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Physiologic concentrations of leptin increase collagen production by non-immortalized human hepatic stellate cells

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Abstract

The effects of leptin, in concentrations seen in obesity, on collagen production and turnover in non-immortalized human hepatic stellate cell (HSC), were unknown. The profibrogenic effects of leptin in these cells were studied. Hepatic stellate cells were obtained from resected livers. Collagen I/III gene expression and protein production were measured by quantitative real-time polymerase chain reaction and sodium dodecyl sulfate-polyacrylamide gel electrophoresis, respectively. The signal transduction pathways involved were evaluated by specific blockers of the phosphatidylinositol 3-kinase (PI3K), mitogen-activated protein kinase kinase (MEK), and Janus kinase 2 (JAK2). The effects on matrix metalloproteinase 1 (MMP-1) and tissue inhibitor of metalloproteinase 1 (TIMP-1) were assessed by their gene transcript levels, collagenolytic activity of cell culture supernatants, and MMP-1 protein levels. At concentrations seen in nonobese individuals ([leptin] < 10 ng/mL), leptin did not affect collagen production. At concentrations seen in obesity (30-50 ng/mL), leptin increased collagen I and III messenger RNA (mRNA) transcript levels by 286% ± 55% ($P < .001$) and 167% ± 62% ($P < .007$) and protein production by 45.8% ± .02% and 84.39% ± .01%, respectively. These effects were blocked by JAK2 inhibition as well as PI3K inhibition. Although MEK inhibition blocked leptin-induced procollagen I and III mRNA levels, there were no significant effects on collagen I and III protein levels. Leptin (10-50 ng/mL) had no significant effects on MMP-1 or TIMP-1 mRNA levels, collagenolytic activity, or MMP-1 protein levels. In conclusion, leptin, at levels seen in obese individuals, produces an increase in collagen production by HSC acting through the JAK and PI3K pathways. At these concentrations, leptin does not affect MMP-1 or TIMP-1 expression or collagenolytic activity of HSC.

1. Introduction

Cirrhosis of the liver is a major cause of morbidity and mortality worldwide. The progression of chronic liver diseases to cirrhosis is characterized by the increasing deposition of a collagenous matrix, that is, fibrosis in the liver. Two of the most common causes of chronic liver disease in North America are nonalcoholic steatohepatitis and HCV infection. In both conditions, the concomitant presence of obesity is associated with increased hepatic fibrosis [1-3]. Obesity is associated with hyperleptinemia [4,5].

In a mouse model of liver injury, leptin was found to be important for the development of hepatic fibrosis [6]. At pharmacologic doses (100-1000 ng/mL), leptin also increased collagen production by immortalized hepatic stellate cells (HSCs) and primary cultures of rat HSC [7,8]. It is however unknown whether leptin, at concentrations seen in obesity, affect collagen production by non-immortalized human HSC. In addition, although the phosphatidylinositol 3-kinase (PI3K), Janus kinase 2 (JAK2), and mitogen-activated protein kinase (MAPK) pathways are all involved in signal transduction after leptin binding to its receptor [9,10], the specific pathways used for leptin's effects on collagen production were unknown.

Matrix production may also be affected by its turnover. In an immortalized LX2 cell line, leptin increased gene expression of the tissue inhibitor of matrix metalloproteinase 1 (TIMP-1) [11]. It was unclear if these effects were also present in non-immortalized activated HSC. Further-

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more, the effects of leptin on matrix metalloproteinase 1 (MMP-1) expression and activity were unknown.

The objectives of this study were to define the effects of leptin, at concentrations typical in obesity, on (1) collagen production and the signaling pathways involved in such effects, and (2) MMP-1 and TIMP-1 expression and collagenolytic activity of non-immortalized human HSC.

2. Materials and methods

2.1. Human HSCs

Human HSCs were obtained from (1) normal liver surrounding resected colon cancer metastases and (2) explants of cirrhotic livers of subjects with hepatitis C at the time of liver transplantation. Hepatic stellate cells were isolated and separated using standard techniques that have been previously described [12]. Cells were maintained in culture using Dulbecco modified eagle medium (DMEM) (Cellgro, Herndon, VA) with 10% calf serum, penicillin (100 U/mL), and streptomycin (100 μg/mL). Primary cultures were frozen down and thawed out for these experiments. The purity of the cultures was confirmed by demonstration of smooth muscle α-actin in more than 95% of cells. The cell viability of all cultures was greater than 90% as assessed by exclusion of propidium iodide.

