

p38 Mitogen-Activated Protein Kinase and Liver X Receptor- α Mediate the Leptin Effect on Sterol Regulatory Element Binding Protein-1c Expression in Hepatic Stellate Cells

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Leptin, a key hormone in regulating energy homeostasis, is mainly produced by adipocytes. Cogent evidence indicates a unique role of leptin in the promotion of liver fibrosis. Hepatic stellate cell (HSC) activation is a pivotal step in the process of liver fibrosis. Sterol regulatory element binding protein (SREBP)-1c, a critical transcription factor for lipid synthesis and adipocyte differentiation, functions as a key transcription factor in inhibition of HSC activation. SREBP-1c is highly expressed in quiescent HSCs and downregulated upon HSC activation. The aim of this study is to examine the effect of leptin on SREBP-1c gene expression in HSCs *in vitro* and *in vivo* and elucidate the underlying mechanisms. The results of the present study demonstrated that leptin strongly inhibited SREBP-1c expression in HSCs *in vivo* and *in vitro*. p38 MAPK was involved in leptin regulation of SREBP-1c expression in cultured HSCs. Leptin-induced activation of p38 MAPK led to the decreases in liver X receptor (LXR)- α protein level, activity and its binding to the SREBP-1c promoter, which caused the downregulation of SREBP-1c expression. Moreover, leptin inhibition of SREBP-1c expression via p38 MAPK increased the expression of α 1(I) collagen in HSCs. Our results might provide new insights into the mechanisms of the unique role of leptin in the development of liver fibrosis and might have potential implications for clarifying the molecular mechanisms underlying liver fibrosis in diseases in which circulating leptin levels are elevated such as nonalcoholic steatohepatitis, type 2 diabetes mellitus and alcoholic cirrhosis.

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INTRODUCTION

Leptin, the product of the *obese* gene, is mainly produced by adipocyte (1). It inhibits free fatty acid uptake and *de novo* fatty acid synthesis, stimulates free fatty acid mobilization and oxidation and reverses adipocyte differentiation (2,3). Accumulating evidence has indicated that leptin plays critical roles in the development of liver fibrosis *in vivo* and *in vitro* (4–7). Hepatic stellate cell (HSC) activation is a pivotal step in the development of liver fibrosis. Leptin can directly target HSCs via activation of its receptor (8)

and stimulate HSC activation and fibrogenesis (8–10). Quiescent HSCs, once called fat-storing cells, are much like adipocytes. It is proposed that HSC transdifferentiation from quiescent to myofibroblastic cells is analogous to adipocyte to preadipocyte (fibroblast) transdifferentiation (11).

The major transcription factors for adipocyte differentiation include the CCAAT/enhanced binding protein family (C/EBP), peroxisome proliferator-activated receptor (PPAR)- γ , liver X receptor (LXR) and sterol regulatory

element-binding protein (SREBP)-1 (12). SREBP-1 belongs to the SREBP family members and exists as two isoforms (SREBP-1a and SREBP-1c). SREBP-1c (also known as adipocyte determination and differentiation-dependent factor 1) is a key transcription factor for the promotion of lipid synthesis and adipocyte differentiation (12). The ratio of SREBP-1a to SREBP-1c mRNA is 1:9 in liver, and SREBP-1c is the predominant transcript in liver (13). A gain of function manipulation for transcription factors such as PPAR γ , LXR α and SREBP-1c by treatment with an adipocyte differentiation cocktail or ectopic transduction of SREBP-1c causes morphologic and biochemical reversal of activated HSCs to quiescent cells (14), indicating that SREBP-1c exerts a critical function in inhibition of HSC activation (14). Interestingly, the process of HSC activation is coupled with the sequential upregulation

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of leptin and downregulation of SREBP-1c (14,15).

Therefore, it is of interest to examine the relationship between leptin and the gene expression of SREBP-1c and elucidate the underlying mechanisms in HSCs, which is still largely unknown. The aim of this study is to examine the role of leptin in SREBP-1c expression in HSCs *in vitro* and *in vivo* and reveal the underlying mechanisms.

MATERIALS AND METHODS

Materials

Leptin was purchased from ProSpec-Tany TechnoGene (Rehovot, Israel) and used to treat cells at 100 ng/mL for 24 h (if no specific indication). SB203580 (a p38 mitogen-activated protein kinase [p38 MAPK] inhibitor) was purchased from CalBiochem (La Jolla, CA, USA) and used at 10 μ mol/L (if no specific indication). Thioacetamide (TAA), isobutylmethylxanthine, dexamethasone and insulin were all purchased from Sigma (St. Louis, MO, USA). All antibodies were purchased from Santa Cruz (Santa Cruz, CA, USA).

Treatment of Animals

Male C57BL/6J ob/ob mice (leptin-deficient) and their lean littermates were obtained from the Model Animal Research Center of Nanjing University (Nanjing, China) and were at 5 wks of age. All animals were housed for an additional week and then used in experiments. The mice were given free access to water and standard chow diet. Animals received humane care, and experimental protocols were conducted according to national and local guidelines.

Mice 6 wks after birth were randomly separated into groups (six mice/group), namely, the TAA group (ob/ob), TAA plus leptin group (ob/ob) and TAA group (lean). The TAA group (ob/ob) and TAA group (lean) were injected intraperitoneally with TAA (200 μ g/g body weight, two times a week) for 4 wks. TAA plus leptin (ob/ob) group were given coadministration of leptin (1 μ g/g

body weight, intraperitoneally) (16) once per day throughout the 4-wk period of TAA treatment.

