosidase activities. Panels show the effect of leptin on the CAT activity of the lysate from cells transfected with p(−3700)ColCAT (A and B), p(−220)ColCAT (C), p(−111)ColCAT (D), or pUCCAT (E). Figures show representative results of three independent experiments. The CAT activity (normalized to β-galactosidase activity) in leptin-treated cells is expressed relative to the CAT activity in untreated cells. Leptin +, Experimental conditions in which leptin was added to the cells. Fold increase over the control levels. AC, Acetyl-[^{14}C]chloramphenicol.

Leptin induces the interaction of *trans*-acting factors Sp1 and Sp3 with footprinting-2 containing sequences (FP2) (−129 to −110 bp) and footprinting-3 containing sequences (FP3) (−161 to −133 bp) of the *Col1a(I)* promoter

Because the 220-bp upstream of the transcription start point in the *Col1a(I)* promoter contains four regions protected from deoxyribonuclease (DNase) I digestion by nuclear proteins from a number of cell types, including HSCs (13, 24, 25), we studied whether treatment of HSCs with 100 ng/ml leptin induced any change in the binding of nuclear proteins to these elements. Gel retardation experiments showed that incubation of a ^{32}P-radiolabeled oligonucleotide containing sequences between −103 bp and −82 bp [footprinting-1 containing sequences (FP1)] (CTGATTGGCTGGGGGCCGGGCT) of the *Col1a(I)* promoter with nuclear proteins extracted from cells untreated or treated with 100 ng/ml leptin for 6 and 12 h led to the formation of two major specific DNA-protein complexes (Fig. 3A). However, treatment of cells with leptin induced little changes in the pattern or intensity of the retarded bands. Thus, these results indicate that leptin does not influence the interaction of nuclear proteins with the FP1 sequence in HSCs.

To examine the effect of leptin on the interaction of nuclear proteins with the element extending from −130 bp to −112 bp (FP2) (CCAAATTGGGGGCCGGGCC) of the *Col1a(I)* promoter, we performed DNA mobility shift assays using as a probe a ^{32}P-radiolabeled oligonucleotide containing the sequence of FP2 region. As Fig. 3B shows, incubation of this probe with nuclear proteins extracted from HSCs also leads to the formation of two specific DNA-protein complexes. However, the intensity of these bands was clearly more pronounced when nuclear extracts were prepared from cells treated with 100 ng/ml leptin for 3–8 h (Fig. 3B). The formation of these complexes was specific because a 200-fold molar excess of the same unlabeled oligonucleotide inhibited these bindings in a competition assay (Fig. 3B, line Comp, lane FP2).

Because it has been shown that the *trans*-acting factor Sp1 interacts with this footprinted region in fibroblasts and HSCs (11, 12, 26), we performed supershift assays including a Sp1 specific polyclonal antibody in the binding reaction. This incubation led to the formation of a supershifted complex (Fig. 3B) that was associated with a decrease in the intensity of the upper band. In an attempt to identify other nuclear factors bound to FP2, supershift

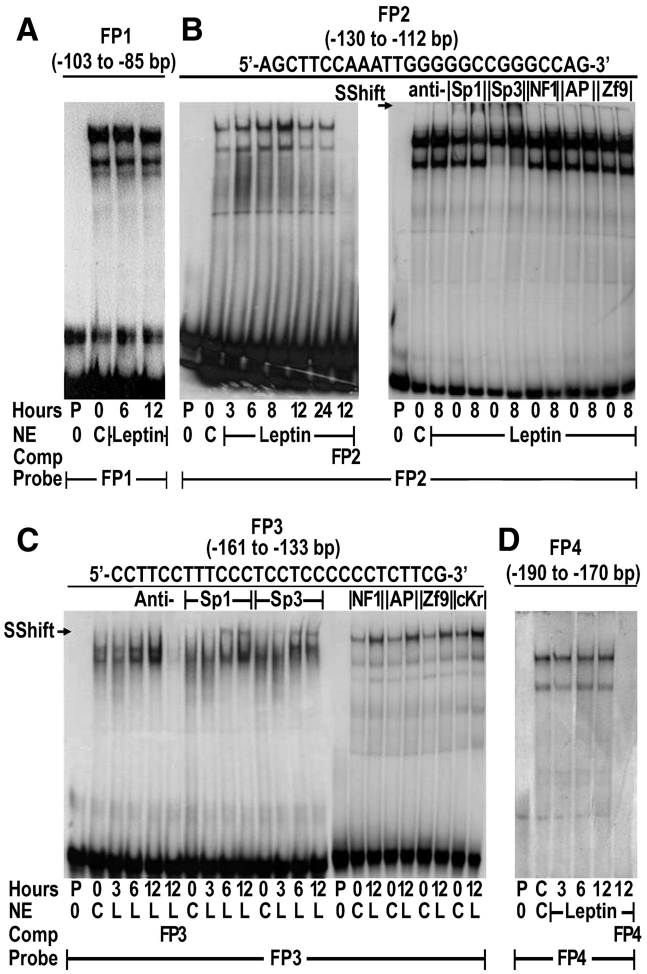

FIG. 3. Leptin increases the binding of Sp1 and Sp3 transcription factors to the *Col1a(I)* gene promoter. DNA binding was analyzed by gel retardation assays as described in *Materials and Methods*. Double-stranded oligonucleotides corresponding to the FP1 (−103 to −82 bp) (A), FP2 (−130 to −112 bp) (B), FP3 (−161 to 133 bp) (C), or FP4 (−190 to −170 bp) (D) of the *Col1a(I)* promoter were radiolabeled with ^{32}P and incubated with 5 μg nuclear protein extracts from confluent cells untreated or treated with 100 ng/ml leptin for the indicated time. Supershift assays were performed including in the binding reaction 3 μl of specific antibodies against Sp1, Sp3, NF1, Zf9, AP2 (AP), or c-Krox (cKr) proteins. C, Control untreated cells; L, leptin-treated cells. NE, nuclear extract; Comp, competitor unlabeled oligonucleotides (lanes FP2, FP3, and FP4 in panels B, C, and D, respectively); SShift, supershifted proteins.

assays were also performed using polyclonal antisera against NF1, a CCAAT binding protein, Sp3, Zf9, and AP2, three GC-rich binding proteins. Although NF1, Zf9, and AP2 antibodies failed to supershift any band, Sp3 antiserum supershifted the lower band almost completely (Fig. 3B). The role played by FP2 in the leptin-induced up-regulation of Col1a(I) was confirmed in HSCs transfected with a ColCAT plasmid containing mutations at FP2. In these cells, leptin did not activated *Col1a(I)* gene expression (Supplemental Fig. 1).

