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Leptin Enhances α1(I) Collagen Gene Expression in LX-2 Human Hepatic Stellate Cells Through JAK-Mediated H_2O_2-Dependent MAPK Pathways

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Abstract Leptin, a liver profibrogenic cytokine, induces oxidative stress in hepatic stellate cells (HSCs), with increased formation of the oxidant H_2O_2, which signals through p38 and extracellular signal-regulated kinase 1/2 (ERK1/2) pathways, stimulating tissue inhibitor of metalloproteinase-1 production. Since oxidative stress is a pathogenic mechanism of liver fibrosis and activation of collagen gene is a marker of fibrogenesis, we evaluated the effects of leptin on collagen I expression. We report here that, in LX-2 human HSCs, leptin enhances the levels of α1(I) collagen mRNA, promoter activity and protein. Janus kinase (JAK)1 and JAK2 were activated. H_2O_2 formation was increased; this was prevented by the JAK inhibitor AG490, suggesting a JAK-mediated process. ERK1/2 and p38 were activated, and the activation was blocked by catalase, consistent with an H_2O_2-dependent mechanism. AG490 and catalase also prevented leptin-stimulated α1(I) collagen mRNA expression. PD098059, an ERK1/2 inhibitor, abrogated ERK1/2 activation and suppressed α1(I) collagen promoter activity, resulting in mRNA down-regulation. The p38 inhibitor SB203580 and overexpression of dominant negative p38 mutants abrogated p38 activation and down-regulated the mRNA. While SB203580 had no effect on the promoter activity, it reduced the mRNA half-life from 24 to 4 h, contributing to the decreased mRNA level. We conclude that leptin stimulates collagen production through the H_2O_2-dependent and ERK1/2 and p38 pathways via activated JAK1 and JAK2. ERK1/2 stimulates α1(I) collagen promoter activity, whereas p38 stabilizes its mRNA. Accordingly, interference with leptin-induced oxidative stress by antioxidants provides an opportunity for the prevention of liver fibrosis. J. Cell. Biochem. 97: 188–197, 2006.

Key words: leptin; collagen; hepatic stellate cells; H_2O_2; p38; ERK1/2

Leptin is a 16-kDa peptide with a structure characteristic of the cytokine family; it controls body weight by regulating food intake and energy expenditure [Friedman, 1998]. Recent studies implicate leptin in liver fibrogenesis [Honda et al., 2002; Ikejima et al., 2002; Saxena et al., 2002]. Current data reveal that leptin has a direct action on hepatic stellate cells (HSCs), the key fibrogenic liver cells upon activation [Mak and Lieber, 1988; Friedman, 1993]. However, investigations have only started to examine the intracellular signaling mechanisms used by leptin to modulate fibrogenesis in HSCs. Thus, while others reported that leptin up-regulates collagen mRNA via an activated signal transducer and activator of transcription 3 (STAT3) mechanism in cultured rat HSC [Saxena et al., 2003], we found that the cytokine has the capacity to stimulate tissue inhibitor of metalloproteinase-1 (TIMP-1) gene expression and to increase TIMP-1 protein production in LX-2 human HSCs [Cao et al., 2004]. This leptin's action on TIMP-1 is mediated, in part, by the Janus kinase (JAK)-STAT pathway via the leptin receptor long form. Furthermore, leptin was found to generate oxidative stress with the formation of the oxidant H_2O_2 via activated JAK1 and JAK2. H_2O_2 in turn signals through the extracellular signal-regulated

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kinase 1/2 (ERK1/2) and p38 of the mitogen-activated protein kinase (MAPK), stimulating TIMP-1 production. The capacity of leptin to produce H_2O_2 with enhanced fibrogenesis is of significant physiological relevance, because oxidative stress caused by reactive oxygen species (ROS) is a pathogenic mechanism of hepatic fibrosis irrespective of etiologies [Parola and Robino, 2001], including that of non-alcoholic steatohepatitis [Reid, 2001].

Enhanced production of ROS results in an activation of the collagen gene, which is a marker of fibrogenesis. Accordingly, we evaluated the capacity of leptin to stimulate collagen production. Specifically, we assessed the effects of leptin on H_2O_2 formation and its signaling through p38 and ERK1/2 pathways affecting α1(I) collagen mRNA expression, its promoter activity and its message stability. These were investigated in LX-2 cells, an immortalized human HSC line, which retains key features of activated HSCs [Cao et al., 2004; Xu et al., 2005].

MATERIALS AND METHODS

Culture and Treatment of HSCs

The human HSC line LX-2 [Xu et al., 2005] was kindly provided by Dr. S.L. Friedman, Mount Sinai School of Medicine, NY. The maintenance of LX-2 cells in Dulbecco's modified essential medium (DMEM) has been described [Cao et al., 2004]. Before treatment, cells were washed in serum-free DMEM, and leptin (Sigma, St. Louis, MO) with or without various inhibitors were added. Leptin was used at concentrations of 25–100 ng/ml. The inhibitors were: JAK inhibitor AG490 (Calbiochem, San Diego, CA); catalase (Sigma); p38 inhibitor SB203580 (Sigma); SB202474, an inactive analog of SB203580 (Calbiochem); ERK1/2 inhibitor PD098059 (Sigma). Except for catalase which was dissolved in the culture medium, others were dissolved in dimethylsulfoxide (DMSO) at a concentration of 2.1 mM.

