

Short communication

### LEPTIN REGULATES MMP-2, TIMP-1 AND COLLAGEN SYNTHESIS VIA p38 MAPK IN HL-1 MURINE CARDIOMYOCYTES

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**Abstract:** A clear association between obesity and heart failure exists and a significant role for leptin, the product of the obese gene, has been suggested. One aspect of myocardial remodeling which characterizes heart failure is a disruption in the balance of extracellular matrix synthesis and degradation. Here we investigated the effects of leptin on matrix metalloproteinase (MMP) activity, tissue inhibitor of metalloproteinase (TIMP) expression, as well as collagen synthesis in HL-1 cardiac muscle cells. Gelatin zymographic analysis of MMP activity in conditioned media showed that leptin enhanced MMP-2 activity in a dose- and time-dependent manner. Leptin is known to stimulate phosphorylation of p38 MAPK in cardiac cells and utilization of the p38 MAPK inhibitor, SB203580, demonstrated that this kinase also plays a role in regulating several extracellular matrix components, such that inhibition of p38 MAPK signaling prevented the leptin-induced increase in MMP-2 activation. We also observed that leptin enhanced collagen synthesis determined by both proline incorporation and picosirius red staining of conditioned media. Pro-collagen type-I and pro-collagen type-III expression, measured by real-time PCR and Western blotting were also increased by leptin, effects which were again attenuated by SB203580. In summary, these results demonstrate the potential for leptin to play a role in mediating myocardial ECM remodeling and that the p38 MAPK pathway plays an important role in mediating these effects.

**Key words:** Leptin, Obesity, Heart failure, Extracellular matrix

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Abbreviations used: AP-1 – activator protein-1; ATF-2 – activating transcription factor-2; ECM – extracellular matrix; HF– heart failure; LV – left ventricular; MAPK – mitogen activated protein kinase; MMP – matrix metalloproteinase; mRNA– messenger ribonucleic acid; PBS – phosphate buffered saline; PCR – polymerase chain reaction; TIMP- tissue inhibitor of metalloproteinase

## INTRODUCTION

Heart failure (HF) is the end-stage pathology of various cardiovascular diseases and is a leading cause of death [1]. HF may result from multiple myocardial remodeling events, some of which occur initially as beneficial adaptive changes but ultimately dictate the transition to heart failure [2]. A manifestation of myocardial remodeling is an increase in extracellular matrix (ECM) remodeling which results from dysregulated levels of collagen synthesis, and its degradation via the delicate balance maintained between matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs) [3]. The characteristics of myocardial ECM remodeling are highly dependent on the disease etiology. For instance, following myocardial infarction, early-phase remodeling is often characterized by an initial degradation of the left ventricular (LV) collagen network [4] and late-phase remodeling is accompanied by excessive collagen accumulation [5]. Whereas, remodeling in pressure overload states includes a slower progression to cardiac fibrosis, specifically interstitial fibrillar collagen deposition, resulting in dysfunctional chamber filling during diastole [6].

The connection between obesity and cardiovascular disease is well-established and is supported by numerous epidemiological studies [7, 8]. In addition, circulating levels of the adipocyte-derived hormone leptin are positively correlated with cardiovascular complications in obese individuals and leptin may serve as a causative link [2]. Concentrations of circulating leptin in lean and obese individuals are between 2-10 ng/ml and 10-100 ng/ml, respectively [35]. Leptin, although primarily produced by adipocytes [9], is additionally synthesized by a plethora of tissues [11-17], including the heart [10]. The cardiac effects of leptin have yet to be fully elucidated, however, leptin regulates cardiomyocyte hypertrophy [13-15, 19] hyperplasia [13], apoptosis [33] and the production of various myocardial matrix components by cardiomyocytes and cardiac fibroblasts [13, 16]

The underlying mechanism through which obesity contributes to the development of cardiac fibrosis in cardiovascular disease, however, remains poorly understood. Moreover, the mechanism through which leptin affects myocardial matrix remodeling events is largely unknown. Thus, the purpose of this study was to examine leptin's effects on the production and secretion of MMPs, TIMPs and collagen, using the HL-1 cardiomyocyte cell line. Leptin acts via a diverse array of signaling mechanisms [18] and here we specifically focus on the role of p38 MAPK that has previously been shown to mediate leptin's hypertrophic effects in cardiomyocytes [13, 14, 19].