Immunofluorescence Staining for Synaptophysin and SREBP-1c

For detection of SREBP-1c gene expression in HSCs in liver, immunofluorescence double staining for SREBP-1c and synaptophysin (SYP), a marker for quiescent and activated HSCs (17), was performed as we described previously (10). Briefly, liver sections were blocked with serum and incubated at 4°C for 48 h with a rabbit primary antibody against SREBP-1 (diluted 1:50) and goat primary antibody against SYP (diluted 1:50) followed by incubation at room temperature for 1 h with a fluorescein isothiocyanate-conjugated chicken anti-rabbit secondary antibody (diluted 1:100) and a Texas Red-conjugated chicken anti-goat secondary antibody (diluted 1:100). The nuclei were counterstained with Hoechst 33342 (Sigma), and images were captured with a fluorescence microscope. Since the ratio of SREBP-1a to SREBP-1c is 1:9 and SREBP-1c is the predominant subtype in liver (13), the protein detected by SREBP-1 antibody is mainly SREBP-1c in liver cells (18).

HSC Isolation and Culture

HSCs were isolated from Sprague-Dawley rats as we described previously (10). After 24 h of serum starvation in Dulbecco's modified Eagle's medium (DMEM) with 0.4% fetal bovine serum (FBS) (if no specific indication), cells were treated with leptin in DMEM with 0.4% FBS for 24 h (if no specific indication).

Western Blot Analysis

Western blot analysis was performed as we described previously (10). Target proteins were detected by primary antibodies against SREBP-1 (diluted 1:500), p38 MAPK (diluted 1:500), phosphorylated type of p38 MAPK (diluted 1:500), LXR α (1:400) and β -actin (diluted 1:2,000), respectively, and subsequently by horseradish peroxidase-conjugated secondary antibodies (diluted 1:4,000).

The level of target protein band was densitometrically determined by using Quantity One 4.4.1 (Bio-Rad) and normalized by the internal control β -actin. The variation in the density was expressed as fold-changes compared with the control in the blot.

RNA Isolation and Real-Time Polymerase Chain Reaction

Total RNA was extracted by using TRI-Reagent (Sigma) following the manufacturer's instructions and treated with deoxyribonuclease I. Real-time polymerase chain reaction (PCR) was carried out as we described previously (19). Fold-changes in mRNA levels of a target gene relative to the endogenous cyclophilin control were calculated as suggested by Schmittgen *et al.* (20) (if no specific indication). The primers used in real-time PCR were as follows: Rat SREBP-1c: (forward) 5'-AGCACAGCAACCAGAACTCAA-3'; (reverse) 5'-AGGTC TTTCAGTGATTGCTTTTGT-3'. Rat α 1(I) collagen: (forward) 5'-TGGTC CCAAAGGTTCTCCTGGT-3'; (reverse) 5'-TTAGGTCACAGGAATCCCAT CACA-3'. Rat cyclophilin: (forward) 5'-TGGATGGCAAGCATGTGGTCTTTG-3'; (reverse) 5'-CTTCTTGCTGGTCTTGCCAT TCCT-3'.

Plasmids and Transient Transfection Assays

The Photinus luciferase-reporter plasmid pSREBP1c-Luc contains the 5'-flanking region (–1,516 bp) of the rat SREBP-1c gene promoter, and Photinus luciferase-reporter plasmid pmutLXRE-Luc contains the mutations of LXR-binding sites in pSREBP1c-Luc (21). Plasmid pdnP38 encodes dominant-negative p38 α MAPK. Plasmid pwtP38 encodes wild-type p38 α MAPK. Plasmid pSP1 encodes rat wild-type specific protein 1 (Sp1). Photinus luciferase-reporter plasmid pSp1-Luc contains three consensus Sp1 binding sites (GGGCGG). Photinus luciferase-reporter plasmid pNFY-Luc contains three consensus nuclear factor-Y (NF-Y) binding sites (CCAAT). Photinus luciferase-reporter plasmid pLXRE-Luc

contains three tandem LXR binding sites (gcttGGGTCActcaAGTTCAagtta). Plasmid pLXR α encodes mouse wild-type LXR α . Plasmid ptwist2 encodes twist2, the repressor for SREBP-1c activity (22).

HSCs in 12-well plastic plates were transiently transfected with a reporter plasmid expressing Photinus luciferase (1 μ g DNA/well) plus 30 ng of control vector expressing Renilla luciferase (pRL-TK; Promega, Madison, WI, USA) by using LipofectAMINE reagent (Life Technologies, New York, NY, USA) following the manufacturer's instructions. A total of 0–0.8 μ g (each well) or 8 μ g (25-cm² flask) of other plasmids or the respective empty vector were used, as indicated in figures. The empty vectors were used to ensure an equal amount of total DNA in transfection assay. Luciferase activity was quantified fluorimetrically by using the Dual-Luciferase Reporter Assay System (Promega). Data were expressed as the ratios of Photinus to Renilla luciferase activity for normalization of Photinus luciferase activity.