α1(I) Collagen mRNA and Collagen Type I Assays

Expression of mRNA for the α1(I) collagen in LX-2 cells was evaluated by Northern blotting as previously described [Cao et al., 2002a,b]. The levels of the mRNA were quantified by measuring the intensity of the bands on X-ray film by imaging densitometry. Collagen type I in the culture media was quantified by enzyme-linked immunosorbent assay (ELISA) as detailed before [Cao et al., 2002a].

JAK Phosphorylation Assay

The level of JAK phosphorylation was determined at 30 min, the time at which maximal phosphorylation by leptin occurred in LX-2 cells [Cao et al., 2004]. A 200 μl aliquot of the cell lysates was incubated with a rabbit anti-p-JAK1 or anti-p-JAK2 antibody (Biosource, Camarillo, CA) and then immunoprecipitated with agarose hydrazide beads [Cao et al., 2004]. The immune complexes (20 μl) were resolved by 12% SDS–PAGE and probed with the respective primary antibodies. Signal intensities were quantified by imaging densitometry.

Intracellular ROS Generation

This was determined by adding the probe 2′,7′-dichlorodihydrofluorescin diacetate (DCFH-DA), obtained from Molecular Probes (Eugene, OR), to LX-2 culture at a final concentration of 20 μM according to Carter et al. [1994]. In the cells, the nonfluorescent DCFH is oxidized to the fluorescent 2′,7′-dichlorofluorescein (DCF) by peroxide in the presence of peroxidases. Increased fluorescence of DCF reflected elevated levels of H_2O_2. The fluorescence was measured at excitation wavelength of 488 nm and emission at 525 nm in a spectrophotometer.

p38 and ERK1/2 MAPK Phosphorylation Assays

The level of MAPK phosphorylation was determined at 2 h, the time shown to be associated with maximal phosphorylation of p38 and ERK1/2 by leptin in LX-2 cells [Cao et al., 2004]. Phosphorylated (p)-p38 and (p)-ERK1/2 proteins were analyzed by Western blots, using the PhosphoPlus p38 MAPK and ERKl/2 MAPK Antibody Kits (Cell Signaling Technology, Beverly, MA) as previously described [Cao et al., 2002b, 2004]. Phosphorylation of p38 was detected using a rabbit phospho-p38 (Thr-180/Tyr-182) antibody and ERK1/2 phosphorylation, a rabbit phospho-ERK1/2 (Thr-202/ Tyr-204) antibody. Signal intensities were quantified by imaging densitometry.

Transfection of α1(I) Collagen Promoter and Luciferase Reporter Gene Assay

Activity of the α1(I) collagen promoter was assessed using the luciferase reporter plasmid (pGLCol2) containing nucleotides −2200 to −220 of the mouse α1(I) collagen gene (kindly provided by Dr. David Brenner, Columbia

University, New York, NY). Transient transfection of LX-2 cells were carried out using the LipofectAmine™ kit obtained from Invitrogen (Carlsbad, CA). One microliter of LF2000 reagent was diluted to 50 μl with DMEM and incubated for 5 min at room temperature. To this was added 320 ng of the reporter plasmid DNA or the promoterless pGLCol2 suspended in 50 μl DMEM. The mixture was incubated for 20 min to allow the formation of DNA–LF2000 complex. Then, the mixture (100 μl) was added to LX-2 cells grown on 24-well plates (3×10^5 cells/well) in DMEM and incubated at 37°C for 24 h. Leptin with or without catalase, SB203580 or PD098059 were added to the transfected cells and incubated for 24 h. Luciferase reporter activity was assayed using the Luciferase Reporter Gene Assay Kit (Roche Molecular Biochemicals, Indianapolis, IN). Cells were lysed in a lysis buffer provided by the kit and 50 μl of the cell extracts were added to the 96-well microtiter plate. Then, 100 μl of the luciferase assay reagent was pipetted into the plate to start the reaction and light emission was immediately measured in a luminescence reader (Bio-Tek Instruments, Winooski, VT). Data are expressed as relative light units/μg protein.

Transfection of p38 Dominant Negative Mutants (p38αdn and p38βdn)

The p38αdn and p38βdn [Wang et al., 1998] were gifts from Dr. J. Han (Scripps Research Institute, La Jolla, CA). The plasmid pCDNA3 (800 ng) containing p38αdn or p38βdn were transiently transfected into LX-2 cells as previously described [Cao et al., 2004], using the LipofectAmine™ kit per the manufacturer's instruction. Transfected cells were treated with leptin for 24 h for analysis.

Collagen mRNA Stability Determination

To assess whether leptin, catalase, p38, and ERK1/2 affect the α1(I) collagen mRNA stability, LX-2 cells were treated with leptin (75 ng/ml) for 24 h followed by actinomycin D (10 μg/ml) treatment for 20 min to block the transcription. The culture medium was changed and fresh medium containing catalase, SB203580 or PD098059 was added. After 4, 12, and 24 h of incubation, total RNA from LX-2 cells was isolated for Northern blot analysis of the α1(I) collagen mRNA levels, and the decay time course in the absence or presence of the inhibitors were analyzed.

