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Direct effects of leptin on size and extracellular matrix components of human pediatric ventricular myocytes

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Abstract

Objective: There is a well-documented association between obesity and heart failure although the mechanistic basis for this correlation is unclear. Both extracellular matrix remodeling and left ventricular hypertrophy are well-defined components of remodeling in heart failure, and here we further investigate the role of leptin, the *obese* gene product, on these parameters.
Methods: We used primary human pediatric ventricular cardiomyocytes combined with gelatin zymography, quantitative PCR analysis, proline and leucine incorporation assays, and investigation of kinase activation by Western blotting.
Results: We show using gelatin zymography that leptin dose-dependently (0–60 nM) increased proteolytic activity at ~72 kDa. Accordingly, upon quantitative PCR analysis we found that leptin increased expression of matrix metalloproteinase-2 (MMP-2). Leptin also caused an increase in collagen type III and IV mRNA expression and a decrease in collagen type I mRNA expression. This was reflected in no significant change in total collagen synthesis, measured by [^{3}H]proline incorporation, in response to leptin. A statistically significant increase in cell size, [^{3}H]leucine incorporation, and expression of well-characterized markers of cardiac hypertrophy, namely cardiac α-actin and myosin light chain, were observed in response to leptin. We demonstrate activation of Janus-activated kinase and mitogen-activated protein kinase pathways by leptin, and using pharmacological inhibitors we show that these signaling pathways play a role in mediating the effects of leptin.
Conclusions: Our findings show that leptin regulates cell size, stimulates MMP-2 expression, and alters the profile, but not the total content, of collagen in human cardiomyocytes. This indicates the potential for altered leptin sensitivity to directly regulate cardiac remodeling in obesity.

Keywords: Leptin; obesity; heart failure; extracellular matrix; hypertrophy

1. Introduction

There is now a well-established association between obesity and cardiovascular disease [1,2] and many studies have highlighted the potential role played by the circulating adipokine leptin, the product of the obese (*ob*) gene [3–6]. Leptin, expressed and secreted primarily by adipocytes, acts via a family of receptor (ob-R) isoforms [7] to mediate an ever growing wide range of physiological effects [8]. These receptors have divergent signaling capabilities, regulating pathways which include JAK/STATs and MAP kinases [7]. Elevated plasma leptin concentrations are found in obese individuals, although whether the heart remains sensitive or becomes resistant to leptin under these circumstances is still uncertain [4,9].

Heart failure can manifest due to multiple etiologies and is a complex process involving a series of adaptive changes within the myocardium, collectively referred to as remodelling, which occur primarily in the left ventricle [10]. Progression of heart failure is now commonly believed to

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result due to a complex interplay of detrimental effects including those affecting size or number of myocytes, changes in metabolic and electrophysiological properties and alterations in the composition and structure of the extracellular matrix (ECM) [10]. For example, the degree of heart failure regardless of differing etiologies is associated with a progressive enlargement of the left ventricle [11,12]. Thus, left ventricular hypertrophy is not only a consequence of but also contributes to heart failure and it is imperative to understand the neurohormonal mechanisms regulating cardiomyocyte hypertrophy. There is a constant turnover of ECM in the myocardium, approximately 0.6% of total per day, and proper maintenance of ECM composition is vital in the healthy heart [10]. Increased MMP activities have commonly been found in failing hearts of diverse etiology and their role is summarized in recent excellent reviews [10,13–15]. Remodeling does not simply involve altered total levels of matrix collagens, the collagen type, organization and cross-linking are of great significance [16]. For example, changes in the ratio of collagens I and III occur in response to a variety of agents [17–20]. Typically, remodeling is associated with elevated collagen I, at the expense of the more compliant collagen III, which promotes myocardial stiffness. Notably, the profiles of collagens and MMPs observed in the left and right ventricles differ in failing hearts [21].

Given the association between obesity and heart failure, here we further investigated the potential role of leptin in heart failure by investigating its effect on cardiomyocyte hypertrophy as well as collagen and MMP production. Importantly, since it is clear that rodent and human obesity contrast significantly, we conducted this study in human pediatric ventricular cardiomyocytes and our results further highlight the potential significance of leptin as a direct mediator of cardiac remodeling in obesity.

2. Methods

2.1. Isolation of human ventricular cardiomyocytes

We have previously described the method used here for isolation of human ventricular cardiomyocytes [22,23]. Briefly, biopsy samples were obtained from children undergoing elective surgical repair of tetralogy of Fallot through a protocol approved by the University of Toronto Human Experimentation Committee as conforms with the Declaration of Helsinki. Myocardial ventricular resection biopsies (20 mg) from children (6 months or older) were immediately washed in PBS then digested by 0.1% collagenase/0.2% trypsin in phosphate buffered saline. Cardiomyocytes were subsequently purified by dilution cloning and maintained at 37 °C (95% air/5% CO_2) in Iscoves modified Dulbeccos medium (IMDM, Canada, Life Technologies Inc., Ontario) containing 10% fetal calf serum, 0.1 mM β-mercaptoethanol, 0.4 mM L-arginine, 100 U/ml penicillin and 100 mg/ml streptomycin. Purification of a cardiomyocyte population was routinely confirmed by fluorescent staining using a monoclonal antibody (MF20, a kind gift from Dr. J.C. McDermott, York University, Toronto) which detects human myosin heavy chain. During the leptin treatment period cells were cultured in media containing 1% FBS (Wisent, Quebec, Canada). Based on previous experience, cells were used for experiments only at passage 3 or passage 4.