## MATERIALS AND METHODS

### Cell culture

Cardiac muscle cells, designated HL-1, which are derived from AT-1 mouse atrial cardiomyocytes were plated in gelatin and fibronectin-coated culture vessels and maintained in Claycomb medium (Sigma-Aldrich, St. Louis, MO)

supplemented with 10% fetal bovine serum, 0.1 mM norepinephrine, 2 mM L-glutamine, 100 units/ml penicillin, 100 units/ml streptomycin, and 0.25 µg/ml amphotericin B. Cells were grown at 37°C in 5% CO<sub>2</sub> and were passaged into fresh media at ~80% confluence.

#### **Preparation of cell lysates and conditioned media**

Cells grown on 6 well plates were treated with recombinant murine leptin (6 nM) for the times indicated in figure legends, then washed quickly with ice-cold PBS. Conditioned media obtained from cells incubated with leptin with or without pretreatment with the p38 MAPK inhibitor, SB203580 (Calbiochem, San Diego, CA), was collected and concentrated using the Centricon-10 system (Millipore, Ontario, Canada). Total protein content was determined using the Bradford assay (BioRad, Mississauga, Canada).

#### **Gelatin zymography**

Cells were stimulated with 6 or 60 nM (100 and 1000 ng/ml, respectively) recombinant mouse leptin (Calbiochem, San Diego, CA) for the times indicated. In the experiments that examined the participation of p38 MAPK in leptin's actions, cells were first pre-treated with 10 µM of SB203580 for 1 h prior to leptin stimulation. The resulting conditioned culture medium was concentrated using the Centricon-10 system (Millipore, Ontario, Canada) and protein content of the culture medium was measured as described previously [16]. Aliquots of culture media containing 25 µg of protein were then resolved on a 10% SDS polyacrylamide gel containing 0.1% gelatin. The gel was rinsed for 1 h in a solution containing 2.5% Triton X-100 in 40 mM Tris-HCl (pH 7.6) to remove SDS and to renature the proteins. The gel was then rinsed for 15 min in 10 mM Tris-HCl (pH 8.0) followed by incubation for 16 h at 37°C with gentle shaking in 0.05 M Tris-HCl (pH 7.5) with 5 mM CaCl<sub>2</sub> to allow activation of MMPs. Gels were then stained for 30 min in 0.5% Coomassie blue R-250, and destained in 40% methanol, 10% acetic acid to highlight protease activity of MMPs. Protease activity was quantified using densitometric scanning and Scion Image software (Scion Corporation, Frederick, MA).

#### **Western blotting**

Equal amounts of protein (~30 µg) were immunoblotted using SP1.D8 (aminopropeptide collagen I, Developmental Studies Hybridoma Bank, Iowa City, USA) and collagen type III H-300 (Santa Cruz Biotechnology, California, USA) as previously described [16]. Quantitative analysis was performed after densitometric scanning using Scion Image Software.

#### **[<sup>3</sup>H] Proline incorporation**

Collagen synthesis was assessed by measurement of cellular [<sup>3</sup>H] proline (Amersham, Quebec, Canada) uptake as previously described [16]. Briefly, cells were pretreated for 1h with 10 µM SB203580 prior to stimulation with 6 nM recombinant mouse leptin for 24 h. [<sup>3</sup>H] proline was added to each well at a final

concentration of 1  $\mu\text{Ci/ml}$  for 24 h. At the end of incubation, cultures were washed 3 times with ice cold PBS and incubated with ice-cold 5% trichloroacetic acid for 30 min at 4°C. After 2 rinses with cold 5% TCA, the acid-precipitate material was solubilized overnight in 0.5 ml of 0.5 N NaOH at 37°C and neutralized with 0.5 ml 0.5N HCl per well. Total protein was determined from an aliquot and the incorporated radioactivity in the remaining cell lysate was measured in a liquid scintillation counter and expressed relative to control.