Statistics

Data are reported as mean ± SE and the significance of difference between mean was assessed using analysis of variance followed by Student–Neuman–Keuls tests. $p < 0.05$ was considered to be significant.

RESULTS

Leptin Increases α1(I) Collagen mRNA and Its Protein

LX-2 cells expressed α1(I) collagen mRNA of 5.8 and 4.8 kb, as analyzed by Northern blot (Fig. 1). Leptin induced a dose-dependent increase of the mRNA, reaching a maximal level with a 3.5-fold rise at 75 ng/ml. This effect was accompanied by a 300% increase of collagen type I protein in the culture media, as quantified by ELISA (6.7 vs. 2.2 μg/ml in the control). The concentration of 75 ng/ml leptin was used in the subsequent experiments.

Leptin Stimulates Phosphorylation of JAK1 and JAK2 and Its Inhibition by JAK Inhibitor AG490

Leptin treatment for 30 min raised the levels of phosphorylated (p)-JAK1 (4.5-fold) and p-JAK2 (4.8-fold), and the rises were prevented by AG490 (Fig. 2), in accordance with the action of the inhibitor [De Vos et al., 2000; Burysek et al., 2002].

Leptin Stimulates ROS Generation and the Increase Is Prevented by JAK Inhibitor AG490

Figure 3A shows that leptin-stimulated ROS generation in LX-2 cells was time-dependent. Peak level of ROS occurred 1 h after leptin (650% of control at 0 time). After 24 h, the ROS level was still 2.9 times higher than in the control. AG490 treatment prevented the ROS formation stimulated by leptin determined at 1 h, reflecting a JAK-mediated process (Fig. 3B). Addition of catalase to the culture medium abolished the increase in DCF fluorescence after leptin, suggesting that H_2O_2 is largely responsible for the ROS increase.

Leptin Increases p38 MAPK Phosphorylation and the Process Is Inhibited by Catalase, p38 Inhibitor SB203580 and p38 Dominant Negative Mutants (p38αdn and p38βdn)

Figure 4 shows that leptin increased the phosphorylation of p38 3.9-fold, determined at 2 h. This effect was totally inhibited by catalase,

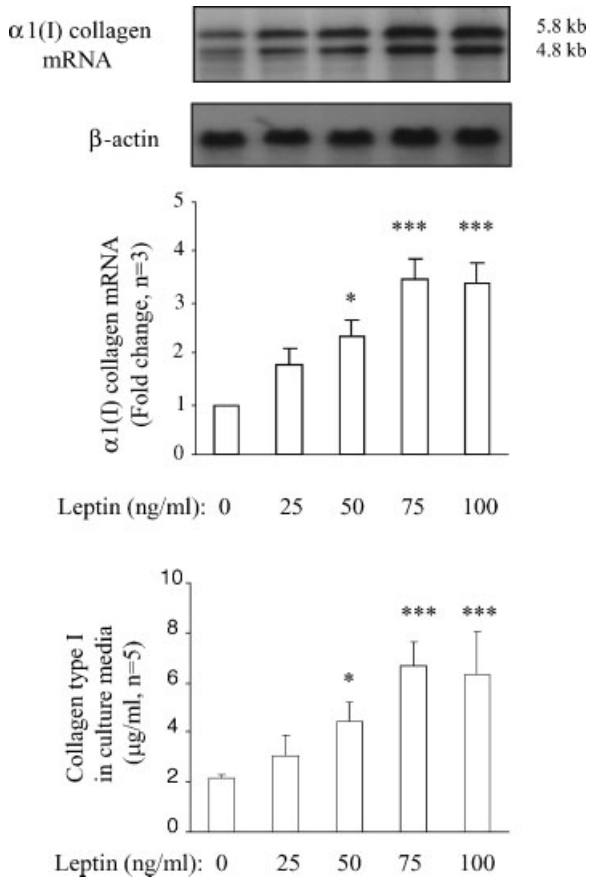

Fig. 1. Leptin stimulation of α1(I) collagen mRNA and its protein production: effects of dose. LX-2 hepatic stellate cells (HSCs) were incubated with leptin at the indicated concentrations for 24 h and the α1(I) collagen mRNA abundance was analyzed by Northern blot. **Upper panels** are representative blots illustrating two mRNA transcripts (5.8 and 4.8 kb) for the α1(I) collagen gene. The **middle panel** shows the corresponding histograms of data of three separate analyses. The mRNA levels were normalized to β-actin, and values are expressed as fold change relative to the control (no leptin) assigned a value of 1. The **lower panel** illustrates type I collagen protein in the culture media of LX-2 cells. $*p < 0.05$ and $***p < 0.001$ versus control (no leptin).

demonstrating a role for H_2O_2 in p38 activation. In the presence of the p38 inhibitor SB203580 [Cuenda et al., 1995], but not of its inactive analog SB202474 [Lee et al., 1994], the level of p38 phosphorylation was reduced to that below the control. A similar change was found in LX-2 cells with transfection of p38αdn and p38βdn. These results demonstrate the contribution of H_2O_2 in the activation of p38 by leptin.