2.2. Gelatin zymography

Cells were pretreated for 1 h with the kinase inhibitors AG490 (JAK, 20 μM), PD98059 (ERK1/2, 20 μM) or SB203580 (p38 MAPK, 10 μM) (Calbiochem, San Diego, CA or Bioshop, Burlington, Ontario) prior to stimulation with leptin (6 nM) for 24 h. The final choice of inhibitor concentrations used here was determined by preliminary experiments and our previous work in these cells [24]. Recombinant human leptin used in this study was purchased from Calbiochem (San Diego, USA) and stock solution prepared according to manufacturers instructions. The resulting conditioned culture medium was concentrated using Centricon-10 system (Millipore, Ontario, Canada). Protein content of the culture medium was measured by the method of Bradford using bovine serum albumin as standard. 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 of 2.5% Triton X-100 in 40 mM Tris–HCl (pH 7.6) to remove SDS and 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 $CaCl_2$ to allow activation of MMPs. Gels were then stained for 30 min in 0.5% Coomassie blue R-250 then destained in 40% methanol, 10% acetic acid to highlight protease activity of MMPs. Protease activity was quantified using densitometric scanning and Scion Image software.

2.3. Real-time quantitative RT-PCR

Total RNA was prepared from cardiomyocytes using Trizol (Invitrogen, Burlington, Canada) according to the manufacturer's instruction. The integrity of RNA was verified by ethidium bromide staining of agarose gel analyses and by an optical density (OD) absorption ratio $OD_{260\ nm}/OD_{28\ 0nm} > 1.9$. One microgram of total RNA was reverse transcribed with Super script II RNAse H-reverse transcriptase using random hexamers (Invitrogen, Burlington, Canada) according to the manufacturer's instructions. Real time quantitative RT-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 μl using the SYBR Green PCR core reagent (Invitrogen, Burlington, Canada) in an ABI PRISM 7700 Sequence

Detection System Instrument (Applied Biosystems). RT-PCR products were also analyzed on ethidium bromide stained agarose to ensure that a single amplicon of the expected size was indeed obtained. 18S ribosomal subunit and/or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) amplification were used in each experiment 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 $2^{-\Delta CtA-CtB)}$. PCR was performed using specific primers as detailed in Table 1. 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 for 30 s, 72 °C for 30 s and a final extension at 72 °C for 10 min.

2.4. [^{3}H] Proline incorporation

Collagen synthesis was assessed by measurement of cellular [^{3}H] proline (Amersham, Quebec, Canada) uptake. Cells were pretreated for 1 h with the kinase inhibitors AG490 (20 μM), PD98059 (20 μM), SB203580 (10 μM) prior to stimulation with leptin (6 nM) for 24 h in continued presence of inhibitors. [^{3}H] proline was added to each well at a final concentration of 1 μCi/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 (TCA) 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 0.5 N 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.

2.5. Analysis of cardiomyocyte size

Cell size was measured in quiescent (serum-starved for 24 h) or leptin-treated (6 nM, 24 h) cells grown on cover slips in 6-well plates. In each experiment, the surface area of 20 randomly selected cells was determined using a phase-contrast microscope equipped with a Polaroid digital camera to allow capture of cell images at a 20× magnification. The area of the cells was measured quantitatively using Image J software.

2.6. Leucine incorporation

Protein synthesis was assessed by measurement of cellular [^{3}H]leucine uptake as described previously [25]. [^{3}H]leucine (L-[4,5-^{3}H(N)]-Leucine, Amersham, St-Foy, QC) was added to each well at a final concentration of 1 μCi/ml for the last 24 h. Cells were pretreated for 1 h with or AG490 (20 μM), PD98059 (20 μM) or SB203580 (10 μM) prior to stimulation with leptin (6 nM) for 24 h in the continued presence of inhibitor. At the end of incubation, cultures were washed 3 times with ice cold PBS and incubated with ice-cold 5% trichloroacetic acid (TCA) 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 0.5 N HCl per well. Total protein was determined from an aliquot and