#### **Fibrillar collagen content**

Conditioned media from cells incubated with leptin with or without pretreatment with SB203580 was concentrated and analyzed as previously described [20]. Briefly, conditioned media samples (50  $\mu\text{g}$ ) were loaded into 96-well plates and allowed to evaporate for 48 h at 37°C in 5%  $\text{CO}_2$ . Following staining with Picosirius Red for 1 h and washing with 10 mM HCl, the stain was solubilized with 0.1 M NaOH. Conditioned media samples and the collagen standard curve were then analyzed via spectrophotometry at an absorbance of 540 nm.

#### **Real-time RT-PCR**

Cells were pretreated with the p38 MAPK inhibitor SB203580 (10  $\mu\text{M}$ ) for 1 h prior to stimulation with recombinant mouse leptin (6 nM or 60 nM) for 24 h. Total RNA was prepared from cardiomyocytes using Trizol (Invitrogen, Burlington, Canada) according to the manufacturer's instruction. RNA integrity was verified by ethidium bromide staining of agarose gels and by an optical density (OD) absorption ratio  $\text{OD } 260 \text{ nm}/\text{OD } 280 \text{ nm} > 1.9$ . One  $\mu\text{g}$  of total RNA was reverse transcribed with Superscript II RNase H-reverse transcriptase using random hexamers (Invitrogen, Burlington, Canada) according to the manufacturer's instructions. Real-time PCR analyses were performed using 20 ng of reverse transcribed total RNA with 200 nM of both sense and antisense primers in a final volume of 20  $\mu\text{l}$  using the SYBR Green PCR core reagent (Invitrogen, Burlington, Canada) in an ABI PRISM 7700 Sequence Detection System Instrument (Applied Biosystems, Foster City, CA). To ensure that a single amplicon of the expected size was produced by RT-PCR, samples were also analyzed on ethidium bromide stained agarose. 18S ribosomal subunit amplification was used routinely to control for variability in the initial quantities of cDNA. Relative quantitation for any given gene, expressed as fold variation over control, was calculated after determination of the difference between cycle threshold (Ct) value of the given gene according to manufacturers protocol using the formula  $2^{-\Delta(\Delta\text{CtA}-\Delta\text{CtB})}$ . Cycling conditions consisted of an initial denaturation step of 95°C for 3 min as a 'hot start' followed by 40 cycles of 95°C for 30 s at the noted annealing temperature of each primer set (see Tab. 1) for 30 s, 72°C for 30 s and a final extension at 72°C for 10 min.

Tab. 1. Sequence of oligonucleotide primers used to detect leptin receptors, TIMP-1 and procollagen type I and III.

Gene	Primer type	Sequence (5' to 3')	Ann. temp.
ObRa	Forward	TGTTTTGGGACGATGTTCCA	62°C
	Reverse	GATACATCAAAGAGTGCCGCTCTC	
ObRb	Forward	TGTTTTGGGACGATGTTCCA	62°C
	Reverse	AAAGATGCTCAAATGTTTCAGGC	
Procollagen type I	Forward	AAGGTCATGCTGGTCTTGCT	62°C
	Reverse	GACCCTGTTACCTTTTCCA	
Procollagen type III	Forward	CACAGCCTTCTACACCTGCT	57°C
	Reverse	CACTCCAGACTTGACATCAT	
TIMP-1	Forward	GTCACTCTCCAGTTTGCAAG	55°C
	Reverse	GACCACCTTATAACCAGCGTT	
$\beta$ -actin	Forward	TTTGATGTCACGCACGATTT	58°C
	Reverse	AGCCATGTACGTAGCCATCC	

### Statistical analysis

Statistical analyses were performed using a one-way ANOVA or paired student's T-test, as appropriate (SigmaStat 3.1, Chicago, IL). Tukey post-hoc comparisons were made when statistical significance ( $p < 0.05$ ) was detected between observations. Data are presented as means  $\pm$  SEM.