Leptin Increases ERK1/2 MAPK Phosphorylation and the Process Is Inhibited by Catalase, and ERK1/2 Inhibitor PD098059

Leptin increased p-ERK1/2 level 4.3-fold above the untreated control, assayed at 2 h, and the rise was prevented by catalase (Fig. 5), demonstrating an involvement of H_2O_2 in the process. In the presence of PD098059, no p-ERK1/2 was detected, consistent with the action of the inhibitor [Dudley et al., 1995].

Leptin Induction of α1(I) Collagen mRNA Is Inhibited by JAK Inhibitor AG490, Catalase, p38 Inhibitor SB203580, p38 Dominant Negative Mutants, and ERK1/2 Inhibitor PD098059

Figure 6 shows the effects of JAKs, H_2O_2, p38, and ERK1/2 on leptin induction of α1(I) collagen mRNA. AG490 and catalase prevented the 3.6-fold increase of the mRNA after leptin. SB203580, but not its inactive analog SB202474, and overexpression of p38αdn and p38βdn halved the level of the mRNA. PD098059 also reduced the rise of the mRNA by 50%. These results suggest that the up-regulation of collagen mRNA by leptin involves the activation of JAK1 and JAK2 and the formation of H_2O_2, which signal through p38 and ERK1/2 pathways.

Leptin Stimulates α1(I) Collagen Promoter Activity and Its Inhibition by Catalase and ERK1/2 Inhibitor PD098059, but not by p38 Inhibitor SB203580

The promoter activity of the α1(I) collagen gene was stimulated sixfold by leptin (Fig. 7). The stimulation was blocked by catalase. Whereas PD098059 almost completely prevented the increase, SB203580 had no effect on the promoter activity. These results implicate that leptin-induced collagen gene expression is mediated by ERK1/2 via H_2O_2 formation and suggest that p38 is not involved in the transcriptional regulation of the α1(I) collagen gene.

Effects of p38 Inhibitor SB203580 and Catalase on Leptin-Induced α1(I) Collagen mRNA Stability

Because p38 activation had no effect on α1(I) collagen promoter activity, we evaluated whether it affects the mRNA stability induced by leptin (Fig. 8). Leptin increased the half-life ($t_{1/2}$) of α1(I) collagen mRNA to 24 h from 14 h in untreated control cells. SB203580 decreased the $t_{1/2}$ from 24 to 4 h in the presence of leptin, suggesting a stabilization of the message by p38. A similar effect was observed after catalase. By contrast, in cells treated with PD098059, no change in the mRNA decay was observed.

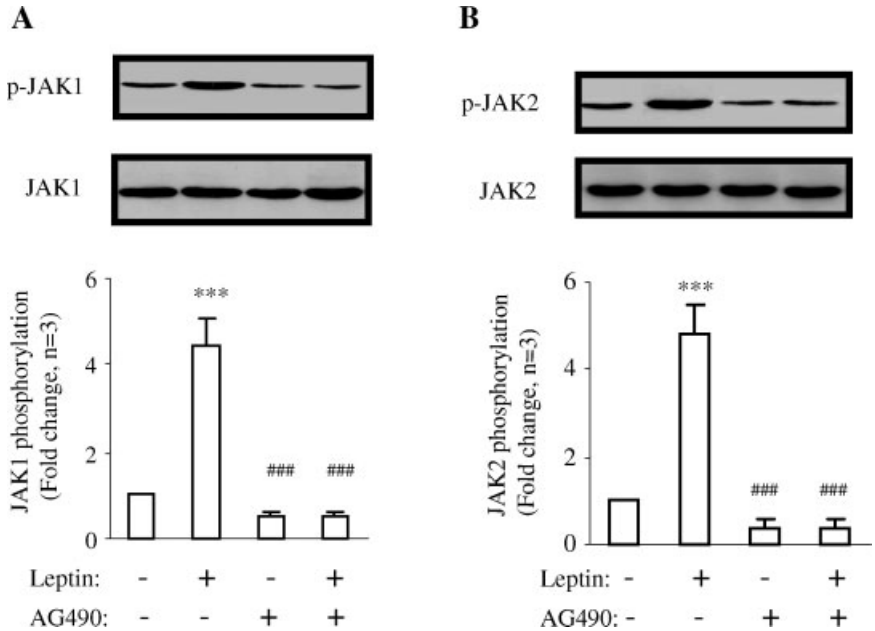

Fig. 2. Increased phosphorylation of JAK1 and JAK2 by leptin and its inhibition by JAK inhibitor AG490. LX-2 cells were treated with leptin, AG490 (50 μM) or both for 30 min. Immunoprecipitates of phosphorylated (p)-JAK proteins were analyzed by Western blots. **Upper panels** are representative blots of p-JAK1 **(A)** and p-JAK2 **(B)**. **Lower panels** are the corresponding histograms of results of three separate analyses showing the ratio of p-JAK1 and JAK2 to the corresponding nonphosphorylated JAKs relative to the control (no leptin and AG490). $^{***}p < 0.001$ versus control (no leptin and AG490) and $^{\#\#\#}p < 0.001$ versus leptin alone.