Table 1
Sequence of oligonucleotide primers used for quantitative PCR analyses

Gene	Primer type	Sequence (5′ to 3′)	An. temp. °C
ObRs	Forward	TGTTTTGGGACGATGTTCCA	62
	Reverse	GATACATCAAAGAGTGTCCGCTCTC	
ObRL	Forward	TGTTTTGGGACGATGTTCCA	62
	Reverse	AAAGATGCTCAAATGTTTCAGGC	
MMP-2	Forward	TGTGTCTTCCCCTTCACTTT	60
	Reverse	GATCTGAGCGATGCCATCAA	
MT1-MMP	Forward	GGATACCCAATGCCCATTGGCCA	64
	Reverse	CCATTGGGCATCCAGAAGAGAGC	
Collagen type I	Forward	AAGGTCATGCTGGTCTTGCT	62
	Reverse	GACCCTGTTCACCTTTTCCA	
Collagen type III	Forward	AAGAAAAATGATACTTCTCT	50
	Reverse	TATCTTTTACTGGTGAGCAC	
Collagen type IV	Forward	ACTCTTTTGTGATGCACACCA	60
	Reverse	AAGCTGTAAGCGTTTGCGTA	
Cardiac α-actin	Forward	TGCTGATCGTATGCAGAAGG	62
	Reverse	GCTGGAAGGTGGACAGAGAG	
Myosin light chain-2	Forward	CCTCTCAATGTTTGGGGAGA	62
	Reverse	CCTCCTCCTTGGAAAACCTC	
GAPDH	Forward	ACCACAGTCCATGCCATCAC	64
	Reverse	TCCACCACCCTGTTGCTGTA	
18S	Forward	CGCCGCTAGAGGTGAATTC	65.5
	Reverse	TTGGCAAATGCTTTCGCTC	

the incorporated radioactivity remaining in the cell lysate was measured in a liquid scintillation counter. The data were calculated as cpm/mg protein and control values assigned an arbitrary value of 1.

2.7. Analysis of ERK1/2, p38 MAP kinase and JAK phosphorylation

Cells grown on 6 well plates were treated with leptin (6 nM, up to 60 min as indicated in figure legends) then washed quickly with ice-cold PBS. Lysates to assess ERK1/2, p38 MAPK and JAK phosphorylation were prepared as previously described by us [26]. Equal amounts of protein (~30 μg) were then immunoblotted using phospho-specific ERK1/2, p38 MAPK and JAK antibodies (Cell Signaling Technology, Beverly, MA), again as previously described [24]. Quantitative analysis was performed by densitometric scanning using NIH Image software.

2.8. Statistical analysis

Data analysis was conducted by one way analysis of variance with Tukey-Kramer multiple comparison test. The level of significance was set at $p<0.05$.

3. Results

We first isolated RNA and used PCR to establish that the human pediatric ventricular cardiomyocytes used in this study express both long (obR_L) and short (obR_s) isoforms of leptin receptor (data not shown). MMP activity was then examined using gelatin zymography and Fig. 1A shows that leptin significantly enhanced gelatin degradation at an apparent molecular weight corresponding to native MMP-2 (72 kDa) in a dose-dependent manner (0–60 nM). A statistically significant stimulation of up to 1.5-fold was