### RESULTS

We first confirmed mRNA expression of both the long (obRb) and short (obRa) forms of the leptin receptor in HL-1 cardiomyocytes (data not shown). We then investigated the effects of leptin on MMP activity by gelatin zymography and found that MMP-2 activity was significantly increased following 6 nM or 60 nM leptin treatment (Fig. 1A). These concentrations were chosen based on initial dose dependent response experiments, published data and the fact that 6nM represents circulating leptin levels in obese patients. In addition, 6 h or 24 h of 6 nM leptin treatment resulted in a similar significant 1.3-fold or 1.4-fold increase above basal in MMP-2 activity, respectively (Fig. 1B). p38 MAPK has previously been shown to be activated by leptin in a variety of cardiac cell types [13,14,19]. Therefore, to investigate if p38 MAPK participates in the leptin-induced increase in MMP-2 activity, HL-1 cells were pretreated with the p38 MAPK inhibitor (SB203580) prior to leptin stimulation. As shown in Fig. 1C, SB203580 alone had no effect on basal, while the stimulatory effect of leptin on MMP-2 activity was attenuated in cells pre-treated with SB203580. We observed that SB203580 inhibited leptin-induced MMP-2 activity below that of basal condition but when compared with SB203580 alone there was no

significant difference (Fig. 1). In contrast, inhibition of PI3-kinase (LY294002) or ERK1/2 (PD98059) did not alter leptin's effects on MMP-2 activity (data not shown).