DISCUSSION

In this study, we demonstrate that leptin stimulation increases α1(I) collagen mRNA expression and its protein production in a dose-dependent manner in LX-2 human HSCs. The effect is mediated, in part, by the generation of the oxidant H_2O_2 via activation of JAK1 and JAK2. H_2O_2 in turn signals through the ERK1/2 and p38 pathways. Whereas ERK1/2 stimulates α1(I) collagen promoter activity, p38 stabilizes the collagen mRNA, promoting the production

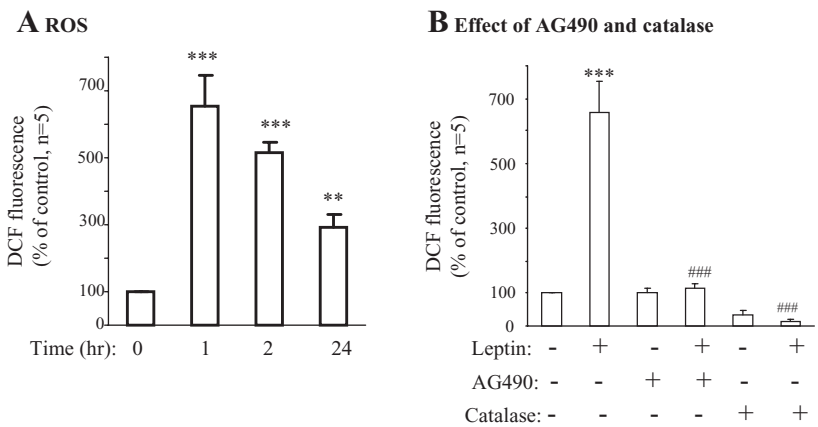

Fig. 3. Generation of intracellular ROS by leptin and its prevention by JAK inhibitor AG490 and catalase. **A**: LX-2 cells were treated with leptin for the times indicated. Following the leptin treatment, 2,7-dichlorodihydrofluorescin diacetate (DCFH-DA) was added to the culture with incubation for 30 min at 37°C in the darkness. Values for the dichlorofluorescein (DCF) fluorescence are expressed as percentage of control at 0 time. $^{**}p < 0.01$ and $^{***}p < 0.001$ versus 0 time. **B**: LX-2 cells were treated with leptin in the presence or absence of AG490 (50 μM) or catalase (1,000 U/ml) for 1 h, followed by addition of DCFH-DA as in (A). DCF fluorescence is expressed as percentage of untreated control. $^{***}p < 0.001$ versus control and $^{\#\#\#}p < 0.001$ versus leptin alone.

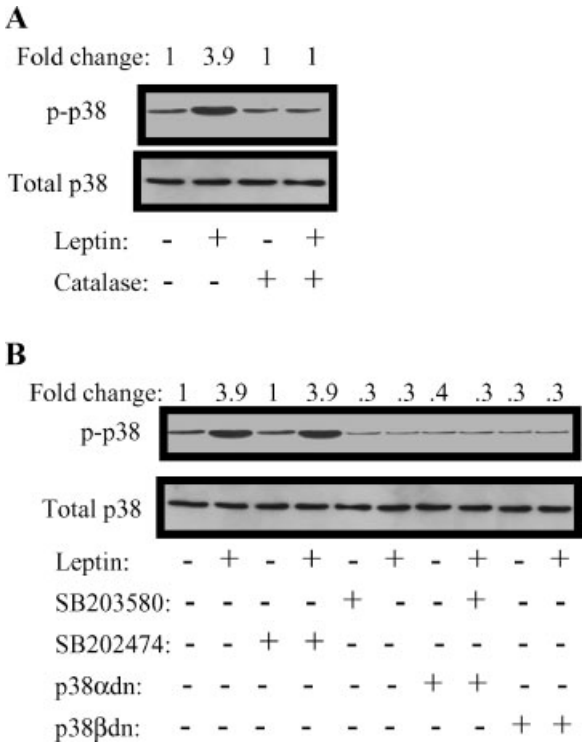

Fig. 4. Activation of p38 MAPK by leptin and its inhibition by catalase, p38 inhibitor SB203580 and p38 dominant negative mutants (p38αdn and p38βdn). LX-2 cells were incubated with leptin for 2 h without or with various inhibitors: **A**: Catalase (1,000 U/ml), and (**B**) SB203580 (20 μM), SB202474 (20 μM), and transfection of p38αdn and p38βdn. The phosphorylated (p)-p38 protein content was analyzed by Western blot using the primary antibody, as described in ''Materials and Methods.'' The signal intensity of total p38 detected with a nonphosphorylated p38 antibody was used to show equal protein loading. The levels of p-p38 were normalized to total p38 and values are expressed as fold change relative to the control (no leptin and inhibitor) assigned a value of 1. The numbers above the blots refer to the mean values of three separate analyses.