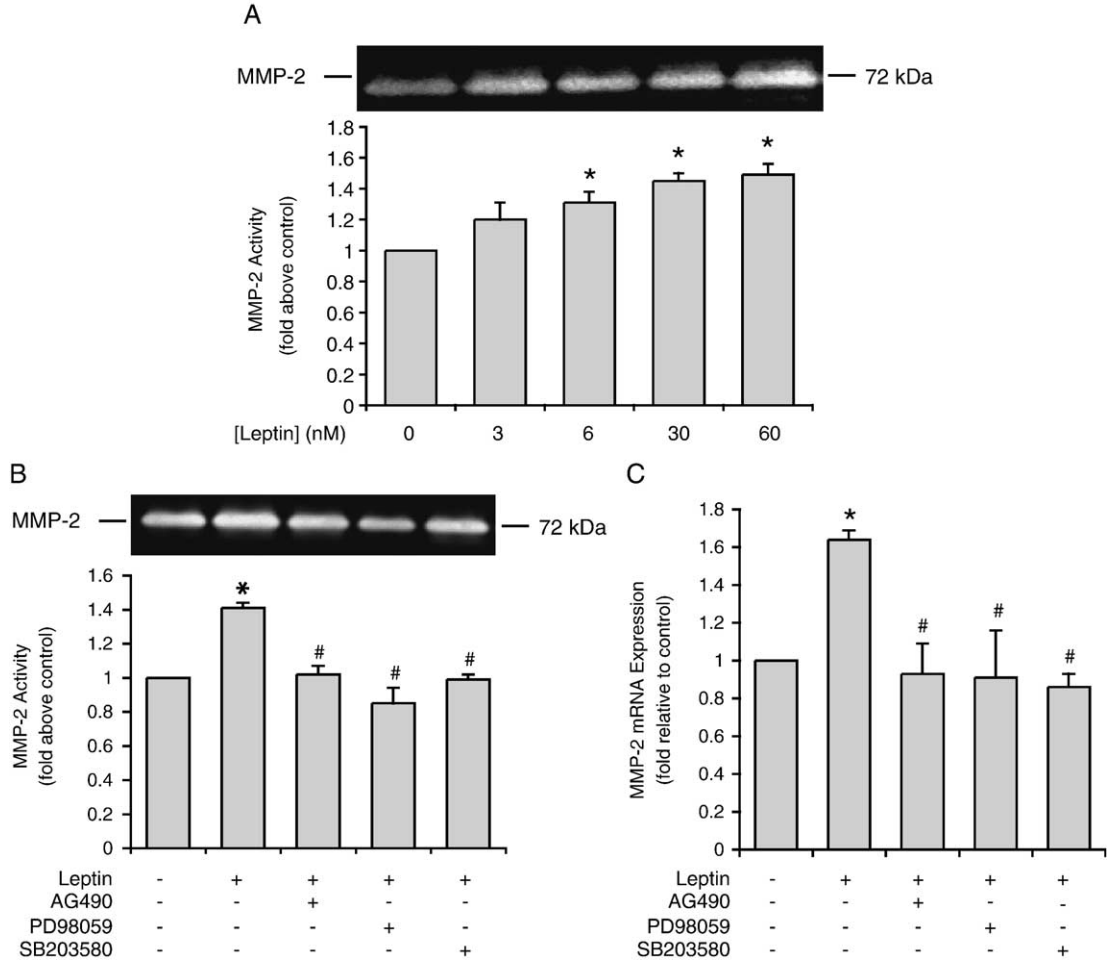

Fig. 1. Analysis of the effect of leptin on matrix metalloproteinase. Gelatin zymogarphy analysis was conducted using conditioned media from control and leptin-treated (3, 6, 30 and 60 nM, for 24 h) cells. The representative zymography gel together with quantitative analysis shown in (A) demonstrates that leptin increased protease activity at ~72 kDa in human cardiomyocytes in a dose-dependent manner. In (B), cells were treated with AG490 (20 μM), PD98059 (20 μM) or SB203580 (10 μM) for 1 h prior to stimulation with leptin (6 nM) for 24 h. Total proteolytic activity was quantitatively assessed by densitometric scanning. After similar incubation conditions RNA was isolated and, as shown in (C), analysis of MMP-2 mRNA expression was determined by quantitative real-time PCR using primers listed in Table 1. Results are expressed relative to control samples and are mean±SEM of at least three independent experiments where * indicates $p<0.05$ from control and # indicates $p<0.05$ compared to leptin alone.

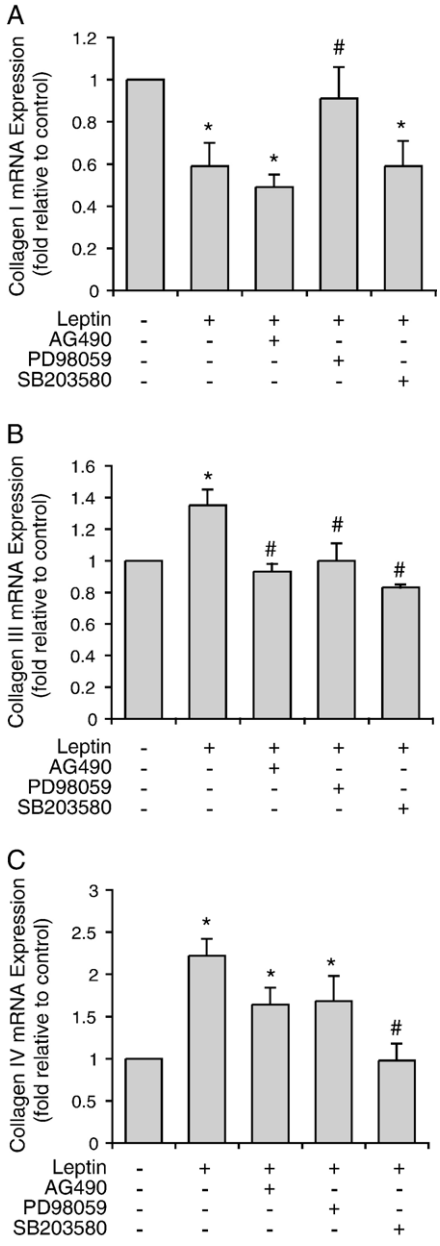

Fig. 2. Effects of leptin on collagen isoform mRNA expression. Cells were treated for 1 h with AG490 (20 μM), PD98059 (20 μM), SB203580 (10 μM) prior to stimulation with leptin (6 nM) for 24 h. RNA was isolated and analysis of collagen isoform mRNA expression was determined by quantitative real-time PCR using primers listed in Table 1. Results shown are expressed relative to control samples and are mean ± SEM of at least four independent experiments where * indicates $p < 0.05$ from control and # indicates $p < 0.05$ compared to leptin alone.