Chromatin Immunoprecipitation (ChIP) Assays

Chromatin immunoprecipitation (ChIP) assays were performed by using a Pierce Agarose Chip Kit (Pierce, Rockford, IL, USA) following the manufacturer's instructions. Briefly, after cultured HSCs were cross-linked with 1% formaldehyde, the extracted nuclei were incubated with Micrococcal Nuclease and lysed. A total of 10% of the supernatant containing the digested chromatin was preserved as input control. The rest of the supernatant was incubated with LXR α antibody. DNA from immunoprecipitation and the input samples was analyzed by real-time PCR. Primers (forward, 5'-TGTTGCCTGT GCGGCAG-3'; reverse, 5'-TCAGGCCCG CCAGGCTTTAA-3') were used to amplify a fragment (260 bp) of the SREBP-1c promoter between nucleotides –267 and –8 containing the two LXR-binding sites. According to the method described by Mastrogiannaki *et al.* (23), the amplification by real-time PCR was quantified as

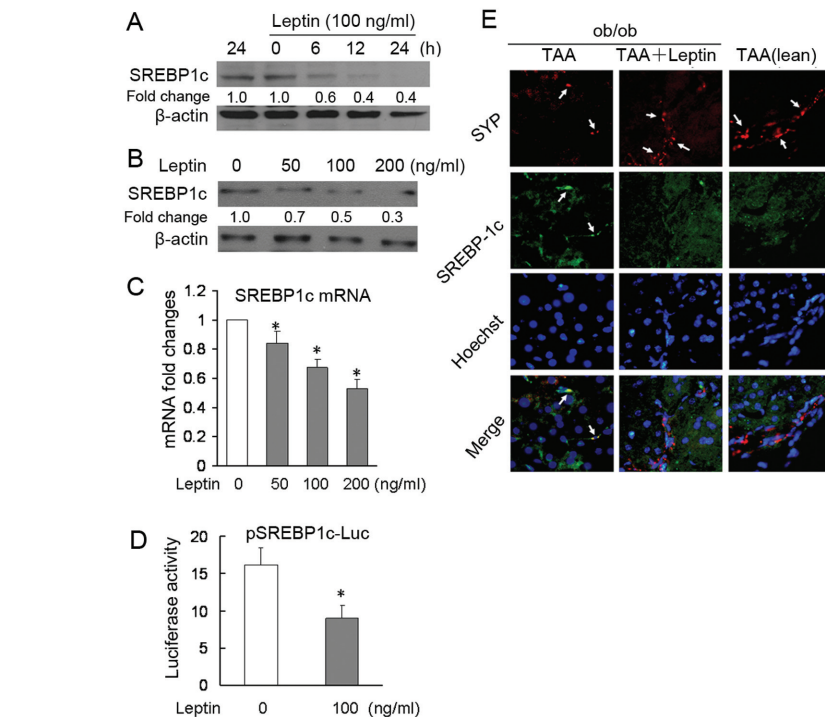


Figure 1. Leptin reduces SREBP-1c gene expression in HSCs *in vitro* and *in vivo*. (A–C) Western blot and real-time PCR analysis of the protein and mRNA levels of SREBP-1c, respectively ($n = 3$). HSCs were stimulated with leptin at the indicated doses for different periods of time (A) or 24 h (B, C). * $P < 0.05$ versus cells without leptin. (D) Transfection assay for analysis of SREBP-1c promoter activity. HSCs in 12-well plastic plates were transfected with pSREBP-1c-Luc and treated with or without 100 ng/mL leptin for 24 h ($n = 6$). * $P < 0.05$ versus cells without leptin. (E) Immunofluorescence staining of liver sections for SREBP-1c in HSCs. C57BL/6J ob/ob mice and lean littermates were received TAA (200 μ g/g body weight, two times a week) or TAA plus leptin (leptin, 1 μ g/g body weight) for 4 wks. Double immunofluorescence staining of liver sections for synaptophysin (SYN, red fluorescence) and SREBP-1c (green fluorescence) were performed ($n = 6$). The nuclei were counterstained with Hoechst 33342 (blue fluorescence) (original magnification, 200 \times).

the ratio: $[2^{-(Ct_{input} - Ct_{ChIP})_{treatment}}] / [2^{-(Ct_{input} - Ct_{ChIP})_{without treatment}}]$, where Ct ChIP is the Ct value corresponding to the immunoprecipitated DNA, and Ct input is the Ct value of an aliquot of digested chromatin sample before immunoprecipitation.

Statistical Analysis

Differences between means were evaluated using an unpaired two-sided Student *t* test ($P < 0.05$ was considered significant). Where appropriate, comparisons of multiple treatment conditions with controls were analyzed by analysis of variance with the Dunnett test for *post hoc* analysis.

RESULTS

Exogenous Leptin Reduces SREBP-1c Gene Expression in HSCs *In Vitro* and *In Vivo*

To investigate whether leptin regulates SREBP-1c gene expression in HSCs, the cultured HSCs were stimulated with or without leptin. Results indicated that leptin clearly downregulated SREBP-1c protein level in a time-dependent manner (Figure 1A) and a dose-dependent manner (Figure 1B) and was also shown to reduce SREBP-1c mRNA level (Figure 1C) in cultured HSCs. Leptin at 100 ng/mL reduced SREBP-1c protein level and SREBP-1c mRNA level, respectively,

by about 50% (see Figure 1B) and 30% (see Figure 1C) compared with the respective control without treatment (the first band on the left in Figure 1B and the first column on the left in Figure 1C). To elucidate whether leptin has an effect on SREBP-1c promoter activity, HSCs were transfected with a SREBP-1c promoter luciferase reporter plasmid pSREBP1c-Luc and incubated with or without leptin. As shown in Figure 1D by luciferase assays, leptin at 100 ng/mL reduced the luciferase activity by 45% (see Figure 1D) compared with the control (without leptin treatment).