Gel retardation experiments were also performed to analyze the effect of leptin on the interaction of nuclear proteins with the third DNase I-protected region. Incubation of nuclear extracts from HSCs with a ^{32}P-radiolabeled oligonucleotide containing the sequence spanning nucleotide −161 to −133 (FP3) (CCCTTC-CTTTCCCTCCTCCCCCCTCTTCG) led to the formation of two major DNA-protein complexes, the intensity of which increased markedly with nuclear proteins extracted from cells treated with 100 ng/ml leptin for 3–12 h (Fig. 3C). Competition with a 200-fold molar excess of unlabeled FP3 oligonucleotide demonstrated the specificity of these complexes (Fig. 3C, line Comp, lane FP3). A supershift assay using Sp1 antibody resulted in the formation of a supershifted band and in a decrease in the intensity of the upper band (Fig. 3C). Likewise, the Sp3 antibody led to the complete disappearance of the lower complex and the formation of a supershifted band. The inclusion of antisera against NF-1, AP2, Zf9, or c-Krox transcription factors in the binding reaction failed to supershift any band (Fig. 3C).

Finally, gel retardation experiments using a ^{32}P-radiolabeled oligonucleotide containing sequence spanning nucleotide −190 to −170 [footprinting-4 containing sequences (FP4)] (GGGAGGGGGGGCGCTGGGTGG) of the *Col1a(I)* promoter as a labeled probe also demonstrated two major complexes formed with nuclear extracts from HSCs. However, the pattern and the intensity of these complexes were not modified by the treatment of cells with 100 ng/ml leptin for 3–12 h (Fig. 3D).

Leptin increases Sp1 gene expression and Sp1 phosphorylation in primary culture of HSCs

Because the preceding results indicated that leptin induced the binding of Sp1 and Sp3 to the *Col1a(I)* gene promoter, we wanted to know whether this binding is involved in the mediation of the effect of leptin on *Col1a(I)* gene expression. Therefore, we pretreated cells for 1 h with 0.1 μM mithramycin, a drug that interferes with the binding of Sp proteins to DNA (27) (Fig. 4A) and determined its effects on leptin-induced *Col1a(I)* gene expression. As Fig. 4, B and C, shows, this pretreatment reduced the basal expression of *Col1a(I)* gene and abrogated the effect of leptin on this gene.

To corroborate that the stimulatory effect of leptin was mediated by the Sp1, we determined whether leptin also increases expression of other genes containing Sp1 binding sites. Therefore, we transiently transfected HSCs using either pΔSLuc or p(−126)Luc reporter plasmids. Whereas the addition of 100 ng/ml leptin to HSCs transfected with pΔSLuc, a deletion construct lacking Sp1 binding site, did not change significantly the expression of this gene, the addition of the same amount of leptin to HSCs transfected with p(−126)Luc, a plasmid containing two consensus

