The Obesity-Associated Peptide Leptin Induces Hypertrophy in Neonatal Rat Ventricular Myocytes
Venkatesh Rajapurohitam, Xiaohong Tracey Gan, Lorrie A. Kirshenbaum, Morris Karmazyn

One of the major manifestations of obesity is increased production of the adipocyte-derived 16-kDa peptide leptin, which is also elevated in heart disease, including congestive heart failure. However, whether leptin can directly alter the cardiac phenotype is not known. We therefore studied the effect of leptin as a potential hypertrophic factor in cultured myocytes from 1- to 4-day-old neonatal rat heart ventricles. Using RT-PCR, we demonstrate that these cells express the short-form (OB-Ra) leptin receptor. Twenty-four hours of exposure to leptin (0.31 to 31.3 nmol/L) produces a significantly increased cell surface area that peaked at 0.63 nmol/L. Subsequent experiments were done with 3.1 nmol/L leptin, which significantly increased cell area by 42%, protein synthesis by 32%, and α-skeletal actin and myosin light chain-2 expression by 250% and 300%, respectively. These events occurred in the absence of any increased cell death. Hypertrophy was preceded by rapid activation of the mitogen-activated protein kinase system including p38 and p44/42 as early as 5 minutes after leptin addition, whereas hypertrophy was inhibited by the p38 inhibitor SB203580 but not by the p44/42 inhibitor PD98059. Our results demonstrate a direct hypertrophic effect of leptin and may offer a biological link between hypertrophy and hyperleptinemic conditions such as obesity.

Obesity is associated with increased production of leptin, a 16-kDa peptide that is a product of the obesity gene (ob) and produced primarily by adipocytes. The effects of leptin are mediated by distinct receptors (OB-R) belonging to the class 1 cytokine receptor family. It has been suggested that leptin may contribute to cardiovascular disease, independently of obesity such as in hypertension, where elevated levels of the peptide could be a contributing factor due to its ability to stimulate the sympathetic nervous system. Recent clinical evidence has implicated leptin as a potential independent risk factor for coronary heart disease, and increased plasma leptin levels have been found in patients with congestive heart failure. Heart failure is generally preceded by myocardial remodeling, involving cardiomyocyte hypertrophy and other maladaptive responses, although whether leptin contributes to these events has not been studied. Accordingly, we examined leptin’s effects in cultured cardiomyocytes and sought to identify potential mechanisms underlying these effects.

Materials and Methods
Experiments were done on primary cultures of rat neonatal cardiomyocytes exposed to leptin for 24 hours in the absence or presence of mitogen-activated protein kinase (MAPK) inhibitors. Hypertrophy was determined by measuring cell area, leucine incorporation, and gene expression of molecular markers. Cell viability was determined by vital staining and MAPK activation with Western blotting.

An expanded Materials and Methods section can be found in the online data supplement available at http://www.circresaha.org.

Results
The leptin receptors are generally classified into two groups, those with short intracellular domains of 40 or fewer amino acid residues (OB-Ra, -Rc, -Rd, -Re) and a family of receptors having a long intracellular domain (302 residues) termed OB-Rb. As shown in Figure 1A, only OB-Ra mRNA was identified in cardiomyocytes, while brain, as expected, expressed both forms of the receptor.

As shown in Figure 1B, at the lowest concentration studied (0.31 nmol/L), leptin increased cell surface area by about 32% whereas peak effects (42% increase) were seen at 0.63 nmol/L and did not increase with higher concentrations. Subsequent experiments were done with a leptin concentration of 3.1 nmol/L (see Discussion). Figure 1C shows phase-contrast images whereas Figures 1D and 1E illustrate cells staining for sarcomeric myosin heavy chain and cell viability, respectively. Approximately 95% of cells demonstrated myosin staining, indicating relatively low nonmyocyte contamination. Leptin had no effect on cell death as determined with vital dye staining; the percentage of positive staining for dead cells was 5.46±0.52 and 5.40±0.8 for control and leptin-treated cells, respectively.

Since MAPK is an important mediator of cardiac hypertrophy, and because leptin can activate MAPK in noncardiac cell lines, we determined the role of MAPK as a potential mediator of leptin’s effects. As shown in Figure 2, both phospho-p38 (Figures 2A and 2B) and phospho-p44/p42 (Figures 2C and 2D) levels were rapidly increased with leptin with peak stimulation after 5 and 10 minutes of leptin treatment. The stimulation in MAPK completely reversed to control values after 24-hour leptin exposure (not shown).