elicited by leptin at 6, 30 and 60 nM when compared with control. We also examined regulation of the effect of leptin on MMP activity by inhibitors of previously characterized leptin signaling pathways including AG490 for JAK, PD98059 for ERK1/2 and SB203580 for p38 MAPK. Results shown in Fig. 1B indicate that inhibiting each of these pathways can significantly attenuate the ability of leptin to increase MMP-2 activity. Since the results of gelatin zymography analyses suggested regulation of MMP-2, we next directly examined whether leptin altered the expression level of this protein using real-time quantitative PCR. We found an increase in MMP-2 mRNA expression of approximately 1.6-fold (Fig. 1C). Again, inhibitors of JAK, ERK1/2 and p38 MAPK also attenuated this effect of leptin (Fig. 1C). We also examined the effect of leptin on MT1-MMP expression by quantitative RT-PCR and observed no change (data not shown).

We next examined the effect of leptin on collagen isoform mRNA expression levels. Collagen type I content was significantly decreased (41%) by leptin (Fig. 2A) whereas the amount of type III and type IV isoforms were increased 1.35-fold and 2.22-fold, respectively (Fig. 2B and C). The ability of leptin to decrease collagen type I mRNA levels was sensitive only to the ERK1/2 inhibitor PD98059 and was unaffected by inhibitors of JAK and p38 MAPK (Fig. 2A). The small but significant increase in collagen type III mRNA was prevented by all inhibitors tested (Fig. 2B) whereas increased collagen type IV mRNA expression was significantly attenuated by SB203580 and only modestly by AG490 or PD98059 (Fig. 2C). As shown in Fig. 3, treatment with 6 nM leptin for 24 h caused a small, but not significant, increase in [^{3}H]proline incorporation, a marker of total collagen synthesis. Despite the small magnitude of reduction in collagen type I synthesis and larger increases in collagen type III and IV mRNA expression, the fact that proline incorporation did not change significantly is understandable given that collagen type I normally represents over 85% of total myocardial collagen [10,27].

Fig. 4A demonstrates that human pediatric cardiomyocyte size is significantly increased (60%, $p < 0.05$) by treatment with 6 nM leptin for 24 h. Protein synthesis is an essential component of myocyte hypertrophy and as shown in Fig. 4B, treatment with 6 nM leptin for 24

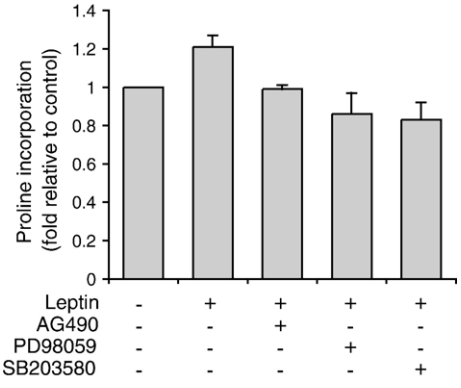

Fig. 3. Effects of leptin on [^{3}H] proline incorporation. Cells were pretreated for 1 h with AG490 (20 μM), PD98059 (20 μM), SB203580 (10 μM) prior to stimulation with leptin (6 nM) for 24 h in the continued presence of inhibitor. Analysis of [^{3}H] proline incorporation was determined and results are expressed relative to values of [^{3}H]proline incorporation seen in control samples and are mean ± SEM of three independent experiments. Statistical analysis showed no significant difference between any of the conditions and control.