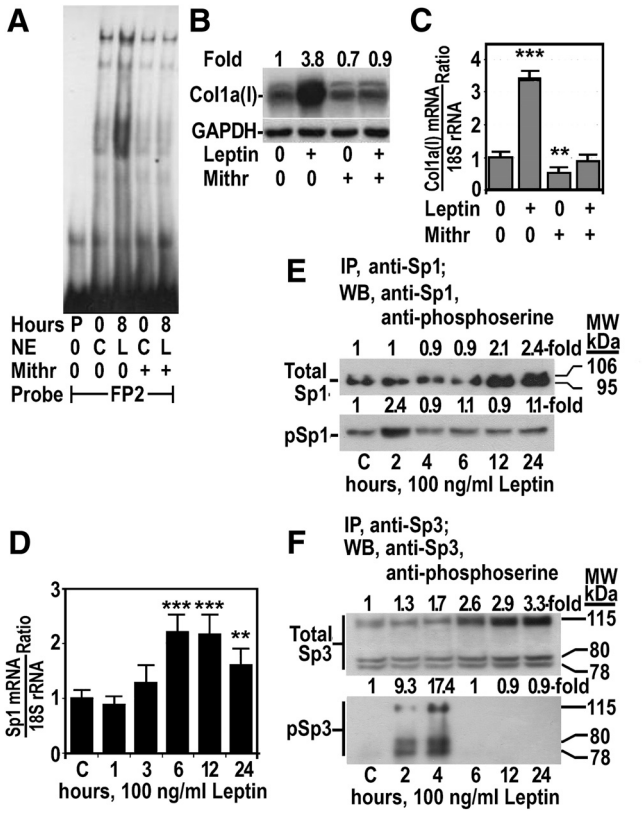

FIG. 4. Binding of Sp1 to FP2 mediates the leptin-induced increase in *Col1a(I)* gene expression. A, DNA binding was analyzed by gel retardation assays using a ^{32}P-radiolabeled FP2 oligonucleotide as a probe. The HSCs were pretreated with 0.1 μM mithramycin (Mithr) for 60 min and incubated for 8 h with (L) or without (C) 100 ng/ml leptin. NE, Nuclear extract. B and C, Effects of 100 ng/ml leptin on *Col1a(I)* gene expression in the absence or presence of 0.1 μM mithramycin (Mithr). Gene expression was evaluated by Northern blotting (B) using GAPDH mRNA as a control for sample loading or by RT-PCR (C) measuring the Col1a(I) mRNA to 18S rRNA ratio. Blots are representative of three separate experiments. D, Time-response effects of 100 ng/ml leptin on *Sp1* gene expression. RT-PCR experiments were done by quintuplicate. Data are shown as mean ± SD. **, $P < 0.01$; ***, $P < 0.001$. E and F, Effects of leptin on Sp1, phospho-Sp1 (pSp1) (E), Sp3, and phospho-Sp3 (pSp3) protein expression (F) in HSC lysates. Sp1 or Sp3 were immunoprecipitated as described in *Materials and Methods* and total Sp1, total Sp3, phospho-Sp1 (pSp1), and phospho-Sp3 (pSp3) were analyzed by Western blotting using, respectively, anti-Sp1, anti-Sp3, or antiphosphoserine. These results are representative of three independent experiments. IP, Immunoprecipitation. WB, Western blot.

Sp1 binding elements, resulted in a 4.2-fold increase in gene expression at 8 h (Supplemental Fig. 2).

Measurement of *Sp1* gene expression by quantitative RT-PCR and Sp1 protein content in immunoprecipitated proteins from cells treated with 100 ng/ml leptin for 1–24 h showed that leptin increased significantly *Sp1* gene expression between 6 and 12 h of treatment (Fig. 4D) and cellular Sp1 protein after 12 h (Fig. 4E). Likewise, leptin increased immunoprecipitated Sp3 after 6 h of treatment (Fig. 4F). Because phosphorylation of Sp proteins plays an important role in the regulation of target genes (28), we determined the effect of leptin on serine phosphorylation of Sp1 and Sp3. As Fig. 4 shows, leptin transiently increased phospho-Sp1 and phospho-Sp3 2.4- and 17-fold above the baseline after 2 and 4 h, respectively, of treatment.

In an attempt to determine the kinase involved in Sp1 phosphorylation, Sp1 was immunoprecipitated from HSCs untreated or treated with 100 ng/ml leptin in the presence or absence of 50 μM AG490, an inhibitor of JAK2; 50 μM SP600125, a Jun-N terminal kinase (JNK) inhibitor; 0.1 μM wortmannin, a phosphatidylinositol-3-kinase (PI3K) inhibitor; 1 μM SB203580, an inhibitor of p38-MAPK; or 25 μM PD098059, an ERK1/2 MAPK inhibitor. Likewise, we inhibited NADPH oxidase with 10 μM of diphenylene-iodonium (DPI), the binding of Sp1 to the DNA with 0.1 μM mithramyin, and reduced hydrogen peroxide with 1000 U/ml catalase. All these inhibitors were added to the cells 1 h before adding leptin. As Fig. 5A shows, leptin-induced Sp1 phosphorylation was suppressed by inhibiting JAK2, PI3K, ERK1/2, NADPH oxidase or by treating cells with catalase but not by blocking p38 MAPK, JNK, or the binding of Sp1. Likewise, inhibition of the same enzymes, including p38 MAPK and the Sp binding, prevented the leptin-induced *Col1a(I)* gene expression (Fig. 5B). The role played by ERK1/2 in mediating the effects of leptin on *Col1a(I)* gene expression was also confirmed by silencing theses kinases with specific small interfering RNAs (Fig. 5B). Western blot using antiphosphorylated ERK1/2 demonstrated that leptin increased markedly ERK1/2 phosphorylation and that this effect was abolished, not only by their specific inhibitor, PD098059, but also by inhibiting JAK2, PI3K, or NADPH oxidase or by pretreating cells with catalase, suggesting that these enzymes and hydrogen peroxide are located upstream to ERK1/2 in the leptin signaling pathway (Fig. 5C).

Collagen gene expression induced by leptin is mediated by oxidative stress

Treatment of HSCs with 100 ng/ml leptin for 1–12 h led to a marked increase in the cellular levels of TBARS (Fig. 6A), to a progressive decrease in the cellular content in reduced glutathione (Fig. 6B), two indexes of oxidative stress, and to a significant increase in the aldehyde-protein adduct formation, a consequence of the oxidative stress (29) (Fig. 6C). This leptin-induced adduct formation was avoided by treating cells not only with 100 μM pyridoxal-5-phosphate (P5P), 4 μM parahydroxymercuribenzoate (pHMB), or 10 μM methylene blue, three inhibitors of aldehyde-protein adduct formation, but also with 1000 U/ml catalase, an enzyme that degrades hydrogen peroxide, and by inhibiting PI3K with 0.1 μM wortmannin or