2.2. Effects on collagen production

Human recombinant leptin (Sigma, St Louis, MO) was used for these studies. Its purity was more than 97% based on sodium dodecyl sulfate (SDS) electrophoresis with negligible contamination with endotoxin (<0.1 ng/μg leptin). Hepatic stellate cells were grown to 90% confluence and then exposed to DMEM, ascorbic acid (0.1 mmol/L), penicillin (100 U/mL), and streptomycin (100 μg/mL) with or without leptin (10-50 ng/mL). After incubation for 18 hours, the supernatant was removed and the cells isolated for procollagen messenger RNA (mRNA) and collagen protein quantification. Cells exposed to serum-free media were used as controls for all sets of experiments.

2.3. Quantification of procollagen α(I) and (III) mRNA

Quantitative real-time polymerase chain reaction (qPCR) was used to measure mRNA in HSC. Total RNA was initially isolated using a Trizol (Invitrogen, CA) extraction protocol and quantified spectrophotometrically (Lambda 25 UV/VIS spectrophotometer, Perkin Elmer, Wellesley, MA) at 260- to 290-nm wavelength. Complementary DNA was prepared and then amplified using specific primers for collagen I (forward—TCAGAATTTCACCAAACGAAGGT, reverse—GCTGGAAAGTGGAGAAGGTCTT) and collagen III (forward—TCTTGGTCAGTCCTATGCGATA, reverse—CATCGCAGAGAACGGATCCT). The levels of procollagen I and III mRNA were normalized to 18S ribosomal RNA and expressed in this manner.

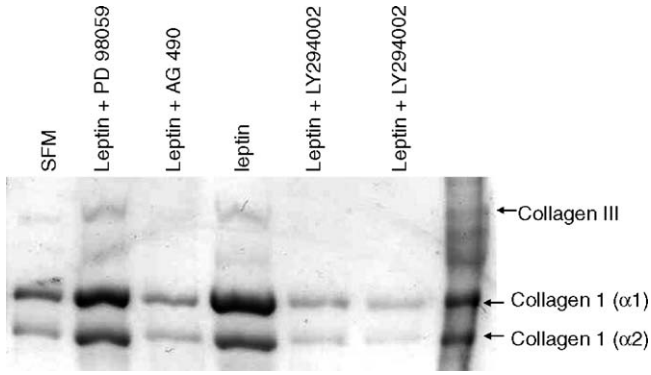

Fig. 1. Collagen I and III proteins stained with Coomassie blue after separation in 7% SDS page gel are shown. The loaded acid-soluble, pepsin-resistant proteins were subjected to delayed reduction with dithiothreitol midway through the running process for separation of the collagen bands. Compared with serum-free media (SFM), leptin (30 ng/mL) increased both collagen I and III, an effect that was abrogated after inhibition of the PI3 kinase pathway with LY294002 and JAK2 by AG490 but not by MEK inhibition with PD98059. Pure human collagen I and III run simultaneously served as internal controls.

2.3.1. Collagen estimation

After overnight incubation with serum-free media or serum-free media and leptin, the media was collected and one portion treated with 0.1 N acetic acid and another with 0.1 N acetic acid and pepsin. The sample treated with acetic acid alone was incubated overnight and then centrifuged at 7000*g* for 40 minutes. Total protein was quantified from the supernatant using the Bio-Rad microplate assay protocol (Bio-Rad Laboratories, Hercules, CA) [13]. Procollagens were extracted from the pepsin-treated media by addition of an equal volume of isopropyl alcohol and centrifugation (7000*g* × 40 minutes). The collagen-containing pellet was air-dried and dissolved in gel loading buffer (100 mmol/L Tris-Cl, pH 6.8, 1% SDS, and 5% glycerol) after discarding the supernatant. Procollagens I and III were separated using SDS page electrophoresis. Collagen proteins were resolved by a delayed reduction technique using dithiothreitol as described previously [14]. The gels were stained with Coomassie blue, and the optical density of the individual bands were measured using Psion image software (version beta 4.0.2, National Institutes of Health, Bethesda, MD) (Fig. 1). Equal protein loading of each lane was achieved by adjusting the columns based on protein concentrations of the supernatants.