The p38 inhibitor SB203580 completely prevented the leptin-induced hypertrophy (Figure 3A), [3H]leucine incorporation (Figure 3B), and the increase in both α-skeletal actin (Figure 3C) and myosin light chain-2 (MLC-2) expression (Figure 3D). The p44/p42 inhibitor PD98059 was without effect on all indices, although it slightly reduced leucine incorporation such that values were not significantly greater from control (Figure 3). Neither drug exerted direct effects on its own or on any parameter.

Discussion
The basis for the increased incidence of cardiovascular-related diseases including cardiac hypertrophy in obese individuals is unknown, although increased plasma levels of leptin in obesity as well as cardiovascular disorders suggest that the peptide could be a contributing factor. Our findings demonstrate the
presence of leptin receptors in neonatal rat ventricular myocytes. We also demonstrate that leptin can directly increase cell surface area and expression of α-skeletal actin, a fetal gene, and MLC-2, a constitutive gene, which are upregulated in cardiac hypertrophy.\textsuperscript{15} We also show that leptin, at least at a concentration of 3.1 nmol/L, is devoid of a direct toxic influence as demonstrated by the lack of effect on cell death. Importantly, the hypertrophic effects of leptin occurred at concentrations well within plasma levels in obese individuals, which can exceed 100 ng/mL (6.1 nmol/L).\textsuperscript{11} Thus, the cardiac cell may be a target for circulating leptin: indeed leptin has been shown to inhibit myocyte shortening.\textsuperscript{16} Our novel observation that leptin produces cardiomyocyte hypertrophy may be important in providing a basis linking obesity and heart failure. Although it is not known how p38 mediates these effects, they likely occur as a consequence of phosphorylation of a downstream transcriptional factor. We should add that the antibodies we used for Western blotting analysis do not permit us to distinguish between the various p38 isoforms, and thus at present it is not possible to comment on the nature or specificity of the p38 isoform mediating the effect of leptin.

Results using cultured myocytes should be interpreted cautiously. However, the ability of leptin to produce hypertrophy at concentrations well within plasma levels of obese individuals suggests a potential direct link between hyperleptinemia seen in obesity and some cardiovascular disorders and increased risk of cardiovascular disease, particularly associated with a hypertrophic phenotype. At present, a clear cause-and-effect relationship linking leptin to heart disease is difficult to demonstrate with certainty because of the...
unavailability of leptin receptor antagonists. A recent study has demonstrated an association between plasma leptin levels greater than 3.1 nmol/L and left ventricular hypertrophy.19 The prospect of leptin antagonism or inhibition of leptin synthesis as a therapeutic target for treating heart disease is potentially attractive and warrants further investigation aimed at determining the precise physiological or pathophysiological role of the peptide.

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References

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Methods

Primary culture of cardiomyocytes

Primary cultures of cardiomyocytes were prepared from 1-day old Sprague-Dawley neonatal rat heart ventricles as described previously.\textsuperscript{1,2} Briefly, the hearts were isolated, ventricles separated, minced and subjected to 4 sequential collagenase digestions at 370 C. All digestions were pooled, centrifuged for 5 min at 500 x g. The cell pellet was suspended in culture medium and plated on Primaria dishes.

Cell surface area analysis

The cells were cultured for 24h in serum-containing medium after which they were serum-starved for 24h before treatment. Cells were treated with increasing concentrations of leptin or its vehicle (control). Subsequent experiments were done with 3.1 nmol/L leptin in the absence or presence of the p38 inhibitor SB203580 (10 µmol/L) or the p44/42 inhibitor PD98059 (10 µmol/L) for 24h. To assess cell surface area, at least 50 cells from each experiment were randomly selected for analysis using a Leica inverted microscope equipped with a Polaroid digital camera at 20 x magnification. Cell area was measured using Mocha software.

RNA isolation and RT-PCR

Total RNA was extracted using Trizol according to the manufacturer’s instructions. 5 µg RNA were used to synthesize first strand of cDNA using SuperScript\textsuperscript{TM} II RNase H-Reverse Transcriptase (Invitrogen, CA) according to the manufacturer’s protocol and was used as template in the following PCR reactions:

Amplification of leptin receptors
Amplification of 160 bp product of OB-Rb was carried out using the 5’-GCTGAGAGCACCAGGGAACC–3’ forward and 5’-GTTTCCTGGCGATGCACTGGC –3’ reverse primers. The PCR cycle conditions were 950 C for 1min; 600 C for 45 sec and 720 C for 1min for 30 cycles. For amplification of OB-Ra with a product size of 446 bp, the forward primer 5’-CCTGAGCAGCAGCTGCG–3’ and reverse primer 5’-TCAAGAGTGTCGGCTCT –3’ were used at the following PCR cycle conditions: 950 C for 1min; 550 C for 45 sec and 720 C for 1 min for 30 cycles. The products were resolved on 3% agarose gel followed by ethidium bromide staining.