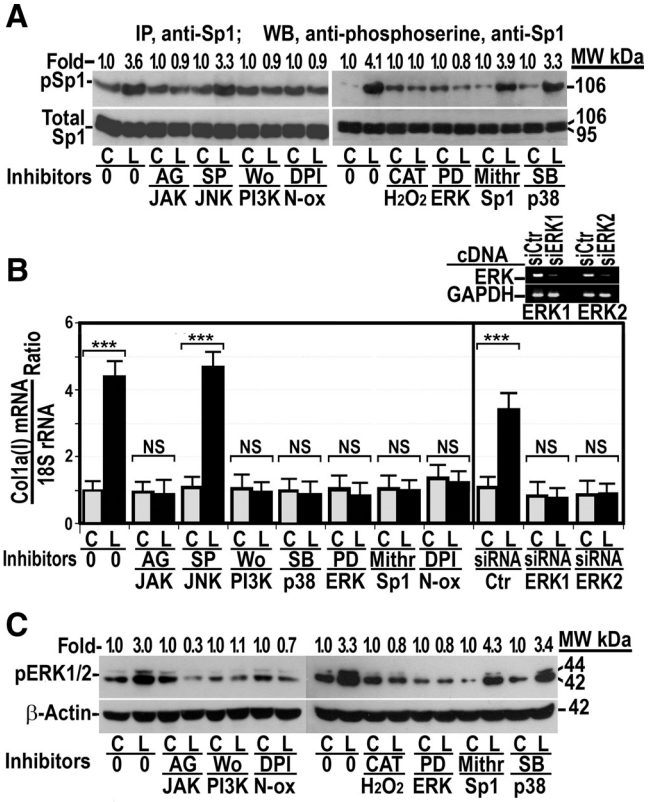

FIG. 5. Leptin-induced Sp1 phosphorylation is mediated by JAK2, PI3K, ERK1/2, and NADPH oxidase. A, HSCs were exposed to 100 ng/ml leptin for 120 min in the presence or absence of 1000 U/ml CAT or inhibitors of JAK2 (50 μM AG490) (AG), JNK (50 μM SP600125) (SP), PI3K (0.1 μM wortmannin) (Wo), NADPH oxidase (N-ox) (10 μM DPI, p38 MAPK (1 μM SB203580) (SB), ERK1/2 (25 μM PD098059) (PD), or 0.1 μM mithramycin (Mithr). Inhibitors were added to the cells 60 min before leptin. Cellular proteins (300 μg) were immunoprecipitated using anti-Sp1 antibody and blotted for phosphoserine or Sp1 to evaluate equal loading. B, HSCs were exposed to 100 ng/ml leptin for 12 h in the presence or absence of catalase or inhibitors of JAK2 (AG), JNK (SP), PI3K (Wo), p38 (SB), ERK1/2 (PD), Sp1 (Mithr), or NADPH oxidase (N-ox) (DPI) as previously indicated. ERK1 and ERK2 RNAs were silenced using appropriated siRNAs as described under *Materials and Methods*. The [Col1a(I)]mRNA to 18S rRNA ratio was measured by RT-PCR. These results are representative of three separated experiments that were done by quintuplicate. ***, $P < 0.001$. NS, Not significant. This panel also shows RT-PCR-amplified cDNA fragments corresponding to ERK1, ERK2, and GAPDH. Expression of these genes was decreased by about 80% in cells with silenced genes. C, Effects of 100 ng/ml leptin for 120 min on ERK1/2 phosphorylation in the presence or absence of 1000 U/ml CAT or inhibitors of JAK2, PI3K, NADPH oxidase (N-ox), ERK1/2, Sp1, or p38 MAPK. Western blot was done using anti-ERK1/2 antibody and anti-β-actin as loading control.

NADPH oxidase with 10 μM DPI or 100 μM apocynin. On the contrary, avoiding Sp binding with mithramycin, or suppressing ERK1/2 or p38 MAPK by pretreating cells with PD098059 or SB203580 did not decrease leptin-induced adduct formation (Fig. 6D).

The oxidative stress mediated the effects of leptin on *Col1a(I)* gene expression as indicated by the fact that these effects were prevented by preincubating cells for 60 min with three different antioxidants, namely, 2 mM glutathione ethyl ester, a cell-permeable glutathione derivative; 1 mM melatonin, a well-known antioxidant; or 150 μM 5,10,15,20-tetrakis (4-benzoic acid) porphyrin, an analog of the superoxide dismutase (Fig. 6E). This effect seems to be mediated by the formation of hydrogen peroxide and aldehyde-protein adducts because pretreated cells with 1000 U/ml catalase, 100 μM P5P, 4 μM pHMB, or 10 μM methylene blue clearly abrogated the stimulatory effect of leptin on *Col1a(I)* gene expression (Fig. 6E). Finally, inhibition of NADPH oxidase with either 10 μM DPI or 100 μM apocynine also blocked the stimulatory effect of leptin on *Col1a(I)* gene expression (Fig. 6E), indicating that this enzyme is also implicated in the stimulatory effects of leptin on *Col1a(I)* gene expression.

To determine whether aldehyde-protein adduct formation are involved in the leptin-induced ERK1/2 phosphorylation, and consequently in Sp1 activation, we inhibited adduct formation by pretreating HSCs with 100 μM P5P or 4 μM pHMB 60 min prior to the addition of 100 ng/ml leptin. As shown in Fig. 6F, these inhibitors, as well as glutathione and apocynin, prevented the leptin-induced phosphorylation of ERK1/2.

To confirm that NADPH oxidase mediated the effects of leptin on *Col1a(I)* gene expression, we first measured the effects of 100 ng/ml leptin on NADPH oxidase gene expression. As Fig. 7A shows, leptin induced gene expression of several components of the NADPH oxidase, particularly of NOX2 and RAC1. The effects of leptin on $p22^{phox}$ and $p47^{phox}$ gene expression were less pronounced and appeared later, at 12 h. The critical role of NADPH oxidase on *Col1a(I)* gene expression was corroborated by showing that silencing NOX2, RAC1, or $p47^{phox}$, three components of the NADPH oxidase complex, with appropriated small interfering RNAs (siRNAs) suppressed completely leptin-induced *Col1a(I)* gene expression (Fig. 7B). Moreover, silencing NOX2 or RAC1 also resulted in an absence of Sp1, Sp3, and ERK1/2 phosphorylation by leptin (Fig. 7C) and prevented the effects of this hormone on the binding of nuclear proteins to FP2 (Fig. 7D) and FP3 (Fig. 7E) ^{32}P-radiolabeled oligonucleotides. Likewise, silencing ERK1 or ERK2 had similar effects on the binding of these proteins to FP2 and FP3 (Fig. 7, D and E).