2.4. Characterization of leptin-induced signaling pathways

The signaling pathways used by leptin to influence collagen production were defined by examining the effect of specific blockers of the PI3K, JAK2, and the downstream effector of the MAPK pathway MEK [9,10,15]. Phosphatidylinositol 3-kinase was blocked by LY294002 (Calbiochem, San Diego, CA), whereas JAK2 and MEK were blocked by AG490 (Calbiochem) and PD98059 (Calbiochem), respectively [15-17]. When HSC (passage 1-2) reached 90% confluence, the media was changed to DMEM,

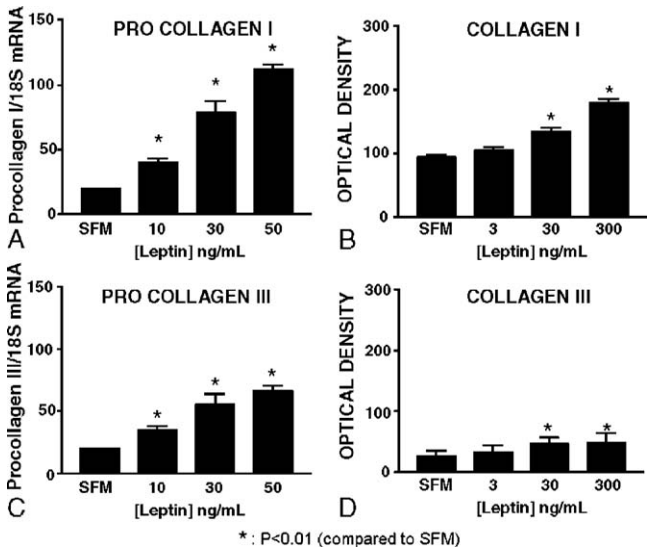

Fig. 2. Leptin induced increase in collagen gene expression (A and C) and protein production (B and D) in human HSC. A, Procollagen I gene expression as measured by real-time PCR and expressed as a ratio of procollagen I gene normalized to 18S mRNA after exposure to leptin in increasing dosage (10-50 ng/mL). B, Optical density of collagen I bands after delayed reduction in SDS gel after exposure to increasing leptin dosage (3-300 ng/mL). C, Procollagen III gene expression as measured by real-time PCR and expressed as a ratio of procollagen III gene normalized to 18S mRNA after exposure to leptin in increasing dosage (10-50 ng/mL). D, Optical density of collagen III bands after delayed reduction in SDS gel after exposure to increasing leptin dosage (3-300 ng/mL). Leptin increased procollagen I and III gene expression over a progressively increasing dose range with maximal effect seen at 50 ng/mL ($P < .01$). Similarly, collagen I and III protein production was affected maximally at a leptin concentration of 300 ng/mL ($P < .001$) with no significant changes observed at less than 10 ng/mL. Data from 5 normal and cirrhotic livers, respectively, performed in triplicate are shown as mean ± SEM.

2% calf serum, ascorbic acid (0.1 mmol/L), and leptin (30 ng/mL) along with either no additional substances (control) or one of the blocking agents noted above to specifically block the JAK, PI3K, and MAPK pathways. After overnight incubation (18 hours), procollagen mRNA and collagen production were quantified as noted above.

2.5. Effects of leptin on matrix metalloproteinases

The potential impact of leptin on MMP-1 and TIMP-1 was assessed by measurement of their transcript levels, collagenolytic activity of cell culture media, and protein levels of MMP-1 after exposure to leptin.