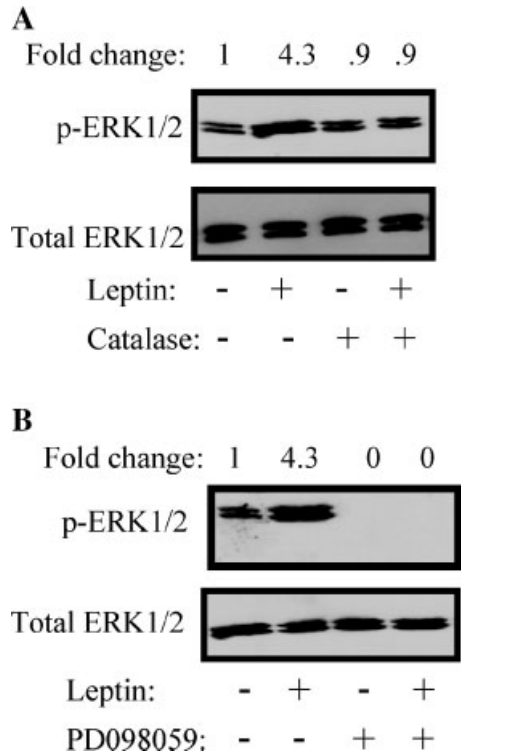

Fig. 5. ERK1/2 MAPK phosphorylation by leptin and its inhibition by JAK inhibitor AG490, catalase, and ERK1/2 inhibitor PD098059. LX-2 cells were incubated with leptin, inhibitors or both for 2 h. **A**: 1,000 U/ml catalase, and (**B**) 30 μM ERK1/2 inhibitor PD098059. The phosphorylated (p)-ERK1/2 protein content was analyzed by Western blot using the primary antibody, as described in ''Materials and Methods.'' The signal intensity of total ERK1/2 detected with a nonphosphorylated ERK1/2 antibody was used to show equal protein loading. The levels of p-ERK1/2 were normalized to total ERK1/2 and values are expressed as fold change relative to the control (without leptin and inhibitor) assigned a value of 1.

of collagen. These signaling pathways have been described to participate in the stimulation of TIMP-1 production by leptin in LX-2 cells [Cao et al., 2004].

LX-2 cells express two transcripts (5.8 and 4.8 kb) of mRNA for the α1(I) collagen gene as observed in primary cultured human [Svegliati-Baroni et al., 1999] and rat [Rippe et al., 1995; Cao et al., 2002b] HSCs. Leptin up-regulates α1(I) collagen mRNA in LX-2 cells in a concentration-dependent manner, with a maximal 3.5-fold increase over untreated control cells at 75 ng/ml assayed by Northern blot. This level of stimulation in LX-2 cells is of the same order of magnitude as that of α2(I) collagen mRNA in primary cultured rat HSCs and in rat HSC-T6 cell line at 100 ng/ml leptin, assayed by ribonucleas protection analysis [Saxena et al., 2002, 2003]. Furthermore, the 3.5-fold increase of collagen mRNA by leptin in LX-2 cells compares favorably with that elicited by TGF-β1 (a potent liver profibrogenic cytokine) in rat HSC cultures [Cao et al., 2002b] and in human HSC cultures treated with insulin-like growth factor or acetaldehyde [Svegliati-Baroni et al., 1999, 2001]. In addition, the up-regulation of collagen mRNA was accompanied by a parallel increase of secreted collagen protein in the culture media. These results validate that the LX-2 HSC line is an appropriate tool for the study of fibrogenesis in vitro.

Previous studies with LX-2 cells showed that leptin induction of oxidative stress was time-dependent (with a peak at 1 h) and was

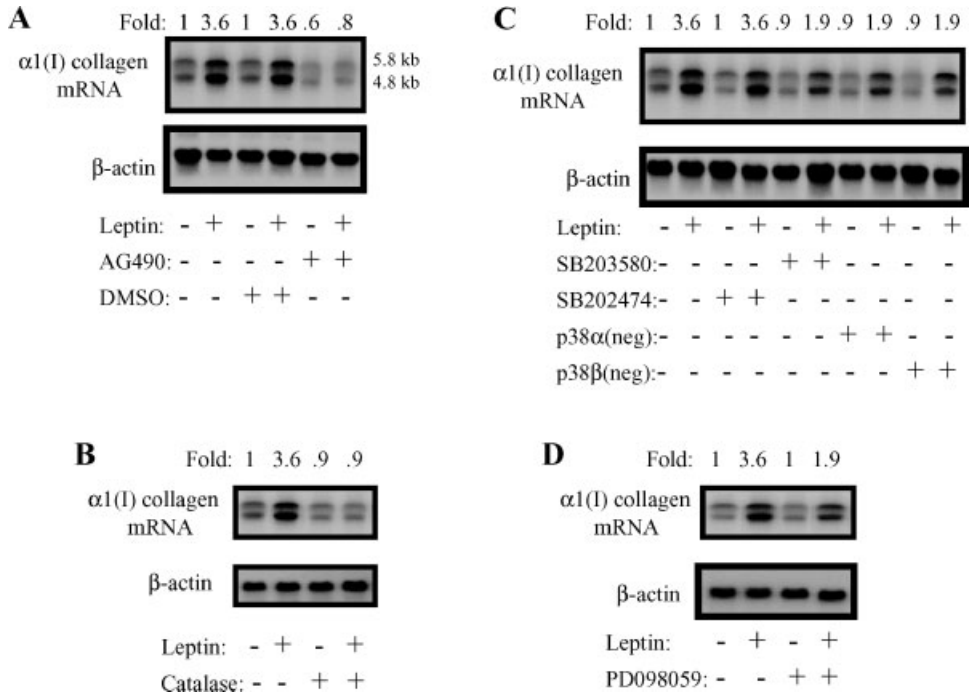

Fig. 6. Inhibition of leptin-induced α1(I) collagen mRNA by JAK inhibitor AG490, catalase, p38 inhibitor SB203580, p38 dominant negative mutants, and ERK1/2 inhibitor PD098059. LX-2 cells were incubated with leptin alone or with the addition of various inhibitors for 24 h. **A**: AG490 (50 μM); (**B**) catalase (1,000 U/ml); (**C**) SB203580 (20 μM); and SB202474 (20 μM); and (**D**) PD098059 (30 μM). LX-2 cells with transfection of p38αdn and p38βdn were treated likewise with leptin. The mRNA levels were normalized to β-actin, and values are expressed as fold change relative to the control (no leptin and inhibitor) assigned a value of 1.