Discussion

There is some controversy concerning the expression of OB-Rb leptin receptor in HSCs. Although some authors were not able to detect the long form of leptin receptors in HSCs (5, 23), others have demonstrated that the expression of this receptor increase progressively as the cells be-

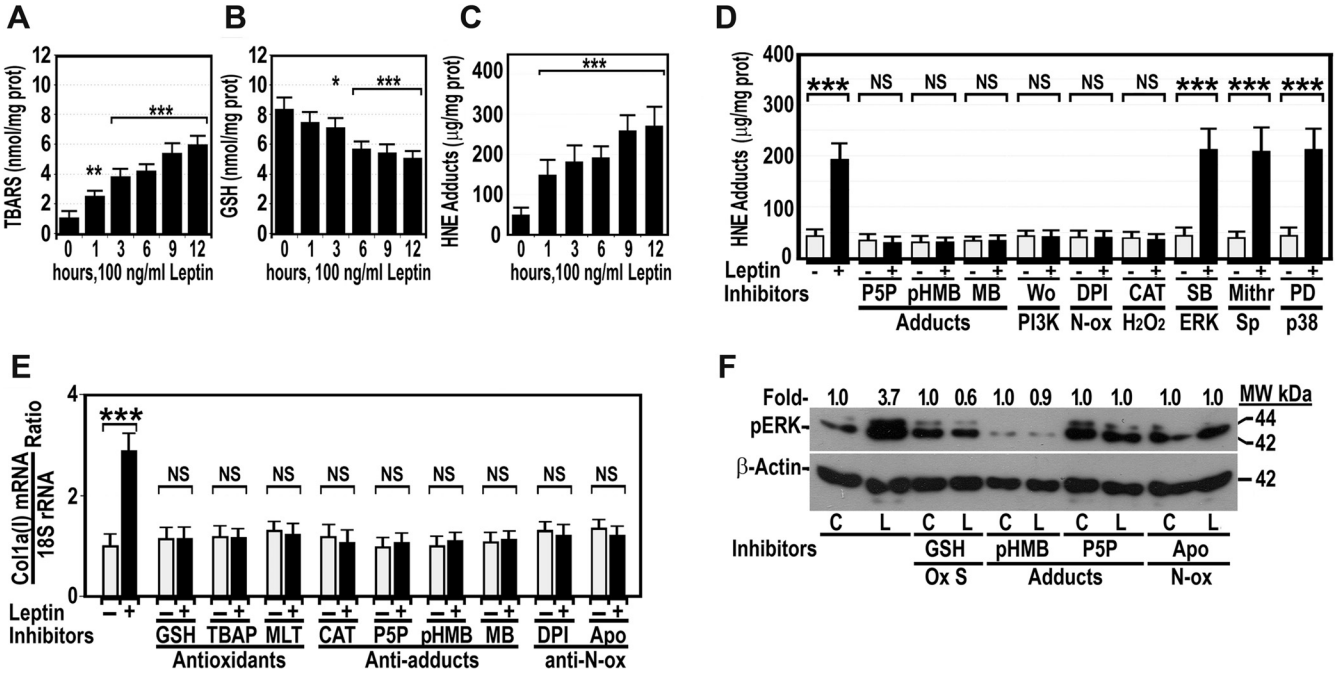

FIG. 6. Leptin produces oxidative stress and stimulates *Col1a(I)* gene expression by activating NADPH oxidase and through the formation of aldehyde-protein adducts. A–C, Effects of 100 ng/ml leptin on the content in TBARS (A), reduced glutathione (B), and aldehyde-protein adducts (C) in cultured HSCs. TBARS, reduced glutathione, and adducts were measured as indicated under *Materials and Methods*. These results are representative of three separate experiments that were done in quintuplicate. Data are shown as mean ± SD. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. D, Effects of 100 μM P5P, 4 μM pHMB, 10 μM methylene blue (MB), 0.1 μM wortmannin (Wo), 10 μM DPI, 1000 U/ml CAT, 1 μM SB203580 (SB), 0.1 μM mithramycin (Mithr), or 25 μM PD098059 on the cellular content in aldehyde-protein adducts in the absence (−) or presence (+) of 100 ng/ml leptin for 6 h. Inhibitors were added to the cells 60 min before leptin. These results are representative of two separate experiments that were done in triplicate. Data are shown as mean ± SD. ***, $P < 0.001$. NS, Not significant. E, Effects of 100 ng/ml leptin on the *Col1a(I)* gene expression [Col1a(I) mRNA to 18S rRNA ratio (RT-PCR)] in the presence or absence of antioxidants [2 mM glutathione ethyl ester (GSH); 5,10,15,20-tetrakis (4-benzoic acid) porphyrin (TBAP); melatonin (MLT), CAT], blockers of aldehyde-protein adducts formation [P5P; pHMB; methylene bleu (MB)], and inhibitors of the NADPH oxidase [DPI; apocynin (Apo)]. Inhibitors were added to the cells as indicated in panel D. ***, $P < 0.001$. NS, Not significant. F, Effects of glutathione ethyl ester (GSH), inhibitors of aldehyde-protein adduct formation (pHMB; P5P), or blockers of the NADPH oxidase (N-ox) (Apo) on the leptin-induced ERK1/2 phosphorylation (pERK). Ox S, Oxidative stress (Western blot using antibody against phosphorylated ERK or β-actin as loading control).

come activated (4). We confirm latter results and show that, at the time the experiments were performed, OB-Rb receptors were fully expressed in HSCs (Fig. 1A).

In the present study, we show that leptin stimulates *Col1a(I)* gene expression and collagen I protein production in primary culture of HSCs (Fig. 1, B–D), reaching its maximal effect with 100 ng/ml at 12 h. These results concur with those reported by others, who also found that leptin increased transcriptional activation of *Col1a(I)* gene in HSCs (4, 7, 8, 30).

Mechanisms of these effects are not completely understood. However, the increase in procolagen mRNA levels induced by leptin was blocked by inhibiting protein synthesis (Fig. 1E), indicating that the *de novo* synthesis of a protein may be required for this effect. Some authors have suggested that leptin up-regulates collagen synthesis by increasing TGFβ1 production from Kupffer and other cells, including HSCs (5, 23). Our study performed in a HSC population 98% pure (14), with less than 2% positive for Kupffer cell markers, shows that leptin at the dose used in our experiments (100 ng/ml) did not increase significantly *TGFβ1* gene expression (Fig. 1F). These results concur with those reported by others (4, 7), who also showed that leptin did not change significantly TGFβ1 mRNA in HSC-T6 cells and primary cultures of HSCs. Moreover, Saxena *et al.* (7) found that anti-TGFβ1 antibody failed to prevent the effects of leptin on collagen-α2(I) gene and Cao *et al.* (8) have shown that leptin up-regulated the *Col1a(I)* gene expression in LX-2 human HSCs lacking Kupffer cell contamination. All these studies indicate that the effects of leptin on collagen gene expression are direct and, in primary cultures of HSCs, are not mediated by TGFβ1. Nevertheless, there are evidences showing that leptin and TGFβ1 share some intracellular signaling pathways and that a cross talk between signaling of both factors does exist (31–33).