2.5.1. MMP-1 and TIMP-1 transcript levels

After exposure to cell culture media alone (control) or with additional leptin (30 ng/mL), MMP-1 and TIMP-1 mRNA expression was assessed using a quantitative RNase protection assay [18]. Matrix metalloproteinase 1 transcript levels were determined using a 356-nucleotide probe corresponding to nucleotides 827 to 1182 (GI:30125) of the MMP-1 transcript. Tissue inhibitor of metalloproteinase 1 transcript levels were determined using a 207-nucleotide probe corresponding to nucleotides 354 to 560 (GI:182482) of the TIMP-1 transcript. Briefly, linearized plasmids containing MMP-1, TIMP-1, or cyclophilin-specific sequences were used as templates for synthesis of phosphorus 32–labeled uridine monophosphate (^{32}P-UMP)–labeled antisense transcripts. The RNase protection assay was performed using the RPA III kit (Ambion, Austin, TX) using the manufacturer's protocol. Labeled probes were co-precipitated with RNA isolated from HSCs, resuspended in hybridization buffer, and after denaturation, allowed to anneal overnight at 42°C. After digestion with ribonuclease T1 to remove single-strand RNA, products were resolved by electrophoresis on 6% urea-acrylamide gels. Probes specific for cyclophilin were included in each reaction to normalize for RNA input.

2.5.2. Collagenolytic activity

Collagenolytic activity was assessed by measuring cleavage of ^{14}C-labeled type III collagen. This assay takes advantage of the ability of MMP-1 to preferentially use soluble type III collagen as a substrate [19]. Medium fractions were incubated with radiolabeled collagen in collagenase assay buffer (25 mmol/L Tris-Cl, 200 nmol/L NaCl, 3 mmol/L $CaCl_2$, 0.03% Brij-35, pH 7.5) at 30°C for 24, 48, and 96 hours. In some reactions, 1 mmol/L *p*-aminophenyl mercuric acetate (APMA) was included. APMA is an activator of MMP zymogens. Use of APMA allows the measurement of actual and potential collagenolytic activity in a sample. Products were resolved on 6% SDS-polyacrylamide gels. The gels were acid-fixed, dried, and exposed to film. Collagenase activity was determined by quantifying the TC^A products generated by cleavage of the type III collagen. After electrophoresis, gels were prepared for fluorography by fixing in 10% acetic acid and 25% isopropanol for 15 minutes and soaking it in Amplify (Amersham, Arlington Heights, IL) for 15 minutes. Dried gels were exposed to Hyperfilm-MP (Amersham) at −80°C. Reference gels containing known amounts of ^{14}C-labeled collagen were used to generate equations for converting scanned units into micrograms of degraded collagen.

2.5.3. Matrix metalloproteinase 1 levels

Matrix metalloproteinase 1 levels were measured by use of sandwich enzyme-linked immunosorbent assay (ELISA) kits (Sigma Aldrich, St. Louis, MO) using known concentrations of MMP-1 obtained from wound fluids as internal standards. The absorbance was measured at 340 nm following standard ELISA protocol with all experiments done in triplicate.

2.6. Statistical analysis

Data from the experiments were compared using Student *t* test when 2 groups were being compared and by using analysis of variance when more than 2 groups were analyzed. A *P* value of less than .05 was considered to be significant, and individual sample data are presented as mean ± SEM, where n represents the number of experiments.

3. Results

The effects of leptin were studied in HSC derived from 5 normal and 6 cirrhotic human livers. All subjects with cirrhosis had hepatitis C. The studies were performed in consecutive subjects with HCV-related cirrhosis who underwent liver transplant. Tissue was obtained from the explants and from normal liver adjacent to metastatic colon cancer at the time of hepatic resection for the latter. The effects of leptin on collagen production from HSC derived from cirrhotic livers were higher than from normal livers, but this did not reach significance. Moreover, the effects of leptin on HSC from normal livers were qualitatively identical to those from cirrhotic livers. The data from normal and cirrhotic livers have therefore been pooled for presentation below.