accompanied by significant changes of several oxidative parameters, including increased formation of the oxidants H_2O_2 and superoxide anions, increased lipid peroxidation and depletion of cellular reduced glutathione, an antioxidant [Cao et al., 2004]. These parameters were normalized by treatment with the JAK inhibitor AG490, indicating that the leptin-induced oxidative stress is mediated by activation of JAK1 and JAK2. The present study focused on the effect of leptin on H_2O_2 formation, because this oxidant has been shown to serve as a signaling molecule between oxidative stress and α1(I) collagen gene up-regulation [Greenwel et al., 2000; Chin et al., 2001; Cao et al., 2002b]. It is noteworthy that the capacity of leptin to generate oxidative stress in LX-2 cells equals that of the profibrogenic cytokine TGF-β1 in rat HSC cultures [Cao et al., 2002b]. Interestingly, both cytokines use H_2O_2 signaling mechanism to mediate collagen mRNA expression through the p38 MAPK pathway, although they transduce their signals via distinct cell surface receptors [Chin et al., 2001; Cao et al., 2004].

Yamagishi et al. [2001] reported, in aortic endothelial cells, that leptin induces monocyte chemoattractant protein-1 (MCP-1) expression and that transfection of manganese superoxide dismutase (MnSOD) resulted in the prevention of the expression, suggesting an involvement of mitochondrial superoxide in the process. On the other hand, Bouloumie et al. [1999] observed that leptin stimulates MCP-1 protein through an increase of H_2O_2 formation in venous endothelial cells. We found that catalase prevents the expression of α1(I) collagen gene expression induced by leptin in LX-2 cells, suggesting an H_2O_2-dependent regulation. However, it will be of interest to determine whether or not superoxide generation in the mitochondria also participates in leptin stimulation of collagen production by leptin in LX-2 cells. The requires the insertion of MnSOD into LX-2 cells, which is the subject of an ongoing investigation.

Activation of p38 MAPK in LX-2 cells after leptin is dependent on H_2O_2 formation, because the process is inhibited by catalase, an antioxidative enzyme that degrades H_2O_2. The finding

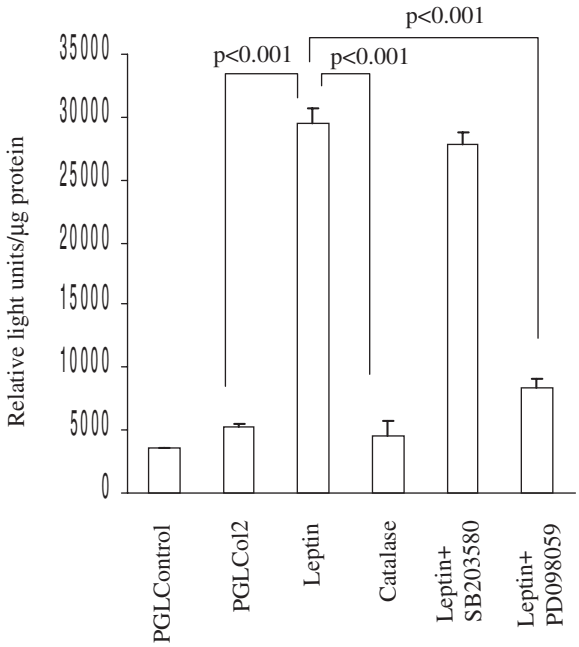

Fig. 7. Stimulation of α1(I) collagen promoter activity by leptin and its inhibition by catalase and ERK1/2 inhibitor PD098059, but not by p38 inhibitor SB203580. LX-2 cells were transiently transfected with the luciferase reporter plasmid (pGLCol2) containing nucleotides −2200 to −220 of the mouse collagen α1(I) promoter. Transfected cells were treated with leptin alone or leptin + 1,000 U/ml catalase, 20 μM SB203580, or 30 μM PD098059. After 24 h, cell extracts were prepared for luciferase reporter activity assay according to the protocol provided in the Luciferase Reporter Assay Kit. pGLcontrol = empty vector; pGLCol2 = unstimulated collagen promoter transfected cells. Data are expressed as relative light units/μg protein.

that p38 had no effect on the collagen promoter activity suggests that the mouse α1(I) collagen promoter may not have the necessary sequences to respond to p38. The kinase appears to up-regulate collagen mRNA posttranscriptionally by conferring stability on the leptin-induced collagen mRNA, because treatment with SB203580 reduced the message half-life sixfold from 24 to 4 h. There are no studies on the mechanisms by which p38 stabilizes the collagen gene, although it was reported that, in activated HSC, mRNA stabilization for the type I collagen gene occurs through binding of αCP protein to the C-rich sequence in the α1(I) collagen 3′untranslated region (UTR) [Stefanovic et al., 1997]. However, several studies on mRNA encoding for proinflammatory genes, including cyclooxygenase-2, IL-6, IL-8, and TNF-α, implicate that their stabilization by p38 is dependent on the A + U-rich elements in the 3′ UTRs of the respective genes [Winzen et al., 1999; Brook et al., 2000; Kishore et al., 2001]. Whether this mechanism could explain the p38 stabilization of the collagen mRNA is not known. Enhanced mRNA stability by p38 has also been observed for TIMP-1 after leptin in LX-2 cells [Cao et al., 2004]. Thus, it appears that it is an attribute of p38 to up-regulate fibrogenic genes through a posttranscriptional mechanism.