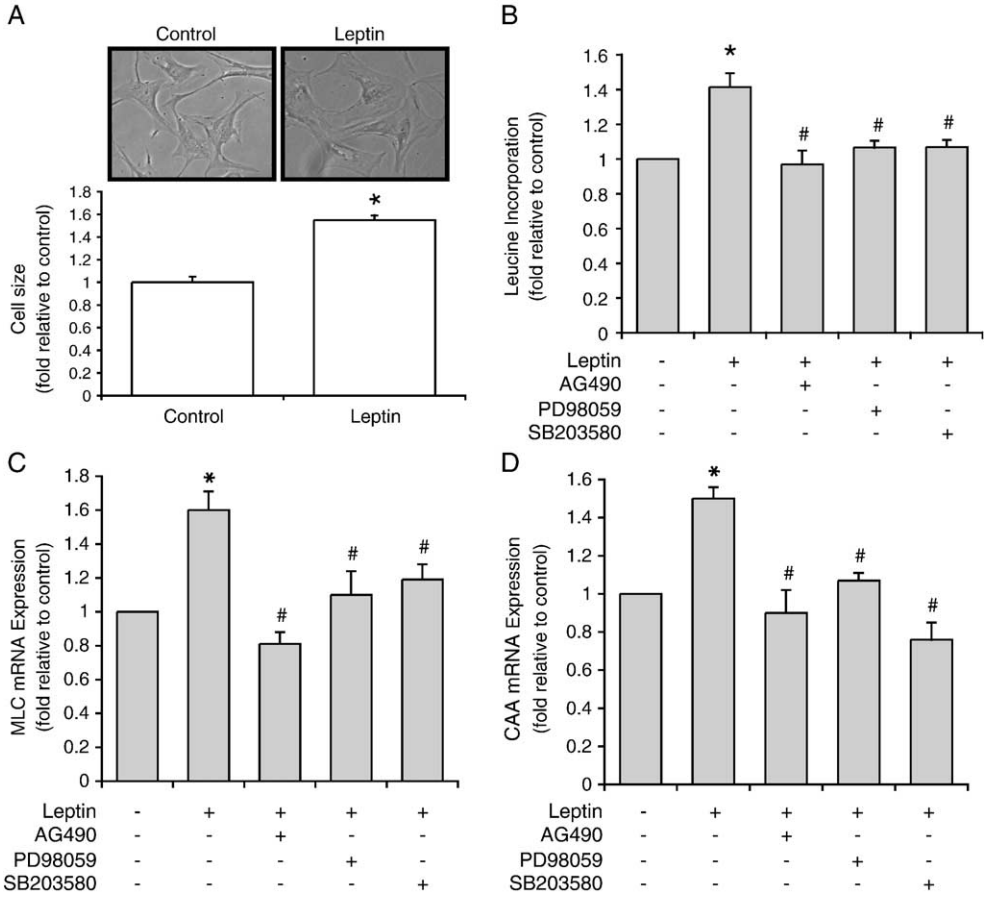

Fig. 4. Effects of leptin on cardiomyocyte hypertrophy. Human pediatric ventricular myocytes were treated with leptin (6 nM) for 24 h prior to analysis of cell size. Representative images are shown (A) above the quantitation of >20 individual cells from 3 independent experiments. In (B), cells were pretreated for 1 h with or AG490 (20 μM), PD98059 (20 μM) or SB203580 (10 μM) prior to stimulation with leptin (6 nM) for 24 h in the continued presence of inhibitor and leucine incorporation determined. Myosin light chain-2 (C) and cardiac α actin (D) mRNA expression was determined in human pediatric ventricular myocytes pretreated after similar incubation conditions as above and results expressed as fold changes relative to values of mRNA expression of either genes seen in control samples. Results are mean ± SEM of at least three independent experiments where * indicates $p < 0.05$ with respect to control and # indicates $p < 0.05$ with respect to leptin alone.

h increased (~42%, $p < 0.05$) [^{3}H]leucine incroporation in human cardiomyocytes. We also examined the effect of leptin in cells pretreated for 1 h with AG490, PD98059 and SB203580. Each inhibitor prevented the ability of leptin to increase leucine incorporation (Fig. 4B) whereas none of the inhibitors significantly altered basal leucine incorporation when used alone (data not shown). We also show using quantitative real-time PCR that leptin (6 nM, 24 h) induced mRNA expression of two markers of cardiomyocyte hypertrophy; myosin light chain-2 (Fig. 4C) and cardiac α-actin (Fig. 4D) by approximately 60 and 50 % ($p < 0.05$), respectively. Again, we also demonstrate that leptin-induced cardiac α-actin and myosin light chain-2 expression is prevented by AG490, PD98059 and SB203580 (Fig. 4C and D). Incubation of cells with inhibitors alone in the absence of leptin gave no significant differences compared with control (data not shown). Since our results suggested that the ability of leptin to induce hypertrophy and regulate MMP and collagen synthesis is mediated by signaling pathways involving JAK, ERK1/2 and p38 MAPK it was important to demonstrate the ability of leptin to activate these kinases in human cardiomyocytes. This was done by preparing cell lysates after treating cells with 6 nM leptin for 5 or 10 min then immunoblotting with phosphospecific antibodies for each kinase. Results shown in Fig. 5A, B and C, confirmed that leptin stimulated JAK, ERK1/2 and p38 MAPK in these cells.

4. Discussion

Given the increasing prevalence of obesity and its association with the development of heart failure [1,2,28], understanding the mechanisms underlying this association is imperative. The ECM provides the support essential for maintaining alignment of myofibrils as well as for maintaining myocyte alignment within the myocardium. Remodeling of the ECM is primarily mediated by MMPs, which can collectively degrade all structural proteins of the ECM and their role in heart failure is summarized in several recent