3.1. Effects of leptin on collagen production

Leptin (10-50 ng/mL) produced a dose-dependent increase in both collagen I and III transcript levels (Fig. 2). Compared with serum-free media, leptin produced a dose-dependent increase in procollagen I mRNA by (mean ± SEM) 105% ± 20.6%, 286.5% ± 55%, and 463% ± 23% ($P < .001$ for all) at concentrations of 10, 30, and 50 ng/mL, respectively. Similarly, procollagen III mRNA levels increased by 78.2% ± 2.1%, 167% ± 6.2%, and 228% ± 3.0% ($P < .001$ for all) after exposure to leptin at the same concentrations. At leptin concentrations of 30 ng/mL and higher, there was a significant increase in collagen I and III protein production. There was a modest additional increment in collagen I formation over a log-dose range (73.2% ± 1.5% at 300 ng/mL of leptin). Similarly, collagen III production increased by 84.4% ± 0.1% and 85.6% ± 0.02% ($P < .01$ compared with serum-free media). In absolute terms, both baseline and leptin-induced collagen I production was greater than collagen III.

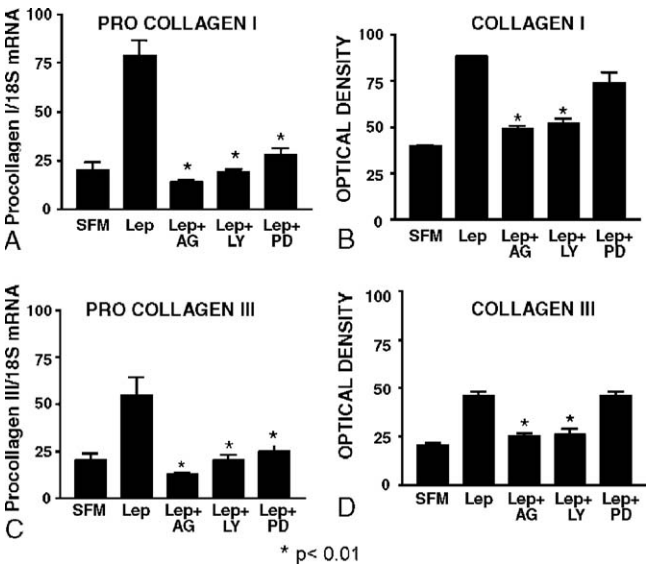

Fig. 3. The effects of inhibition of JAK by AG490 (AG), PI3 kinase by LY294002 (LY), and MEK by PD98059 (PD) on procollagen mRNA and collagen protein are shown. A, Procollagen I gene expression after exposure to leptin (30 ng/mL) in the presence and absence of AG490, Ly2940002, and PD98059. B, Optical density of collagen I bands after exposure to leptin (30 ng/mL) in the presence and absence of AG490. LY294002 and PD98059 (C) procollagen III gene expression after exposure to leptin (30 ng/mL) in the presence and absence of AG490, LY294002, and PD98059. D, Optical density of collagen III bands after exposure to leptin (30 ng/mL) in the presence and absence of AG490, LY294002, and PD98059. Collagen I and III gene expression was measured by quantitative PCR and expressed as a ratio normalized to 18S mRNA. JAK2 and PI3 kinase inhibition abrogated the leptin-induced increase in procollagen I and III mRNA as well as collagen I and III protein production. Although MEK inhibition decreased procollagen I and III mRNA, it did not have any significant effect of collagen protein. Data from 5 normal and 6 cirrhotic livers each performed in triplicate are shown as mean ± SEM.

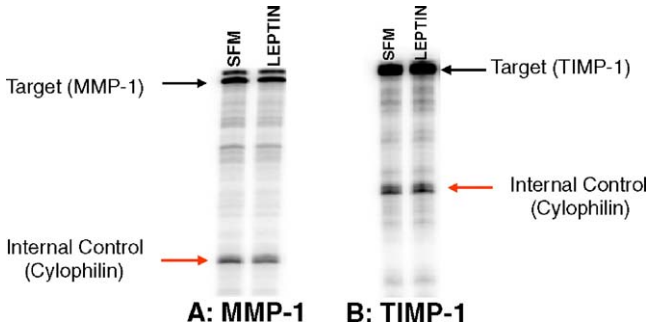

Fig. 4. The effects of leptin on matrix metalloproteinase (MMP-1) and tissue inhibitors of metalloproteinase (TIMP-1) mRNA expression by human HSC are shown. RNase protection assay and subsequent autoradiography were used to determine mRNA expression. Compared with SFM, leptin (30 ng/mL) had no effect on MMP-1 (left panel A) and TIMP-1 (right panel B) mRNA expression. Cyclophilin mRNA expression was used as an internal control for both set of studies.