Next, we adopted a mouse model of TAA-induced liver damage (16) to further examine the effect of leptin on SREBP-1c expression in HSCs in liver. The mice were treated with TAA or TAA plus leptin for 4 wks as described in Materials and Methods. Immunofluorescence staining of liver sections for SREBP-1c in HSCs was performed. Figure 1E showed the representative photomicrographs of immunofluorescence analysis for SREBP-1c and SYP in the liver sections. In the TAA group (ob/ob), the SREBP-1c protein (green fluorescence) was detectable in HSCs (red fluorescence). In the TAA plus leptin group (ob/ob) and TAA group (lean), SREBP-1c-positive HSCs were barely detectable, although there were more HSCs (red fluorescence). These double-staining results indicated that leptin also inhibited SREBP-1c protein expression in HSCs *in vivo*. Collectively, these results strongly suggested that leptin could exert an inhibitory role in SREBP-1c expression in HSCs *in vitro* and *in vivo*.

p38 MAPK Mediates the Inhibitory Effect of Leptin on SREBP-1c Gene Expression in Cultured HSCs

p38 MAPK promotes HSC activation (24,25) and the time course showed that leptin treatment led to the activation of p38 MAPK (Figure 2A). To examine whether p38 MAPK was involved in the effect of leptin on SREBP-1c expression, SB203580 (a specific p38 MAPK inhibitor) was used to interrupt the leptin-

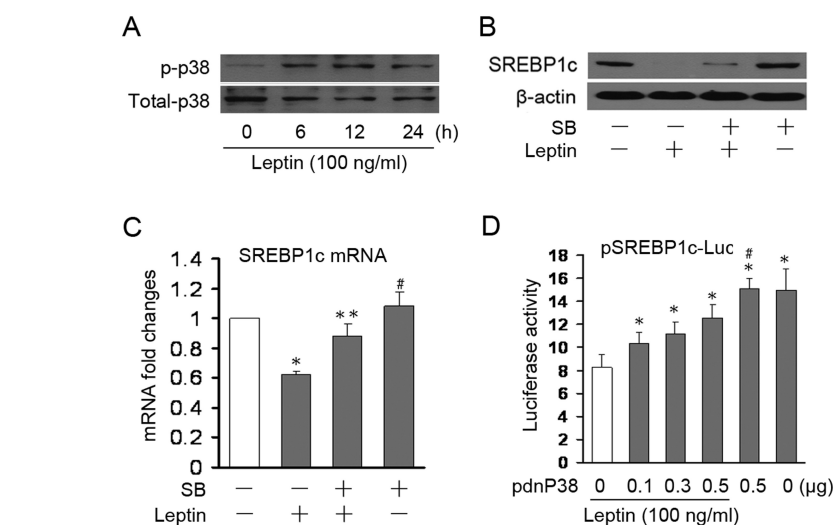


Figure 2. p38 MAPK mediates the inhibitory effect of leptin on SREBP-1c gene expression in cultured HSCs. (A) Western blot analysis showed leptin-induced activation of p38 MAPK (p-p38) (n = 3). (B, C) Western blot and real-time PCR analysis of SREBP-1c protein (B) and mRNA (C) levels, respectively (n = 3). HSCs were pretreated with SB203580 (SB) at 10 μmol/L for 30 min before incubation with or without leptin for an additional 24 h. **P* < 0.05, #*P* > 0.05 versus cells without treatment; ***P* < 0.05 versus cells with leptin alone. (D) Transfection assay for analysis of SREBP-1c promoter activity. HSCs in 12-well plastic plates were transfected with a fixed amount of a DNA mixture (including pSREBP1c-Luc, pdnP38, pRL-TK and the empty vector) per well and treated with 100 ng/mL leptin for 24 h (n = 6). The empty vector was used to ensure the equal amount of total DNA in transfection assay. **P* < 0.05 versus the control with leptin alone (the first column on the left). #*P* > 0.05 versus the cells without pdnP38 and leptin (the first column on the right).

induced p38 MAPK signaling pathway. HSCs were pretreated with or without SB203580 before incubation with or without leptin for an additional 24 h, and the protein and mRNA levels of SREBP-1c were evaluated by Western blot and real-time PCR, respectively. Figures 2B and 2C indicate that the inhibitory effects of leptin on the protein and mRNA levels of SREBP-1c were partially counteracted by blockade of p38 MAPK pathway with SB203580. To further test the result, a dominant-negative p38α MAPK expression plasmid pdnP38 MAPK were cotransfected into HSCs with plasmid pSREBP1c-Luc. Figure 2D demonstrated that the inhibition of leptin-induced activation of p38 MAPK pathway by a different concentration of pdnP38 increased SREBP-1c promoter activity in a dose-dependent manner. Compared with the control without treatment (the first column on the right),

pdnP38 alone had no effect on SREBP-1c promoter activity.

Taken together, the results shown in Figure 2 revealed that leptin reduced SREBP-1c expression at least through p38 MAPK in cultured HSCs.

Leptin Inhibits SREBP-1c Gene Expression Through Downregulation of LXRα Protein Level and Activity in Cultured HSCs

It has been shown that the transcription factors including NF-Y, Sp1 and LXRα can promote SREBP-1c gene expression through binding to their binding sites in the region of SREBP-1c promoter in hepatocytes (21). On the basis of the observations, we first investigated the effects of leptin on the activities of NF-Y, Sp1 and LXR in HSCs. HSCs were transfected with plasmid pSP1-Luc (containing three consensus Sp1 binding sites), plasmid pNFY-Luc (containing

three consensus NF-Y binding sites) or plasmid pLXRE-Luc (containing three tandem LXR binding sites) and incubated with leptin at increasing doses for 24 h. Transfection assays indicated that leptin evidently increased Sp1 activity (Figure 3A) and reduced LXR activity (Figure 3D), but had no influence on NF-Y activity (Figure 3C) in HSCs.