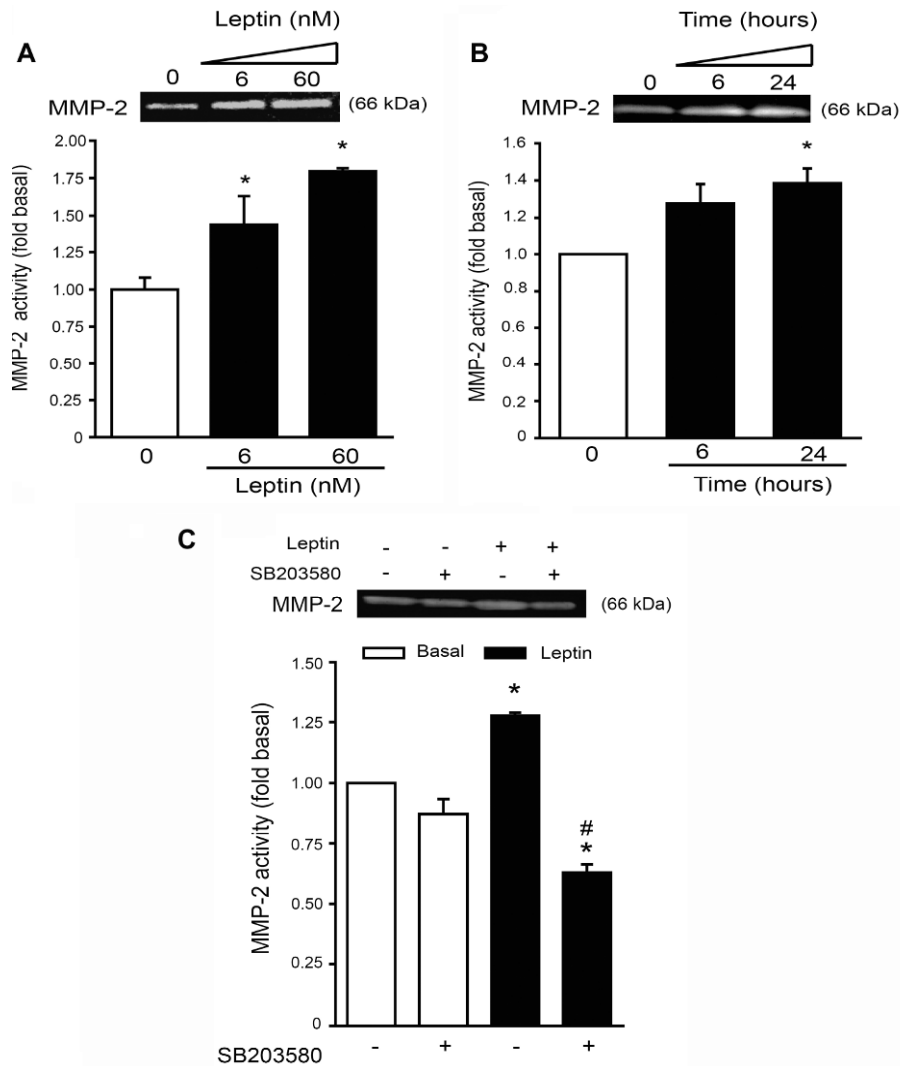


Fig. 1. Leptin's stimulatory effects on MMP-2 activity are mediated via p38 MAPK. Conditioned media from control and leptin-treated cells were analyzed for MMP-2 activity by gelatin zymography as described in Materials and Methods. Shown are representative zymography gels and quantitation of active MMP-2 in cells treated with 6 nM or 60 nM leptin for 24 h (A) or 6 nM leptin for 24 h (B). Representative zymography gel and quantitation of MMP-2 activity (C) in cells treated with 6 nM leptin with or without a 1 h pre-treatment with 10  $\mu$ M SB203580 (present continuously during the treatment periods) as described in Materials and Methods. Data are means  $\pm$  SEM ( $n = 3$  independent experiments), expressed relative to basal. \* $p < 0.05$  vs. basal, # $p < 0.05$  vs. leptin alone.

TIMP-1, 2, 3 and 4 mRNA levels were analyzed following 24 h leptin treatment in the presence or absence of SB203580. HL-1 cells exhibit minimal expression of TIMP-2, 3 and 4 mRNA (data not shown), but we observed that leptin caused a significant reduction in TIMP-1 mRNA (Fig. 2). Pre-treatment with SB203580 prevented the leptin-induced reduction in TIMP-1 mRNA although somewhat surprisingly, SB203580 treatment alone reduced TIMP-1 expression, perhaps suggesting that constitutive endogenous p38 MAPK activity plays a role in tonic TIMP-1 turnover (Fig. 2).

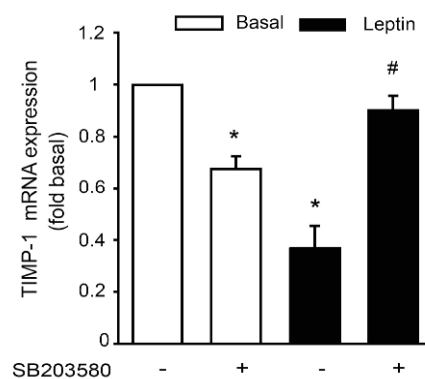


Fig. 2. TIMP-1 mRNA expression in response to leptin and p38 MAPK inhibition. Cells were pre-treated with 10  $\mu$ M SB203580 prior to stimulation with leptin (6 nM, 24 h). Total RNA isolated and analysis of TIMP-1 mRNA expression was determined by quantitative real-time PCR as described in Materials and Methods. Data are means  $\pm$  SEM (n = 3 independent experiments), expressed relative to basal. \*p < 0.05 vs. basal.