3.2. Signaling pathways used by leptin to increase collagen production

The signaling pathways used by leptin to increase collagen production were evaluated by blocking JAK2, PI3K, and MAPK with their specific blockers. In preliminary studies, cell viability was confirmed when they were exposed to specific inhibitors of these pathways. AG490, a JAK2 inhibitor, essentially abrogated the leptin-induced increase in procollagen I and III mRNA levels (Fig. 3). AG490 also blocked the effects of leptin on collagen protein levels. Similarly, the leptin-induced increase in procollagen I and III mRNA transcript and protein levels was blocked by LY294002, an inhibitor of PI3K. Although the inhibition of the MAPK pathway by PD98059 blocked the effect of leptin on procollagen mRNA levels, there were no significant effects on leptin-mediated increments in collagen I and III protein formation. Similarly, U0126 (10 μmol), a potent MEK inhibitor [20], also failed to block the effects of leptin on collagen I and III protein production by the HSC. Effective blockade of MEK was confirmed in these experiments by noting a 2-fold decrease in MEK phosphorylation with PD98059 and leptin compared with leptin alone. These

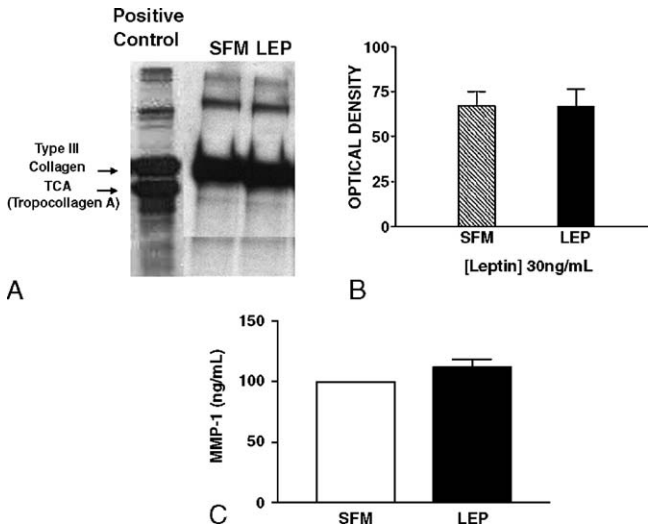

Fig. 5. Collagenolytic activity of cell culture supernatants in the presence and absence of leptin are shown. Collagenolytic activity was measured by the production of TC^A fragment after cleavage of ^{14}C-labeled type III collagen by MMP-1 in the cell culture media. Autoradiography (panel A) with subsequent densitometric analysis (panel B) was used to quantify the TC^A bands after cleavage. Compared with SFM, leptin (30 ng/mL) had no effect on collagenolytic activity. The effects of leptin on MMP-1 protein levels in the cell culture supernatants after 18 hours of incubation are shown (panel C). Matrix metalloproteinase 1 levels were measured by ELISA. Compared with SFM, leptin (30 ng/mL) had no significant effect on MMP-1 protein levels. Data from 5 normal and cirrhotic livers performed in triplicate are shown as mean ± SEM.

results were not dependent on the duration of exposure to leptin because identical results were obtained after variable periods of exposure (1-48 hours).

3.3. Effects on matrix metalloproteinases

Leptin (30 ng/mL) had no effect on MMP-1 transcript levels compared with serum-free media (Fig. 4A). Leptin also did not have any significant effect on TIMP-1 transcript levels (Fig. 4B). Leptin had no effect on the collagenolytic activity of the supernatants of cell cultures (Fig. 5). This lack of effect was noted after 24, 48, and 96 hours of incubation, and was unaltered by the addition of 1 mmol/L of APMA to the samples. Finally, leptin also did not have any significant effects on MMP-1 levels in the media as measured by ELISA.