Real-time PCR analysis for MLC-2, α-skeletal actin and 18S rRNA.

The expression of α-skeletal actin, MLC-2 and 18S rRNA genes was quantified using real-time PCR analysis (DNA Engine Opticon 2 System, MJ Research). The α-skeletal actin was amplified using forward primer 5’ CACGCGATTATCACCAACTG- 3’ and the reverse primer 5’ CCCAGGACATAGAGACAG- 3’. The MLC-2 was amplified using forward primer 5’ TGCGAACATCTGGTCGATC- 3’ and reverse primer 5’ GCTGCGAACA TCTGGTCGATC-3’. The PCR conditions to amplify α-skeletal actin and MLC-2 were950 C for 20 sec; 600 C for 30 sec and 720 C for 30 sec for 35 cycles. The amplification of 18S rRNA was carried out using forward primer 5'GTAACCCGTGAAACCCCATT-3' and reverse primer 5' CCATCCAATCGGTAGTACGCG-3' with the PCR conditions of 940 C for 20 sec; 570 C for 30 sec and 720 C for 30 sec for 35 cycles. The mRNA levels were quantified using a standard graph of the respective genes. All the quantification values were within the range of standards used.

Incorporation of [³H] leucine

Cardiomyocytes (4x105) were plated into 24-well Primaria culture plates and cultured for 24 h in serum containing media followed by incubation in serum free media for 24 h. The cultures were treated with vehicle or 3.1 nmol/L leptin in presence or absence of SB203580 or PD98059. Two μCi [³H] leucine were added to each well and incubated for
24 h. At end of labeling, cultures were washed 3 times with ice cold PBS and incubated with 5% TCA on ice for 30 min. The cells were washed 2 more times with ice cold 5% TCA and solubilised in 0.5N NaOH. After neutralizing with 0.5N HCl, an aliquot was taken to determine the incorporated radioactivity by liquid scintillation counting.

**Immunofluorescence**

Cardiomyocytes were cultured for 24 h in serum containing medium followed by serum starvation for 24 h. Cells were treated with leptin (3.1 nmol/L) for 24 h and assessed for viability by staining cells with the vital dyes calcein-acetoxyethyl ester (AM) (2 mmol/L) and ethidium homodimer-1 (2 mmol/L) for 30 min (Molecular Probes, Eugene). Cells were washed and mounted on glass slides and visualized using an Olympus AX70 Research microscope equipped with an excitation and emission filter set to simultaneously detect the number of live (green) and dead (red) cells, respectively. The relative number of green vs. red cells was determined from at least 500 cells/condition. to identify the number of dead cells as previously reported. The number of green cells versus red cells was used to determine changes in cell viability. In addition, myocytes were fixed and incubated with a murine antibody directed toward sarcomeric myosin heavy chain (1:5 dilution MF20 hybridoma) and 10 μg/ml rhodamine-conjugated sheep F(ab’)2 anti-mouse IgG (Boehringer Mannheim) followed by Hoechst 33258 dye for nuclear morphology.

**SDS-PAGE and Western blotting.**

Cardiomyocytes were cultured in serum containing media for 48 h followed by serum starvation for 24 h. Cells were treated with 3.1 nmol/L leptin for indicated times. After treatment the cells were washed twice with cold PBS and lysed in buffer containing 50 mM Tris-Cl, pH 7.5, 150 mM NaCl, 10 mM tetrasodium phosphate, 40 mM β-glycerophosphate, 2 mM EDTA, 2 mM EGTA, 1% Triton X, 10% glycerol, 50 mM sodium fluoride, 0.2 mM sodium orthovanadate and protease inhibitors. The cell lysate suspension was centrifuged and clear supernatant was stored at –800 C. Protein
Quantification was done using the Bio-Rad protein assay. Protein (30 µg) was resolved on 10% acrylamide gel and transferred on to 0.45 µM nitrocellulose membrane. Membranes were stained with Ponceau S to check for transfer efficiency and protein quality. After blocking the membranes with 5% skim dry milk (Bio-rad, CA), they were probed with P-p38 antibody (Cell Signaling Technology, MA) or for P-p44/42 (Cell Signaling Technology). After incubating with horseradish peroxidase-coupled to the secondary antibody, the signal was detected with ECL western blotting detection reagents (Amersham Pharmacia, England). The blots were stripped and reprobed with either p38 (Santa Cruz Biotechnology, CA) or p44/42 (Santa Cruz Biotechnology) antibodies, respectively.

**Statistical analysis**

The data were analysed with ANOVA and post ANOVA analysis with Dunnett's. Student’s t-test for unpaired data was used to determine differences in cell viability between control and leptin treated cells. P values of < 0.05 were considered as significant.

**References**