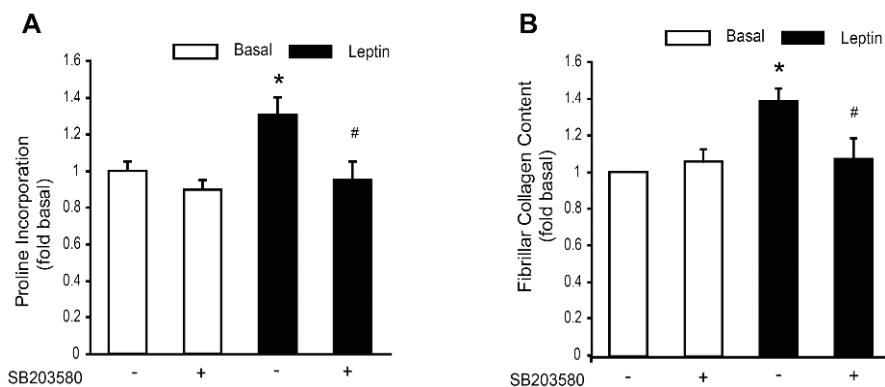


Fig. 3. Leptin-induced increase in intracellular and extracellular total collagen content is reduced by p38 MAPK inhibition. Cells were stimulated with leptin (6 nM, 24 h) with or without a 1 h pre-treatment with 10  $\mu$ M SB203580 (present continuously during the treatment periods) and [ $^3$ H] proline incorporation (A), as well as total fibrillar collagen content (B) were analyzed as described in Materials and Methods. Data are means  $\pm$  SEM (n = 4 independent experiments), expressed relative to basal. \*p < 0.05 vs. basal, #p < 0.05 vs. leptin alone.

Collagen is rich in the amino acid proline and tracing [ $^3\text{H}$ ]-proline uptake is routinely used as a measure of collagen synthesis. Here we demonstrate that in HL-1 cells, leptin (6 nM) treatment for 24 h resulted in a significant 1.3-fold above basal increase in proline incorporation (Fig. 3A). Pre-treatment of cells with SB203580 reduced this stimulatory effect of leptin to basal values, while the inhibitor alone had no effect on proline incorporation. We also examined extracellular content of fibrillar collagen via analysis of HL-1 conditioned media. Leptin treatment for 24 h significantly increased the accumulation of fibrillar collagen in conditioned media by 1.4-fold (Fig. 3B). Moreover, pre-treatment with SB203580 followed by leptin for 24 h resulted in attenuation of leptin-induced stimulation of fibrillar collagen secretion (Fig. 3B).