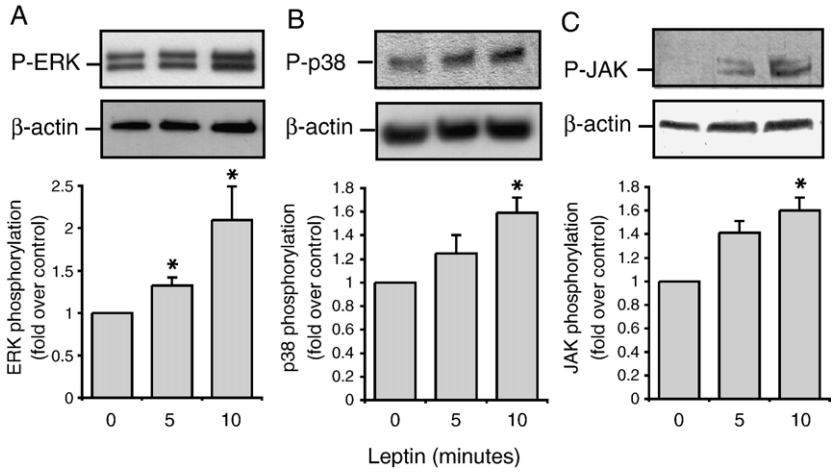

Fig. 5. Stimulation of JAK, ERK1/2 and p38MAPK by leptin. Cell lysates were prepared from human pediatric ventricular myocytes treated with leptin (6 nM) for 5 and 10 min. Equal amounts of protein were resolved by SDS-PAGE and activation of ERK1/2 (A), p38 MAPK (B) and JAK (C) was examined by Western blotting using phosphospecific antibodies. An immunoblot of one representative experiment including analysis of β-actin levels, together with quantitation of $n=3$ or 4 experiments is shown in each case. Statistical analysis was performed and * indicates $p<0.05$.

excellent review articles [10,15]. During the process of remodelling MMPs are initially activated to reduce wall stress and allow dilation in response to increased workload by increasing fibrillar collagen denaturation and degradation. Ultimately, cardiac function is adversely affected since the ultrastructural collagen which is initially degraded by MMPs is replaced by poorly structured collagen. The direct effect of leptin on ECM metabolism in cardiomyocytes is unknown, therefore in this study we examined the effects of leptin on MMP and collagen isoform production in human cardiomyocytes to further investigate the potential role of this adipokine as a mediator of remodeling in obesity [3–6].

As noted above, MMPs are the driving force for degradation of ECM during remodeling and changes in MMP levels have been described in both human and a variety of animal models of heart failure [29–35]. Here we found using gelatin zymography that leptin enhanced proteolytic activity at ~72 kDa. This corresponds with the latent form of MMP2 which is constitutively expressed at low levels in many cell types [36,37]. Using quantitative PCR we also confirmed that leptin enhanced MMP-2 mRNA expression. The latent form of MMP2 is normally activated by membrane type (MT) MMPs [38]. Here we examined the effect of leptin on MT1-MMP expression in human cardiomyocytes but did not observe any change. In addition, it has been demonstrated previously that MMP2 can serve as an activator of other MMPs, although we did not detect additional MMP activity upon zymography analysis of conditioned media from cardiomyocytes. However, there exists the intriguing and likely possibility that paracrine effects of proteins secreted by cardiomyocytes and adjacent cells such as fibroblasts may be of great importance in vivo.

Work in a variety of animal models supports an important role for alterations in MMP activity in the progression of heart failure. Transgenic mice overexpressing MMP1 initially show myocyte hypertrophy and normal heart function but ultimately showed progressive left ventricular remodeling which led to heart failure [39]. A decrease in infarction-induced left ventricular size was observed in mice lacking MMP2 or MMP9 [40–42]. Chemical inhibition of MMPs has also been shown to attenuate left ventricular dilation and preserve function after coronary ligation [40,43]. Some precedent exists for the potential of leptin to regulate MMP synthesis and activity from studies in a variety of tissues. Previous work has demonstrated increased MMP2 activity in response to leptin in human endothelial cells (HUVECs) [44], cytotrophoblastic cells [45] and coronary artery smooth muscle cells [46] and increased MMP9 production by leptin in cytotrophoblastic cells [45]. It is also known that MMP2 activity can be attenuated by all four tissue inhibitor of metalloproteinase (TIMP) isoforms [37]. Our work now demonstrates that leptin can increase MMP2 expression in human pediatric cardiomyocytes. Thus, it might be projected that if leptin enhances MMP2 levels in obese individuals this might play a detrimental role in the function of the heart.

A detrimental consequence of excessive MMP activity during remodeling is the replacement of the normal highly structured matrix collagen profile by fibrous interstitial deposits of poorly cross-linked collagens [10,29]. Collagens I, III and IV are the major forms found in the heart [13]. Collagen type I has the tensile strength of steel and comprises approximately 85% of all collagen in the myocardium [10,27]. The remainder of myocardial collagen is predominantly type III which confers elasticity to the myocardium, and to a lesser extent type IV [10]. Importantly, remodeling does not simply involve altered total levels of matrix collagens. The collagen type, organization and cross-linking are of great significance [16]. Indeed, changes in the ratio of collagens I and III occur in response to a variety of agents [17–20].