The regulatory elements involved in the leptin-induced activation of *Col1a(I)* gene expression are unknown. To our knowledge, no study has been published approaching this issue. Extensive studies of the *Col1a(I)* gene promoter

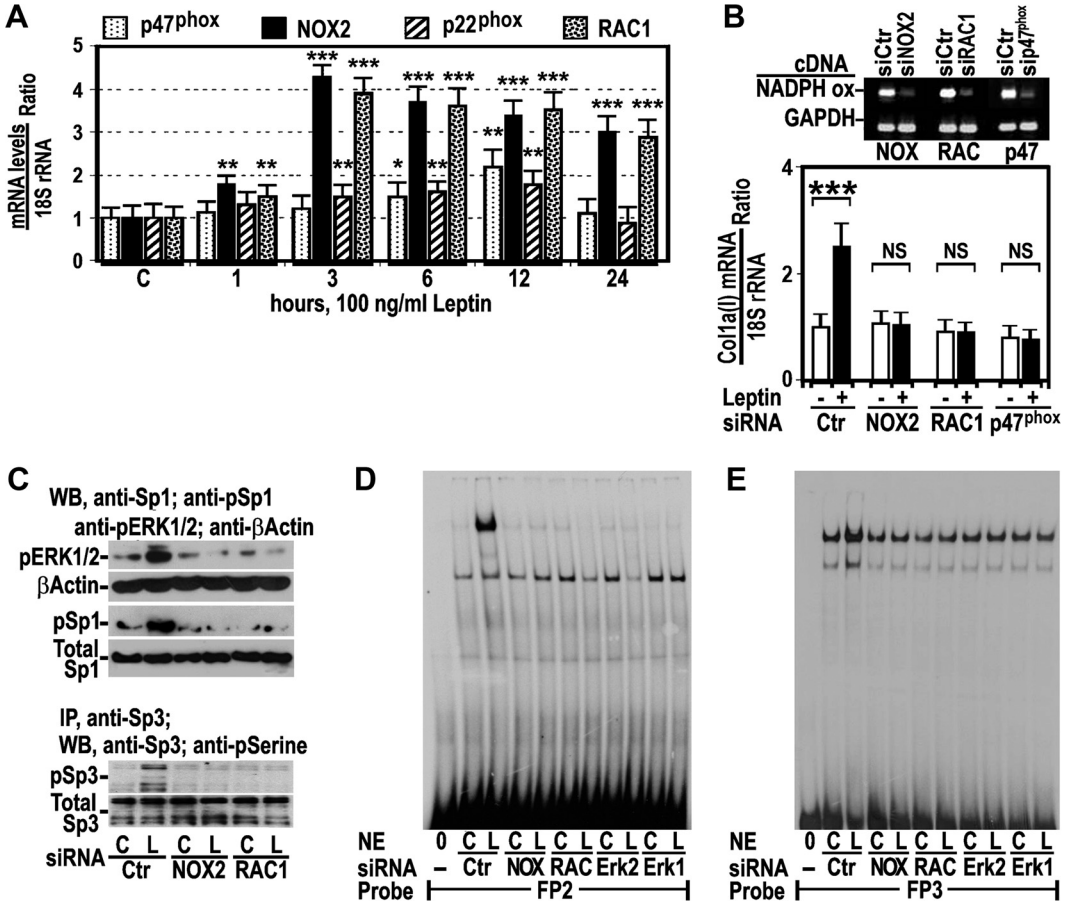

FIG. 7. NADPH oxidase mediates the effects of leptin on *Col1a(I)* gene expression, ERK1/2, and Sp1/Sp3 phosphorylation and the binding of nuclear proteins to *Col1a(I)* promoter. A, Leptin up-regulates gene expression of several components (p47^{phox}; p22^{phox}; NOX2; RAC1) of the NADPH oxidase [mRNA levels to 18S rRNA ratio (RT-PCR)]. a, $P < 0.05$; b, $P < 0.01$; c, $P < 0.001$. All experiments were done in quintuplicate. Data are shown as mean ± SD. B, Silencing NOX2, RAC1, or p47^{phox}, three components of the NADPH oxidase complex (NADPH ox), with appropriated siRNAs, blocked the up-regulating effects of leptin on *Col1a(I)* gene expression [Col1a(I) mRNA to 18S rRNA ratio (RT-PCR)]. The panel also shows RT-PCR-amplified cDNA fragments corresponding to NOX1, RAC2, p47phox, and GAPDH. Expression of these genes was decreased by about 80% in cells with silenced genes. C, Effects of leptin on ERK1/2, Sp1, and Sp3 serine phosphorylation in control cells (Ctr) and cells with silenced NOX2 or RAC1. Phospho-ERK1/2 (pERK1/2) and phospho-Sp1 (pSp1) were measured after 2 h of treatment with 100 ng/ml leptin. Phospho-Sp3 (pSp3) was measured after 4 h of treatment with leptin. Total Sp1, phospho-Sp1, and phospho-ERK1/2 (pERK1/2) were analyzed by Western blotting using specific anti-Sp1, antiphosphorylated Sp1, anti-pERK1/2 antibody, and β-actin. Phospho-Sp3 was analyzed after immunoprecipitating cellular proteins with anti-Sp3 antibody and Western blotting proteins with anti-Sp3 and antiphosphoserine (p-Serine). IP, Immunoprecipitation. WB, Western blot. D and E, Gel retardation assays showing the effects of silencing NOX2, RAC1, ERK1, or ERK2 on the leptin-induced binding of nuclear proteins to FP2 (D) or FP3 (E). The binding of nuclear proteins to FP2 or FP3 was analyzed after 8 or 12 h, respectively, of treatment of cells with 100 ng/ml leptin. NE, Nuclear proteins; C, control, untreated cells; L, cells treated with leptin; siRNA Ctr, nonspecific control RNA.