There are two subtypes of LXR: LXR α and LXR β . LXR α is mainly expressed in some tissues including liver, whereas LXR β is ubiquitously expressed (26). Next, the roles of Sp1 and LXR α in leptin regulation of SREBP-1c activity were explored. After the wild-type Sp1 expression plasmid pSP1 (Figure 3B) or the wild-type LXR α expression plasmid pLXR α (Figure 3E) were cotransfected into HSCs with pSREBP1c-Luc, cells were incubated with or without 100 ng/mL leptin for 24 h and the luciferase activities were measured. Transfection assays revealed that Sp1 had no effect on SREBP-1c promoter activity in HSCs; even the plasmid pSP1 was transfected into HSCs at different doses (see Figure 3B). However, 0.8 μ g pLXR α led to a sixfold increase in the luciferase activity over the control (the first column on the left) (see Figure 3E). This result in Figure 3E suggested that LXR α had a dramatic promotion effect on SREBP-1c promoter activity in HSCs *in vitro*.

Because leptin could inhibit LXR activity (see Figure 3D) and LXR α upregulated SREBP-1c promoter activity (see Figure 3E), it is possible that leptin might downregulate LXR α protein level in HSCs, leading to a decline in LXR α activity and, consequently, the decrease in SREBP-1c gene expression. Therefore, it was investigated whether leptin had an influence on LXR α protein level in cultured HSCs. HSCs were incubated with different doses of leptin for 24 h, and the protein levels of LXR α were determined by Western blot analysis. As shown in Figure 3F, 100 ng/mL leptin reduced LXR α protein level by 40% compared with the control (without treatment, the first band on the left).

Results in Figures 3D–F suggested that leptin might reduce SREBP-1c expression

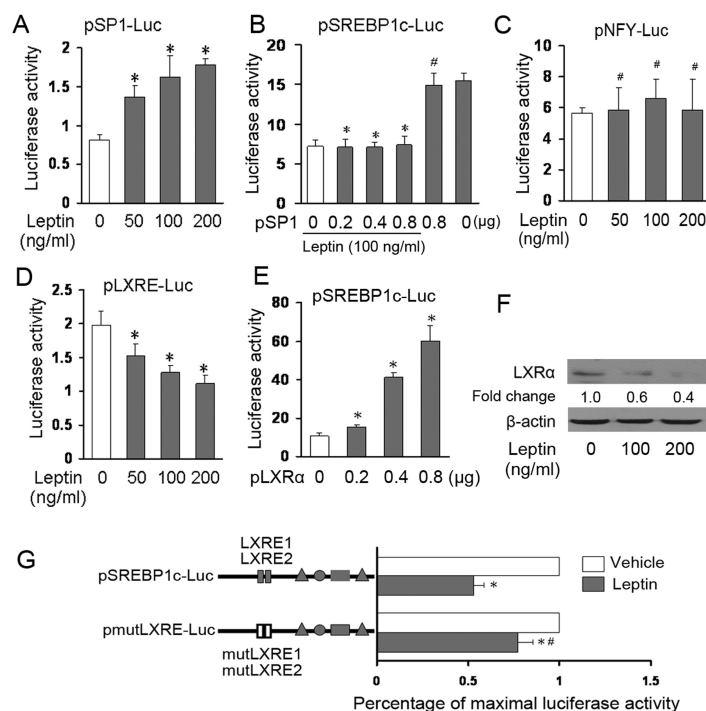


Figure 3. LXR α is involved in leptin inhibition of SREBP-1c expression in cultured HSCs. (A, C, D) Transfection assay for analysis of the activity of Sp1, NF-Y or LXR. HSCs were transfected with plasmid pSP1-Luc (A), pNFY-Luc (C) or pLXRE-Luc (D) and treated with leptin at increasing doses for 24 h (n = 6). *P < 0.05 (A, D), #P > 0.05 (C) versus the respective control without leptin. (B, E) Transfection assay for analysis of SREBP-1c promoter activity. HSCs were transfected with a fixed amount of a DNA mixture (including pSREBP1c-Luc, pSP1 or pLXR α , pRL-TK and empty vector) per well and treated with or without 100 ng/mL leptin for 24 h (n = 6). The empty vector was used to ensure the equal amount of total DNA in transfection assay. (P > 0.05 versus the control (the first column on the left) (B). #P > 0.05 versus cells without pSP1 and leptin (the first column on the right) (B). *P < 0.05 versus cells without pLXR α (E). (F) Western blot analysis of LXR α protein level (n = 3). HSCs were incubated with different doses of leptin for 24 h. (G) Transfection assay for analysis of the role of LXR binding sites in leptin inhibition of SREBP-1c promoter activity. HSCs were transfected with pmutLXRE-Luc or pSREBP1c-Luc and treated with or without 100 ng/mL leptin for 24 h. The luciferase activity in cells without leptin for each construct was expressed as 100% activity after Renilla luciferase activity normalization (n = 6). *P < 0.05 versus cells without leptin for each construct. #P < 0.05 versus cells with pSREBP1c-Luc (leptin treatment).

through inhibition of LXR α expression and activity in HSCs. This result was further tested by mutation of LXR binding sites in the SREBP-1c promoter. The plasmid pmutLXRE-Luc (created by inserting SREBP-1c promoter with mutation of two LXR-binding sites into the luciferase reporter plasmid [21]) or pSREBP1c-Luc was transfected into HSCs, and the cells were stimulated with or without leptin for 24 h. The normalized luciferase activities were expressed as the fold-changes over the respective control value (without

leptin, the empty column). As shown in Figure 3G, the luciferase activity in cells with leptin (the solid column) decreased compared with the respective control, but the luciferase activity in cells with pmutLXRE-Luc (leptin treatment, the solid column) was 1.45-fold over that in cells with pSREBP1c-Luc (leptin treatment, the solid column), suggesting that the LXR binding sites were required for leptin inhibition of SREBP-1c gene expression in HSCs.