4. Discussion

Leptin is an adipocytokine that plays a central role in energy homeostasis by decreasing energy expenditure through its effects on pro-opiomelanocortin neurons in the hypothalamus [21]. Obesity is associated with elevated leptin levels, suggesting the presence of resistance to the central metabolic actions of leptin [4,5]. The biologic implications of higher circulating levels of leptin in obese individuals remain to be fully defined. A growing body of literature indicates that leptin is required for a fibrogenic response in animal models of liver injury [6-8,22]. The current study demonstrates that leptin, at biologically relevant concentrations, increases collagen I and III production by human HSC via a JAK- and PI3K-mediated pathway.

Leptin has been shown to increase procollagen mRNA levels in other studies [7,8]. The increase in procollagen I and III gene transcript levels further corroborates these data and suggest that leptin produces transcriptional activation of procollagen genes. In a mouse model of liver injury, leptin increased the expression of the collagen transcriptional factor activator protein-1 (AP-1) [23]. The specific transcriptional factors or complexes required for leptin-induced activation of the collagen promoter and the specific sites on the promoter that are critical for this activation in humans remain to be defined. It is also known that procollagen mRNA in rat HSC can be affected by posttranscriptional stabilization [24]. Whether the leptin-induced increase in procollagen I and III mRNA involves such a mechanism remains to be examined.

It is interesting to note that leptin increased procollagen mRNA levels to a greater degree than collagen I protein. It is well known that different factors may affect gene transcript levels and protein levels to varying degrees [25]. This has been attributed to posttranscriptional changes in mRNA, varying translational efficiency, and posttranslational changes in the target protein [26]. Although some or all of these may have impacted the procollagen mRNA and protein levels in the current study, the studies were not designed to dissect out these possibilities that remain to be evaluated. Importantly, the direction of change in procollagen mRNA and protein after exposure to leptin was identical. Therefore, although the degree of change produced in procollagen mRNA and protein levels was different, the data are internally consistent.

Inhibition of MEK by PD98059 blocked the leptin-mediated increase in procollagen mRNA, but did not affect the leptin-mediated collagen protein production. Theoretically, this could be due to several possibilities. These include increased translational efficiency, posttranslational stabilization of collagen, and inhibition of collagenolytic pathways. Such effects could result from an unmasking of an opposing effect of MAPK or continued effects mediated by the PI3K and JAK2 pathways. These possibilities also remain to be experimentally verified.

The current study did not find any significant effects of leptin on either the expression of TIMP-1 and MMP-1 or overall collagenolytic activity of human HSC. The failure to notice any increase in collagenolytic activity despite measurable MMP-1 activity probably reflects the presence of other factors in the media that inhibited the collagenolytic activity of the MMP-1 present. Leptin has been found to increase MMP activity in the LX2 HSC line [11]. This difference may reflect innate differences between immortalized cell lines and primary cultures of HSC. In addition, in vivo, leptin may affect matrix turnover by its effects of sinusoidal endothelial cells and Kupffer cells [23].

The biologic importance of hyperleptinemia in the progression of fibrosis in obese subjects with liver disease is obviously a key question. Hepatic fibrosis requires activation of HSC, which then produce a collagenous matrix. In this study, the HSCs were already activated. Recently, activated HSCs have been shown to produce leptin [27,28]. At concentrations typically seen in obesity, leptin directly increased collagen production by the HSC, as shown in the current study. Leptin also enhances the transforming growth factor β–mediated increase in collagen synthesis by immortalized HSC [7]. These considerations raise the possibility that hyperleptinemia contributes, at least in part, to the observation that obese individuals with liver disease, for example, hepatitis C or nonalcoholic steatohepatitis, have more aggressive fibrosis [2,3,29]. This hypothesis now requires validation in appropriately designed studies.

In conclusion, pathophysiologic concentrations of leptin increased procollagen I and III mRNA and protein production by primary cultures of human HSC derived from normal and cirrhotic livers. These effects were mediated by the JAK2 and PI3K pathways. Leptin did not have any significant effects on TIMP-1 and MMP-1 expression or collagenase activity of human HSC.

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