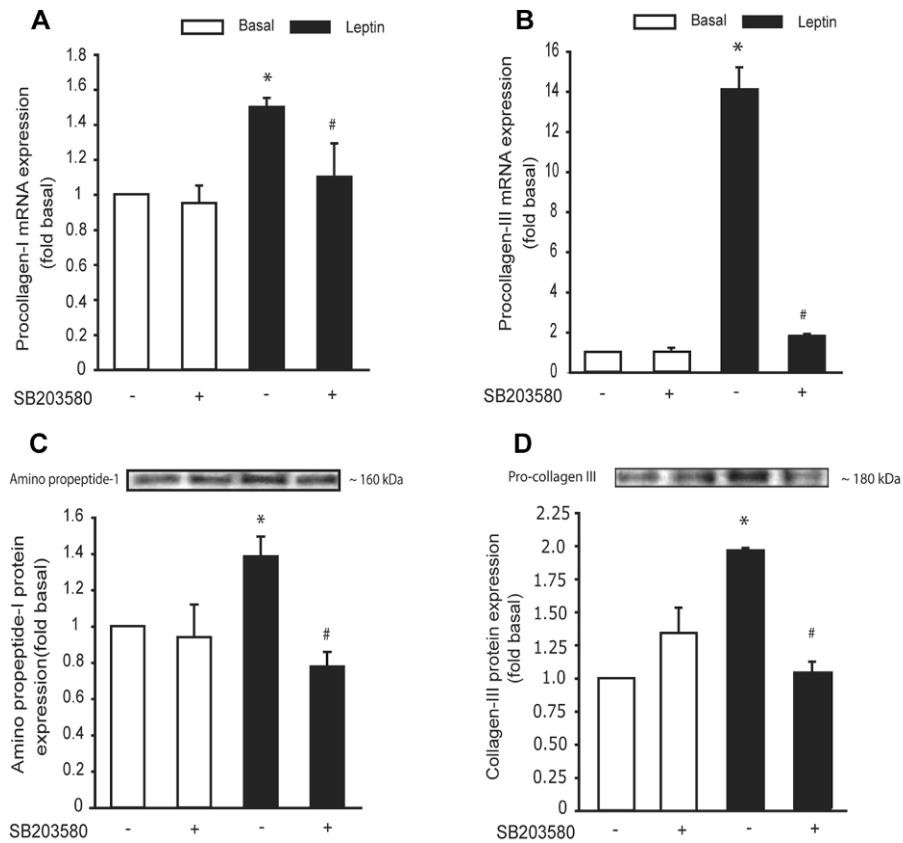


Fig. 4. Pro-collagen I and -III mRNA and protein expression are increased by leptin and inhibited by p38 MAPK inhibition. RT-PCR analysis of HL-1 cells stimulated with 6 nM leptin for 24 h with or without a 1h pre-treatment with 10  $\mu\text{M}$  SB203580 (present continuously during the treatment periods), was utilized to examine pro-collagen I (A) and pro-collagen III (B) mRNA expression. Protein expression of amino propeptide  $\alpha$ -1 (C) and collagen-III (D) were analyzed as described in Materials and Methods. Data are means  $\pm$  SEM (n = 4 independent experiments), expressed relative to basal. \*p < 0.05 vs. basal, #p < 0.05 vs. leptin alone.



We next examined the effect of leptin on collagen-I and -III expression. Leptin treatment for 24 h significantly increased procollagen-I (1.5-fold above basal) and procollagen-III (15-fold above basal) mRNA levels (Figs 4A and 4B). These stimulatory effects of leptin were attenuated in cells pre-treated with SB203580, while SB203580 alone was without effect (Figs 4A and 4B). We also examined the effects of leptin on protein expression of collagen-I and -III isoforms. Amino propeptides are by-products of procollagen cleavage, which is an integral step in the assembly of mature collagen fibers and thus are useful indicators of fibrillar collagen content. As shown in Fig. 4C, leptin enhanced procollagen type I amino propeptide levels 1.4-fold and this increase was abolished following pre-treatment with SB203580. Similar results were obtained upon analysis of collagen  $\alpha 1$  type III levels (Fig. 4D).

## DISCUSSION

Numerous studies suggest that leptin is an important mediator of pathological cardiac remodeling [13, 14, 17, 19, 21-24], which is characterized by cardiomyocyte hypertrophy and a disruption of the ECM resulting in increased collagen deposition [1, 3]. Here we investigated the effect of leptin on regulation of myocardial matrix components, specifically MMP-2 activity, TIMP-1 expression and collagen-I/-III expression, in HL-1 cardiomyocytes.

Altered activation of MMP-2 is known to contribute to the development of cardiac fibrosis in failing hearts [26-28]. We show here that leptin stimulates MMP-2 activation in a dose- and time-dependent manner. We and others have similarly revealed enhanced MMP proteolytic activity by leptin in cardiac fibroblasts [16], ventricular cardiomyocytes [13] and aortic endothelial cells [29]. Functional activity of MMPs is not only dependent on mRNA or protein levels; post-translational regulation via TIMPs is an important component since modifications in the MMP:TIMP ratio can oppose the hydrolytic capacity of MMPs [3]. Our data showed that expression of the endogenous MMP inhibitor, TIMP-1, is decreased following leptin stimulation. Although functional data for TIMP-1 is not available, our data suggests that in HL-1 cardiomyocytes, leptin may regulate the activity of MMP-2 indirectly via attenuating the expression its natural inhibitor, TIMP-1 and altering the MMP:TIMP ratio. Somewhat surprisingly, SB203580 treatment alone reduced TIMP-1 expression, perhaps suggesting that constitutive endogenous p38 MAPK activity plays a role in tonic TIMP-1 turnover.