Previous studies have demonstrated effects of leptin on collagen synthesis in a variety of tissues. Leptin increased collagen type I promoter activity and collagen type I mRNA expression in human and rat hepatic stellate cells [47,48]. Leptin was also found to stimulate collagen type I production in cultured db/db mouse mesangial cells [49] and collagen type IV expression in rat glomerular endothelial cells [50]. However, until now any regulation of collagen synthesis in cardiomyocytes by leptin has not been documented. Here we show that leptin acts to decrease collagen type I but increase type III and IV mRNA in human ventricular myocytes. As indicated above, the collagen isoform profile rather than total collagen content is of great importance in ventricular remodeling. Indeed, here we did not observe a significant change in proline incorporation in response to leptin yet the changes in collagen type production could be of great impact as a contributor to remodeling in obesity. It is now clear that fibrosis may be caused by systemic effects resulting in volume and pressure overload or by direct effects of other factors on the myocardium [10] and our work highlights the potential significance of leptin as one of these factors.

As indicated above, progressive enlargement in the left ventricle over time has been shown to be directly related to deterioration in ventricular performance [11,12]. We directly measured the size of cardiomyocytes grown under control conditions or in the presence of leptin and observed that leptin caused a significant increase in cell size. Increased protein synthesis is commonly used as an indicator of cell hypertrophy and studies conducted in cultured neonatal rat cardiomyocytes have shown an ~30–50% increase in leucine incorporation in response to leptin [25,51]. Here we demonstrate for the first time the importance of this phenomenon in human cardiomyocytes by showing that leptin increased leucine incorporation to a similar extent. Furthermore, we showed that leptin increased expression of mRNA for well characterized indices of hypertrophy, namely cardiac alpha actin as well as myosin light chain. Thus, our studies in human cardiomyocytes correlate with the two published reports showing that leptin can have direct hypertrophic effects in rodent cardiomyocytes [25,51]. One report suggesting that leptin mediates 'antihypertrophic' effects must be interpreted with caution. In this study, *ob/ob* mice which lack leptin and exhibit left ventricular hypertrophy, were infused with leptin which reversed the increase in left ventricular wall thickness [52]. The original hypertrophy in these mice may not have resulted from lack of leptin per se, but instead one of a multitude of other systemic alterations which occur in these animals. Furthermore, the ability of leptin to reverse hypertrophy most likely reflects correction of whole body physiological parameters that influence cardiac structure rather than simply a direct effect on myocytes. This model does not accurately reflect the alterations observed in human obesity and taking our data together with all available previous work [25,51,52] we believe that hyperleptinemia may induce a hypertrophic effect in human obesity.

We also investigated the signaling mechanisms responsible for mediating these effects of leptin. Given the established role of JAK, ERK1/2 and p38 MAPK in leptin signaling [7] we investigated their role in mediating these effects of leptin. We observed that each kinase appeared to attenuate the increased gelatinase activity and MMP-2 mRNA expression elicited by leptin. Collagen isoforms were regulated by distinct leptin-stimulated signaling pathways. For example, ERK1/2 did not play a role in the decreased collagen type I or increased type IV content induced by leptin, but did mediate the ability of leptin to increase collagen type III mRNA. Inhibition of p38 MAP kinase on the other hand prevented the ability of leptin to stimulate collagen type III and IV mRNA expression but did not alter leptin's effect on collagen type I. The fact that inhibition of distinct kinases can selectively regulate the ability of leptin to control expression of distinct collagen isoforms is interesting since it suggests the potential for pharmaceutical manipulation of the direct effects of leptin on cardiac collagen isoform content. Inhibitors of ERK1/2, p38MAPK and JAK signaling also attenuated the increase in leucine incorporation and cardiac α-actin and myosin light chain mRNA expression. Previous work in neonatal rat cardiomyocytes suggested that p38MAPK, but not ERK1/2 mediated the hypertrophic effects of leptin [25]. Importantly, these cells were shown to only express the short isoform of leptin receptor, whereas the human ventricular myocytes used in this study express both long and short leptin receptor isoforms. Therefore, it is possible that the additional role of ERK1/2 and JAK in our study reflects effects mediated by the long form of leptin receptor.

In summary, during the progression of ventricular remodeling leading to heart failure, changes in MMP expression and activation and alterations in collagen content are typically observed. The results of the present study provide evidence that leptin stimulates MMP2 expression and enhances expression of collagen type III and IV mRNA, but decreases that of collagen type I in human ventricular cardiomyocytes. These findings are also the first to demonstrate a direct hypertrophic effect of leptin in human pediatric ventricular myocytes and support results obtained previously in neonatal rodent cardiomyocytes [25,51]. Importantly, whether excessive effects of leptin prevail due to hyperleptinemia in obesity or whether selective leptin resistance that occurs in obesity encompasses effects on the heart is still a matter of debate [4,9,53]. It is unlikely that any single aspect of remodeling itself will adequately explain the progressive decompensation observed during the progression of heart failure. However, understanding the potential contribution of leptin to regulation of cardiac remodeling may aid in the development of therapeutic strategies for the treatment of heart failure in human obesity.

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