have identified several *cis*-acting regulatory elements. Our study indicates that the leptin-induced activation of the *Col1a(I)* gene must be mediated through elements located between nucleotide −220 and −111 of its promoter (Fig. 2). The segment of the *Col1a(I)* promoter located between −220 and −82 bp contains four regions protected from DNase I digestion by nuclear proteins (13, 24–26, 34) named, following that of Brenner and colleagues (26, 35), FP1 through FP4. DNA mobility shift assays using as probe oligonucleotides containing sequences corresponding to these four protected regions showed that treatment of cells with leptin increased the binding of nuclear proteins to oligonucleotides containing FP2 or FP3 sequences but not to FP1 or FP4 oligonucleotides (Fig. 3). These results suggest that leptin increases *Col1a(I)* gene expression acting on these two *cis* regulatory elements.

The FP2 sequence contains a reverse CCAAT box motif and a 12-bp GC-rich repeat (24, 26), to which bind, at least in NIH 3T3 fibroblasts, NF1 and Sp1, respectively (26). As in previous studies (11, 12), we show that Sp1 is involved in the formation of the upper DNA-protein complex observed after incubating nuclear proteins from HSCs with FP2 oligonucleotide and that leptin increases the formation of this complex (Fig. 3B). On the contrary, we were not able to demonstrate the presence of NF1 in any of the two DNA-protein complexes formed after in-

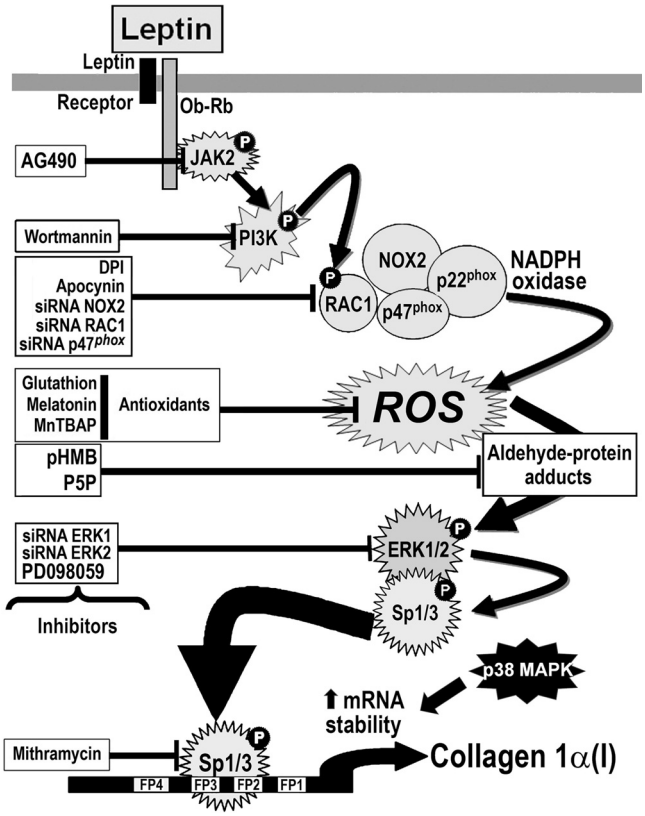

FIG. 8. Schematic diagram for leptin-induced collagen α1(I) gene expression in hepatic stellate cells. Leptin binds to the long form of the leptin receptor (ObRb) leading to JAK2 phosphorylation. Activated JAK2, mediated by PI3K, phosphorylates RAC1 and consequently activates the nonphagocytic NADPH oxidase complex, which in turn increases the production of reactive oxygen species (ROS). In addition, leptin increases gene expression of several components of this oxidase complex. Oxidative stress caused by the NADPH oxidase activity results in the formation of aldehyde-protein adducts, which induce ERK1/2 phosphorylation. These kinases phosphorylate Sp1/Sp3 and determines their nuclear translocation and their binding to the collagen α1(I) promoter.

cubating nuclear proteins with FP2 oligonucleotide. Likewise, supershift assays using specific antibody against AP2 or Zf9, two GC-rich binding proteins, did not demonstrate the interaction of these nuclear factors with FP2 (Fig. 3B). On the contrary, supershift assay using Sp3 antibody demonstrated that this nuclear factor is implicated in the formation of the lower complex (Fig 3B). The leptin-induced interaction of Sp proteins with FP2 has a functional role on the increased expression of *Col1a(I)* gene as indicated by the absence of the leptin effect on cells transfected with ColCAT plasmids containing mutations at FP2 (Supplemental Fig. 1) or by treating cells with mithramycin (Figs. 4, B and C), a drug that interferes with the binding of Sp proteins to DNA (Fig. 4A) (27).

The FP3 is a pyrimidine-rich region that contains TCCCCC and TCCTCC motifs between nucleotide −161 and −149 of the noncoding strand of the *Col1a(I)* promoter. In fibroblasts, these motifs have been shown to be binding sites for transcription factors c-Krox (36, 37), Sp1, and Sp3 (38). In the present study, we were not able to demonstrate that leptin increases the interaction of c-Krox with FP3. However, as Fig. 3C shows, leptin increased clearly the binding of Sp1 and Sp3 to this region of the *Col1a(I)* promoter.