A direct consequence of altered MMP activity is excessive accumulation or degradation of a diverse array of ECM proteins, of which fibrillar collagen is the predominant substrate targeted by cardiac MMP-2 [28]. In the present study, we found that leptin mediates the expression of this triple-helical protein at various steps of the collagen biosynthesis pathway. Intracellular accumulation of total collagen was stimulated by leptin, as well as mRNA expression of the predominant isoforms found in cardiac tissue, collagen-I and -III. Although

collagen-III mRNA expression increased substantially (15-fold), an augmentation of similar magnitude was not observed in the total intracellular collagen pools likely because this isoform makes up a small proportion of total cardiac collagen (< 10%), while collagen-1 accounts for approximately 80% and additional isoforms (ie. collagen-IV) comprise the remaining 10% (1). Furthermore, it is not surprising that collagen-III mRNA levels do not reflect secreted protein levels when the complexity of the various regulatory steps involved in the production, processing, secretion and assembly of collagen are considered. Indeed, asynchronous mRNA and protein expression levels in cardiac tissue have been reported previously [34]. Extracellular collagen accumulation was similarly affected such that collagen-I, -III and total fibrillar collagen were increased, however to a lesser degree when compared to leptin's effects on procollagen expression. This discrepancy may be due to leptin's ability to concurrently enhance MMP-2 proteolytic activity, of which collagen is a potential substrate. Collectively, these results suggest that leptin increases the synthesis and consequently the accumulation of collagen in the extracellular space. One would reason that an enhanced MMP-2 activity would be paralleled by reduction in collagen accumulation in the extracellular space. Yet, as previously mentioned this was not the observation in the present study. We previously found that leptin similarly increases the expression of various MMPs as well as collagen accumulation [13, 16]. A likely explanation may be that the promoter regions of MMP-2 and collagen-I/III mutually utilize AP-1 consensus recognition sites, both of which can be stimulated by leptin (discussed in additional detail below). Additionally, leptin's ability to increase MMP-2 activity, resulting in increased collagen degradation, may obscure any direct effects of leptin on collagen extracellular accumulation.

Leptin is known to engage the p38 MAPK pathway to elicit its physiological and pathological responses in cardiomyocytes including hypertrophy and hyperplasia [13, 14, 19]. In the present study, we show that in HL-1 cardiomyocytes, leptin utilizes the p38-MAPK pathway to alter the expression of TIMP-1, MMP-2, as well as collagen-I and -III. Downstream targets of p38 MAPK include a myriad of transcription factors including activating transcription factor (ATF)-2, which has been implicated in the regulation of MMP-2 gene expression [30]. Since leptin is known to enhance ATF2 activity [31], it is plausible that these transcription factors stimulate MMP-2 promoter activity via leptin-mediated p38 MAPK activation. In a similar manner, leptin is known to stimulate the transcriptional activity of procollagen  $\alpha$ 2(I) via another p38 MAPK downstream target, AP-1, which binds c-Jun and c-Fos and is located upstream of the COL1A2 promoter [32]. As previously mentioned, the concurrent increase in MMP-2 activity with collagen expression in conditioned media is unexpected as increased collagen degradation likely follows upregulation of its primary hydrolytic enzyme, MMP-2. However, various studies demonstrating the utilization of ATF-2 and AP-1 in MMP-2 and collagen-I/III transcriptional activation respectively, provides evidence that transactivation of these factors,

both of which are downstream mediators in p38 MAPK signaling, are potential targets of leptin activation of p38 MAPK. Additional studies are required further address these potential mechanisms.

In summary, our study demonstrates that in HL-1 cardiomyocytes, leptin acts via the p38 MAPK pathway to regulate cellular events which can contribute to myocardial matrix remodeling, specifically, MMP-2 activation, as well as total, type-I and -III collagen expression. Further understanding of the potential contribution of leptin signaling to myocardial matrix remodeling may ultimately aid in generating novel therapeutic strategies targeting cardiac remodeling in the clinical settings of obesity and HF.

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