Collectively, the data in Figure 3 demonstrated that leptin inhibited

SREBP-1c expression, at least partly, through downregulation of LXR α protein level and activity in HSCs.

p38 MAPK Mediates Leptin-Induced Reduction of the Protein Level and the Activity of LXR α and the Binding of LXR α to SREBP-1c Promoter in Cultured HSCs

Because p38 MAPK and LXR α were involved in the inhibitory effect of leptin on SREBP-1c gene expression in HSCs (Figures 2 and 3), the relationship between p38 MAPK and LXR α gene expression was examined. HSCs were pretreated with SB203580 at different doses for 30 min before incubation with or without leptin (100 ng/mL) for an additional 24 h, and the protein levels of LXR α were determined by Western blot. Figure 4A revealed that blockade of leptin-induced p38 signaling pathway by SB203580 evidently increased LXR α protein levels in a dose-dependent manner, and SB203580 alone had no effect on LXR α protein level, suggesting that leptin could downregulate LXR α protein level by activation of p38 MAPK in cultured HSCs. To confirm the result, the plasmid pwtP38 or pdnP38 was cotransfected into cultured HSCs with pLXRE-Luc, and cells were then incubated with or without 100 ng/mL leptin for 24 h. As shown in Figure 4B by luciferase assays, compared with the control (the first column on the left), the activation of p38 α MAPK pathway by pwtP38 plus leptin (the second column on the left) dramatically reduced luciferase activity, whereas inhibition of leptin-induced p38 α MAPK pathway by pdnP38 (the third column on the left) increased luciferase activity. pwtP38 or pdnP38 alone had no effect on the luciferase activity. These results in Figure 4B indicated that p38 α MAPK activation by leptin contributed to the inhibition of LXR activity in HSCs.

Leptin reduced the protein level and activity of LXR α through p38 MAPK (see Figures 4A, B), and LXR α was required for leptin inhibition of SREBP-1c gene expression in cultured HSCs (see Figure 3). These results prompted us to investigate

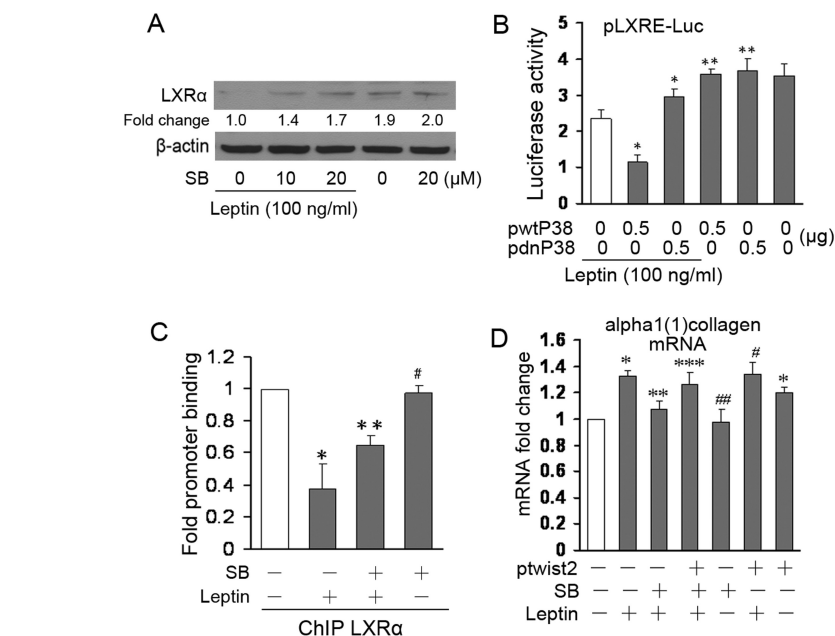


Figure 4. p38 MAPK mediates leptin reduction of LXR α protein level and activity and LXR α binding to SREBP-1c promoter and the effect of leptin-induced inhibition of SREBP-1c expression on α 1(I) collagen transcription in cultured HSCs. (A) Western blot analysis of LXR α protein level. HSCs were pretreated with SB203580 (SB) at different doses for 30 min before incubation with or without leptin (100 ng/mL) for an additional 24 h (n = 3). (B) Transfection assay for evaluating LXR activity. HSCs were cotransfected with pLXRE-Luc, pwtP38 or pdnP38 and the empty vector and treated with or without 100 ng/mL of leptin for 24 h (n = 6). The empty vector was used to ensure the equal amount of total DNA in transfection assay. * P < 0.05 versus the control (the first column on the left). ** P > 0.05 versus cells without pwtP38, pdnP38 and leptin (the first column on the right). (C) ChIP assay for examining the binding of LXR α to SREBP-1c promoter. After HSCs were maintained in DMEM containing the adipogenic differentiation cocktail (MDI: 0.5 mmol/L isobutylmethylxanthine, 1 μ mol/L dexamethasone and 1 μ mol/L insulin) with 0.4% FBS for 24 h, cells were switched to the medium with or without SB203580 (10 μ mol/L) and pretreated for 30 min before incubation with or without 100 ng/mL leptin for an additional 24 h. Chromatin fragments were immunoprecipitated by anti-LXR α antibody. Real-time PCR analysis was used to quantify a 260-bp segment of rat SREBP-1c promoter (n = 3). * P < 0.05, # P > 0.05 versus cells without treatment. ** P < 0.05 versus cells with leptin alone (the second column on the left). (D) Real-time PCR analysis of α 1(I) collagen mRNA level. HSCs in a 25-cm² flask were transfected with 8 μ g plasmid ptwist2 or empty vector. The empty vector was used to ensure the equal amount of total DNA. After incubation with MDI for 24 h, cells were switched to the medium with or without SB203580 (10 μ mol/L) and pretreated for 30 min before incubation with or without leptin (100 ng/mL) for an additional 24 h (n = 3). * P < 0.05, ### P > 0.05 versus cells with no treatment (the first column on the left). ** P < 0.05, # P > 0.05 versus cells with leptin alone (the second column on the left). *** P < 0.05 versus cells with SB plus leptin (the third column on the left).