ERK1/2 MAPK is activated concurrently with p38 in LX-2 cells after leptin. Activation of

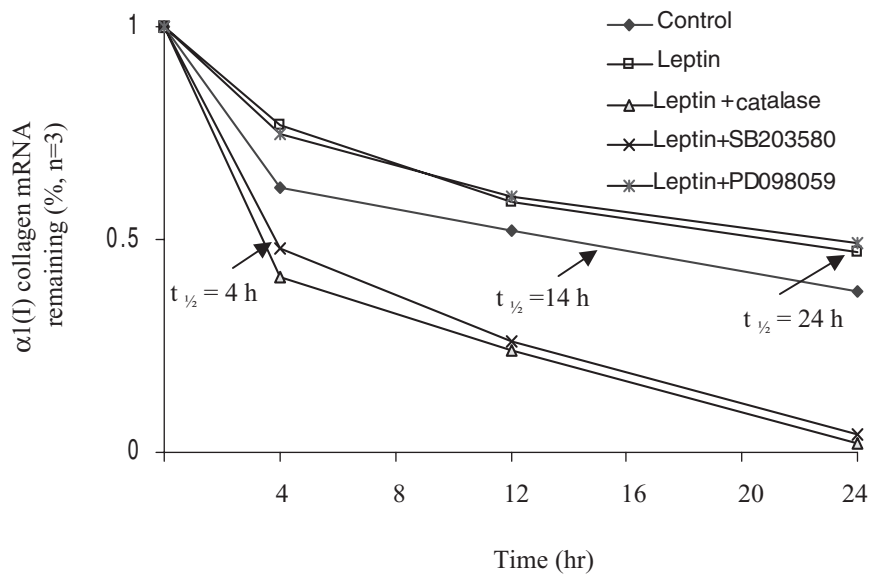

Fig. 8. The p38 inhibitor SB203580 and catalase, but not the ERK1/2 inhibitor PD098059, decrease leptin-induced α1(I) collagen mRNA stability. LX-2 cells were treated with leptin for 24 h to induce α1(I) collagen mRNA expression, followed by actinomycin D treatment for 20 min to block the transcription, as described in ''Materials and Methods.'' The rate of the collagen mRNA decay in the presence or absence of the inhibitors is expressed as percentage of the mRNA remaining relative to the time 0 level at the start of actinomycin D treatment. Values for the control (untreated) cells were also included for comparison.

ERK1/2, like that of p38, is mediated by H_2O_2 formation. Unlike p38, ERK1/2 appears to regulate collagen mRNA expression at the transcriptional level through stimulation of α1(I) collagen promoter activity. In passaged rat HSC, ERK1/2 signaling was found to involve NF-1 and SP-1 sites in the proximal promoter of the α1(I) collagen gene [Davis et al., 1996]. The involvement of ERK1/2 in collagen type I production has been observed after stimulation with insulin-like growth factor [Svegliati-Baroni et al., 1999] or acetaldehyde in human HSCs [Svegliati-Baroni et al., 2001].

It is important to point out that leptin can also use signaling mechanisms other than the p38 and ERK1/2 pathways to elicit fibrogenesis in HSC. Thus, we have described that TIMP-1 gene expression in LX-2 cells is also mediated, in part, by the JAK-STAT pathway, which is activated by phosphorylation of Tyr-1141 in the cytoplasmic domain of the leptin receptor long form [Cao et al., 2004]. Because there are no data (to date) which suggest that the leptin-induced STAT pathway involves H_2O_2 signaling, it was not the focus of the present study. It will be of future interest, however, to analyze the relative contributions of these pathways (namely JAK → STAT and JAK → H_2O_2 → p38/ERK1/2) to collagen production in the same cell systems, since important cross-talk among these signaling pathways to either enhance or inhibit their effectiveness has been recognized [Korzus et al., 1997; Decker and Kovarik, 2000; Burysek et al., 2002].

In conclusion, this study emphasizes the central role of H_2O_2 signaling through the p38 and ERK1/2 MAPK pathways to mediate fibrogenesis induced by leptin in HSCs. The oxidant generated by leptin does not only stimulate collagen gene expression, but it also activates TIMP-1 gene, as shown in our previous study [Cao et al., 2004]. Since increased production of collagen and TIMP-1 favors collagen deposition and since leptin has been implicated in the development of liver fibrosis, including that of non-alcoholic steatohepatitis [Reid, 2001], interference with the leptin-induced oxidative stress provides an opportunity for the prevention of this disease.

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