Leptin may intensify the binding of Sp proteins to *Col1a(I)* gene promoter either by increasing the amount of Sp proteins or by inducing their phosphorylation (28). In the present study, we show that leptin increases Sp1 and Sp3 protein expression (Fig. 4, E and F), but these effects occur later, after having induced the binding of Sp1 to the *Col1a(I)* promoter and the expression of *Col1a(I)* gene (Figs. 1, B and D, and 3, B and C). However, leptin-induced serine phosphorylation of Sp1 and Sp3 occurred as early as 2 and 4 h, respectively, which may be responsible for the enhanced binding to FP2 and FP3 and for the activation of *Col1a(I)* gene expression observed in leptin-treated cells. Although both transcription factors, Sp1 and Sp3, recognize the same GC-rich repeats (39), they do not act competitively on *Col1a(I)* gene as it has been clearly demonstrated by a number of authors (40–42), likely because Sp1 forms a complex with Sp3 and both factors work cooperatively in up-regulating *Col1a(I)* gene (40). Moreover, although Sp1 is phosphorylated at h 2, Sp3 is activated later mainly at h 4 (Fig. 4, E and F). The involvement of phosphorylated Sp1 in the activation of *Col1a(I)* promoter is not specific for leptin because we have previously shown that malondialdehyde and iron-containing solutions increase *Col1a(I)* gene expression using a similar mechanism of action (11, 12).

Although Sp1 phosphorylation seems to be necessary to stimulate *Col1a(I)* promoter, the specific pathways used by leptin for activating Sp1/Sp3 and consequently for increasing *Col1a(I)* gene expression are unknown. Our study shows that leptin-induced Sp1 phosphorylation was prevented by inhibiting JAK2, PI3K, nonphagocytic NADPH oxidase, and ERK1/2, suggesting that these enzymes are implicated in the activation of this transcriptional factor (Fig. 5A). Moreover, inhibiting these enzymes also prevented the up-regulating effects of leptin on *Col1a(I)* gene expression. Our study also shows that inhibition of p38-MAPK prevents leptin-induced up-regulation of *Col1a(I)* gene expression without suppressing ERK1/2 and Sp1 phosphorylation (Fig. 5). This effect may be attributed to the fact that p38-MAPK acts posttranscriptionally by increasing Col1a(I) mRNA stability (8).

In the present study, we also show that leptin induces oxidative stress as indicated by the significant increase in TBARS concentration, by the decrease in reduced glutathione in HSCs exposed to leptin, and by the increased formation of aldehyde-protein adducts (Fig. 6, A–C). The

latter effect of leptin is mediated by H_2O_2, PI3K, and NADPH oxidase because pretreating cells with catalase or with inhibitors of these two enzymes prevented adduct formation (Fig. 6D). The leptin-induced oxidative stress plays a role in the increased expression of *Col1a(I)* gene because the use of four different antioxidants abrogated this effect of leptin (Fig. 6E). In previous studies, we have already demonstrated that oxidative stress stimulates *Col1a(I)* gene expression, like leptin, by inducing the binding of Sp1/Sp3 to elements located between −161 and −110 bp of the *Col1a(I)* promoter (11, 12). In these and other studies (29), we presented evidence supporting the role played by the aldehyde-protein adducts formed as a consequence of lipid peroxidation in the stimulation of *Col1a(I)* gene expression, seeing that oxidative stress increases adduct formation (29) and P5P, pHMB, and methylene blue, agents known to inhibit aldehyde-protein adducts formation (43), abolished the stimulatory effect of iron salts and malondialdehyde on Col1a(I) mRNA levels (11, 12). In the present study, we show that leptin shares the same final mechanism to stimulate *Col1a(I)* gene expression because blocking aldehyde-protein adduct formations also results in the abrogation of these effects (Fig. 6E).

The leptin-induced oxidative stress has been ascribed to the nonphagocytic NADPH oxidase (10), which in turn would activate ERK1/2. In the present study, we provide evidence supporting this mechanism of action because inhibition of this oxidase with DPI or apocynin or by silencing gene expression of its components abolished ERK1/2 (Figs. 5C, 6F, and 7C) and Sp1 (Figs. 5A and 7C) phosphorylation and *Col1a(I)* gene expression (Figs. 5B, 6E, and 7B). Moreover, our study shows that leptin increases gene expression of some components of NADPH oxidase, particularly the expression of NOX2, a membrane-bound component of the catalytic unit of this complex, and the cytosolic RAC1 (Fig. 7A), which activity is crucial for NADPH oxidase function in HSCs (44). These effects of leptin occur early; therefore, the blocking effects exerted by cycloheximide on the effects of leptin (Fig. 1E) may be explained by blocking the synthesis of these components of the NADPH oxidase. ERK1/2-dependent activation of Sp1 that we have observed in the present study might be a consequence of the oxidative stress provoked by leptin. Mechanisms of ERK1/2 activation by the oxidative stress may be ascribed to the effects of aldehyde-protein adducts on ERK1/2 phosphorylation (45) because inhibitors of adduct formation prevented ERK1/2 phosphorylation (Fig. 6F). The inhibitory effects of wortmannin on Sp1 and ERK1/2 activation and leptin-induced *Col1a(I)* gene expression (Fig. 5) may be ascribed to the role played by PI3K in the phosphorylation of RAC1, a component required for NADPH oxidase function (46, 47). Finally, phosphorylated ERK1/2-dependent activation of Sp1 is supported by the fact that phosphorylated ERK interacts with Sp1 and both bind collagen promoter (31).

We conclude that, in HSCs, leptin up-regulates Col1a(I) gene expression after activating NADPH oxidase, inducing oxidative stress, aldehyde-protein adduct formation, and ERK1/2 phosphorylation, which in turn activates Sp1/Sp3 and provokes the binding of these two factors to regulatory elements located between −161 and −110 bp of the *Col1a(I)* promoter (Fig. 8).

Acknowledgments

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This work was supported in part by Grants AP2884-2008, AP7257-2010, and AP8540-2011 from the "Fundación Mutua Madrileña" and Grants PI07/0052, PI10/0312 from the "Fondo de Investigación Sanitaria" (Spain).

Disclosure Summary: The authors have nothing to disclose.

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