the effects of leptin and p38 MAPK on the binding of LXR α to SREBP-1c promoter *in vivo* by using ChIP assay. After HSCs were maintained in DMEM containing the adipogenic differentiation cocktail (MDI) (0.5 mmol/L isobutylmethylxanthine, 1 μ mol/L dexametha-

sone and 1 μ mol/L insulin) with 0.4% FBS for 24 h, which was used to increase the expression of the LXR α gene in cultured HSCs (14), cells were switched to the medium containing SB203580 (10 μ mol/L) for 30 min before incubation with or without 100 ng/mL leptin for an

additional 24 h. ChIP assays were performed as described in Materials and Methods. DNA was recovered after immunoprecipitation with anti-LXR α antibody and used as a template for real-time PCR analysis of a 260-bp segment of the rat SREBP-1c promoter corresponding to nucleotides -267 and -8 containing the two LXR-binding sites. ChIP assays (Figure 4C) demonstrated that leptin strongly inhibited LXR α binding to SREBP-1c gene promoter, which was partially eliminated by the p38 MAPK inhibitor SB203580.

Taken together, these results in Figure 4, combined with the results in Figure 3, suggested that p38 MAPK mediated leptin-induced reduction of LXR α protein level, activity and its binding to SREBP-1c promoter, leading to the decline in SREBP-1c expression in cultured HSCs.

Leptin-Induced Inhibition of SREBP-1c Expression Leads to the Increase in Gene Expressions of Alpha1(I) Collagen in HSCs

Alpha1(I) collagen expression is dramatically improved with HSC activation, which is inhibited by SREBP-1c expression (14). It is of interest to examine the effect of leptin-induced decline in SREBP-1c expression on alpha1(I) collagen expression. HSCs in a 25-cm² flask were transiently transfected with or without the plasmid ptwist2 encoding twist2, a potent repressor for SREBP-1c activity (22). After incubation with MDI (MDI was used to reduce alpha1(I) collagen expression in HSCs [14]) for 24 h, cells were switched to the medium containing SB203580 (10 μ mol/L) and pretreated for 30 min before incubation with or without leptin (100 ng/mL) for an additional 24 h. Figure 4D indicated that leptin alone increased alpha1(I) collagen mRNA level compared with the control (the first column on the left). Pretreatment with SB203580 attenuated leptin-induced increase in alpha1(I) collagen mRNA level (the third column on the left), which was partially counteracted by ptwist2 transfection (the middle column). Compared with the cells treated

with leptin alone (second column on the left), ptwist2 had no effect on alpha1(I) collagen mRNA level in the presence of leptin alone (the second column on the right). These results indicated that leptin-induced decline in SREBP-1c expression via p38 MAPK increased alpha1(I) collagen expression in HSCs.

DISCUSSION

Key transcription factors play pivotal roles in cell transdifferentiation. The changes in the expression and/or activity of key transcription factors induce a global reprogramming of cell gene expression, which results in cell transdifferentiation. Quiescent HSCs contain ample neutral lipids much like adipocyte (11), and their activation, a key step in the development of liver fibrosis, leads to the depletion of lipid droplets and transdifferentiation from quiescent to myofibroblastic phenotype. The process of HSC activation is analogous to the reverse transdifferentiation of adipocyte to preadipocyte (fibroblast) (11). Therefore, it seems that transcription factors associated with upregulation of lipid synthesis might play important roles in inhibition of HSC activation. In fact, SREBP-1c, a key transcription factor for the promotion of lipid synthesis and adipocyte differentiation (12,27), exerts a pivotal role in suppression of HSC activation (14) and is highly expressed in quiescent HSC and downregulated on HSC activation (14). Hence, it is important to find the factors that affect SREBP-1c expression and reveal the corresponding molecular mechanisms in HSCs. Leptin, a key hormone in regulating energy homeostasis, inhibits FA uptake and synthesis, stimulates FA mobilization and oxidation and reverses adipocyte differentiation (2,3). More importantly, leptin plays a unique role in promotion of liver fibrosis (4–10). Our results demonstrated that leptin strongly inhibited SREBP-1c expression in HSCs *in vivo* and *in vitro*. p38 MAPK was involved in leptin regulation of SREBP-1c expression in cultured HSCs. Leptin-induced activation of p38 MAPK led to a decrease in LXR α protein level

and activity and LXR α binding to an SREBP-1c promoter, which caused the downregulation of SREBP-1c expression. Moreover, leptin-induced inhibition of SREBP-1c expression via p38 MAPK led to the expression of alpha1(I) collagen in HSCs. These results demonstrated the relationship between leptin and the gene expression of SREBP-1c in HSCs *in vivo* and *in vitro* and revealed the involvement of p38 MAPK and LXR α in leptin regulation of SREBP-1c expression in cultured HSCs. Nogalska *et al.* (28) found that the increase of leptin gene expression could account for the reduced SREBP-1c gene expression in white adipose tissue of old animals. This result supports the effect of leptin on SREBP-1c expression in HSCs.

Our previous studies indicated that leptin inhibited PPAR γ expression, another important transcription factor for lipid synthesis and adipocyte differentiation (12), in HSCs, leading to HSC activation and alpha1(I) collagen expression (10,19). These findings, together with the data in the current report, seem to suggest that leptin might contribute to liver fibrosis, at least in part, through downregulation of the expression of key transcription factors involved in lipid synthesis in HSCs.

p38 MAPK is associated with promotion of liver fibrosis (24,25). Our results clearly demonstrated that p38 MAPK mediated leptin-induced inhibition of SREBP-1c expression in cultured HSCs. The activation of p38 MAPK by leptin led to a decline in SREBP-1c protein and mRNA levels and SREBP-1c promoter activity in cultured HSCs (see Figure 2). Furthermore, it was indicated that p38 MAPK mediated leptin-induced inhibition of SREBP-1c expression through downregulation of LXR α expression and activity and LXR α binding to SREBP-1c promoter in cultured HSCs. Moreover, the inhibition of SREBP-1c expression by leptin-induced activation of p38 MAPK caused alpha1(I) collagen expression in HSCs (see Figure 4D). The role of p38 MAPK in leptin-induced inhibition of SREBP-1c expression was in line with the

effect of p38 MAPK on liver fibrosis and might provide a new insight into the mechanisms underlying the promotional role of p38 MAPK in liver fibrosis.

LXR α , Sp1 and NF-Y are involved in promoting the expression of SREBP-1c in rat hepatocytes (21). Results in the present report revealed that LXR α , but not Sp1 and NF-Y, appeared to be involved in leptin-induced inhibition of SREBP-1c expression in cultured HSCs. Sp1 activation exerts a positive action on liver fibrosis (29), whereas SREBP-1c plays a key role in inhibition of HSC activation (14). Therefore, it might be reasonable that Sp1 could not cause SREBP-1c promoter activity in HSCs (see Figure 3B). The data in this report could not explain the mechanisms by which leptin-induced Sp1 activation had no effect on SREBP-1c promoter activity in HSCs, whereas Sp1 can contribute to SREBP-1c promoter activity in hepatocytes (21). The mechanisms might be correlated with the different coregulators in hepatocytes and HSCs.

LXR α was found to be involved in leptin-induced inhibition of SREBP-1c expression in HSCs (see Figures 3 and 4). First, leptin-stimulated activation of p38 MAPK reduced both the expression of LXR α and the activity of LXR α in HSCs (see Figures 4A, B). Second, the expression of LXR α induced SREBP-1c promoter activity (see Figure 3E), and LXR binding sites were required for leptin inhibition of SREBP-1c promoter activity (see Figure 3G). Third, ChIP experiments directly revealed that p38 MAPK activation by leptin reduced LXR α binding to SREBP-1c promoter in HSCs (see Figure 4C). These results strongly suggested that leptin inhibited SREBP-1c expression through reduction of LXR α protein level and activity and LXR α binding to SREBP-1c promoter in HSCs. Of interest is that LXR plays a central role in insulin-mediated activation of SREBP-1c transcription in liver (30). In addition, in many mouse tissues including liver, SREBP-1c expression is markedly increased in an LXR-dependent manner by synthetic agonists for both LXR and its heterodimer partner, whereas the expres-

sions of the related gene products, SREBP-1a and SREBP-2, are not increased (31). These findings point to an important role for LXR α , a principal LXR isoform in liver (26), in regulation of SREBP-1c expression in HSCs. Leptin regulation of LXR α might play a key role in its inhibition of SREBP-1c expression in HSCs.

CONCLUSION

In summary, leptin stimulated p38 MAPK activation, leading to the reduction of LXR α expression and activity and LXR α binding to the SREBP-1c promoter, which contributed to the decline in SREBP-1c expression and the consequent increase in α 1(I) collagen expression in HSCs. It bears emphasis that our results and this model do not exclude any other mechanisms of leptin-induced suppression of SREBP-1c expression in HSCs *in vitro* and *in vivo*. The concentration of leptin in liver is still unknown. A total of 100 ng/mL leptin was mainly used in the present experiments. Our studies cannot rule out the possibility that there is a higher or lower concentration of leptin in the patient's liver.

Hepatic fibrosis has been shown to be six times more prevalent in obese individuals compared with general population (32,33), and hyperleptinemia is a common finding in obese patients (34). Leptin is strongly correlated with liver fibrosis (4–10). In humans, an association between serum leptin levels and hepatic fibrosis liver diseases was suggested but never firmly established (35). Unlike the mouse model of obesity (for example, ob/ob mice), obese individuals present elevated leptin levels indicating resistance to leptin rather than a deficiency. Insulin resistance, which shares several similarities with leptin resistance, occurs in a cell-specific fashion. It has not been ascertained whether leptin resistance is extended to human HSCs *in vivo*, but at this stage, no data support the presence of leptin resistance in human HSCs *in vitro* (6,7). The results of the present study might provide new insights into the mechanisms of the unique role of leptin in the development

of liver fibrosis and might have potential implications for clarifying the molecular mechanisms underlying liver fibrosis in diseases in which circulating leptin levels are elevated, such as nonalcoholic steatohepatitis (one of the major consequences of the obesity epidemic) (36), type 2 diabetes mellitus (37) and alcoholic cirrhosis (38). Recombinant leptin has been proposed to be used in common types of obesity or nonalcoholic fatty liver disease (39). On the basis of the results of present research, recombinant leptin administration might have serious adverse therapeutic drawbacks (39), because it may further increase the already elevated serum leptin levels.

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DISCLOSURE

The authors declare that they have no competing interests as defined by *Molec-*

ular Medicine, or other interests that might be perceived to influence the results and discussion reported in this paper.

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