Leptin Signaling in the Failing and Mechanically Unloaded Human Heart

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Background—Increased circulating leptin is present in human heart failure, and leptin deficiency is linked to worse outcomes in chronic ischemic injury. In the present observational study, we tested the hypothesis that cardiac leptin production and signaling are increased in the failing human heart, and that mechanical unloading with a ventricular assist device (VAD) reverses these changes.

Methods and Results—All studies were performed using human cardiac tissue obtained from (1) hearts not matched for transplantation (nonfailing), (2) at the time of cardiac transplant (failing), or (3) paired samples at the time of VAD implant (pre-VAD) and removal (post-VAD). The expression of brain natriuretic peptide, leptin, leptin receptor, and tumor necrosis factor α mRNA was measured, and the protein expression of leptin and its receptor was examined by Western blot and immunofluorescent staining of cardiac sections. The assessment of leptin signaling was performed by measuring the phosphorylation state of the leptin receptor. The phosphorylation state of signal transducer and activator of transcription-3 and AMP-activated kinase proteins were also measured. All data are expressed as mean±SEM with a statistical significance in failing relative to nonfailing groups determined by Student independent t test, and the significance between pre- and post-VAD groups determined by paired t test. In failing human hearts, the mRNA expressions of leptin and its receptor were increased 5.4±0.3-fold (P<0.05) and 4.5±0.3-fold (P<0.05), respectively, with similar changes in protein. The phosphorylation state of both the leptin receptor and signal transducer and activator of transcription-3 proteins were increased 1.4±0.1-fold (P<0.05), and the level of phosphorylated AMP-activated kinase protein was increased 1.9±0.2-fold (P<0.05). Mechanical unloading of the failing human heart with a VAD resulted in no change in tumor necrosis factor α expression but a marked decrease in leptin production to 1.7±0.1% (P<0.05) and leptin receptor expression to 3.0±0.2% (P<0.05) of pre-VAD levels. Phosphorylation of the leptin receptor, signal transducer and activator of transcription-3, and AMP-activated kinase were also decreased to 45±7%, 75±8%, and 58±8% of pre-VAD values, respectively (P<0.05 for all values).

Conclusions—These results indicate that the failing human heart increases expression of leptin and its receptor and that mechanical unloading downregulates this increase. Further, a cardioprotective role for leptin in the failing human heart is suggested through the activation of signal transducer and activator of transcription-3 and AMP-activated kinase signaling. (Circ Heart Fail. 2009;2:676-683.)

Key Words: leptin ■ leptin receptor ■ ventricular assist device ■ AMP kinase ■ heart failure

 Hunman systolic heart failure is characterized by loss of myocardocyte number and decreased contractile performance of the left ventricle (LV).1 The etiology of systolic heart failure is multifactorial, with coronary artery disease linked to the majority of cases in the elderly.2 There are also nonischemic causes of heart failure, resulting in viral, hypertensive, and valvular cardiomyopathies.3 Regardless of the etiology, however, studies have shown that cytokines play an important role in both the progression and mitigation of cardiac remodeling. For example, although leptin-deficient Ob/Ob mice demonstrate age-related cardiac hypertrophy,4 greater mortality in viral myocarditis,5 and worse LV function and survival in response to ischemic injury,6 repletion of leptin mitigates these deleterious effects of leptin deficiency. In addition, cardiac leptin production and signaling are increased in mice after myocardial infarction (MI).6 In humans, serum leptin is increased after MI7 and in advanced heart failure8 independent of weight. Thus, leptin is an important cytokine that mediates beneficial effects in heart failure when present and results in deleterious effects when absent.

Clinical Perspective on p 683

Although serum leptin is increased in human heart failure,8 it is unknown if leptin production or signaling is altered in the failing human heart. Further, if cardiac leptin production or signaling is altered in human heart failure, determination of
whether it is a cause or consequence of pathological changes becomes an important issue. This study was undertaken to test the hypothesis that the failing human heart produces leptin and upregulates beneficial leptin signaling pathways, including signal transducer and activator of transcription (STAT)-3 and AMP-activated protein kinase (AMPK). Additional experiments examining the effect of LV mechanical unloading on cardiac leptin production and signaling were performed to test the hypothesis that alterations in leptin production and signaling are a consequence rather than a cause of myocardial dysfunction.

Methods

Human Heart Samples
Failing human heart tissue was obtained from the University of Pittsburgh after institutional review board approval and with the subjects’ informed consent. Transmural samples of the lateral wall of the LV were obtained from failing human hearts at the time of transplant (n = 10) or from the LV apex at the time of ventricular assist device (VAD) implant and removal (paired samples, n = 8). Nonfailing human heart tissue (NF) was obtained from the Cleveland Clinic Foundation after institutional review board approval and with the subjects’ informed consent. Transmural samples of the lateral wall of the LV were obtained from unmatched donor hearts that did not meet criteria for transplantation, including donor age >50 years (n = 2), positive toxicity screen (n = 1), mild coronary artery disease (n = 2), and 2+ mitral regurgitation detected on echocardiography (n = 1). All cardiac tissue was placed into cold cardioplegic solution (4°C to 8°C) and rapidly transported to the laboratory. Tissues were then divided for fixation and histology or snap frozen in liquid nitrogen and stored at −80°C until biochemical analyses.

Quantitative Real-Time RT-PCR Analysis
Total RNA was isolated and reverse transcribed from whole tissue homogenates, as previously described.6 Real-time RT-PCR was performed using the DNA-binding dye SYBR Green1 and previously validated primers and conditions for the real-time RT-PCR detection of human brain naturetic peptide (BNP),10 human leptin,11 human long form leptin receptor (ObR),12 human tumor necrosis factor (TNF)-α,13 and human GAPDH.14 Specific PCR products were confirmed by the presence of a single first derivative melting peak,15 and by the amplification product size determined by gel electrophoresis.16 Equal amplification kinetics of the target and the reference genes (GAPDH) were confirmed by serial dilutions as described.17 Quantification was then performed using the comparative Ct method (2−ΔΔCt).18

Western Blotting
Protein was extracted from cardiac tissue in RIPA buffer, subjected to SDS-PAGE, and electrotransferred to PVDF membranes in equal amounts, as previously described.6 All gels were run with a broad range molecular weight standard (Biorad; catalog No. 161-0375). Antibodies that were used detected leptin (Santa Cruz Biotechnology; catalog No. sc-842) or anti-ObR (Santa Cruz Biotechnology; catalog No. sc-8325), Y705-phosphorylated STAT-3 (Cell Signaling; catalog No. sc-16419), STAT-3 (Cell Signaling; catalog No. sc-8325), Y935-phosphorylated ObR (Santa Cruz Biotechnology; catalog No. sc-16419), AMPK-α (Cell Signaling; catalog No. 2531), and GAPDH (Research Diagnostics Inc; catalog No. RDI-TRK5G4–6C5). After transfer to membranes, blots were blocked in 5% milk plus tris buffered saline tween (TBST) for 1 hour at room temperature with agitation. Membranes were then cut at the appropriate molecular weight for probing with GAPDH and the protein of interest. The x-ray films were digitized using Image J software (NIH) to determine an integrated density in pixels/unit area. Detection of GAPDH was performed on all blots to control for differences in gel loading, protein transfer, and protein quantitation. Any blots with unequal GAPDH were repeated and not used for analysis. All blots were repeated at least 3 times to assure reproducibility of results. For changes in total protein, the mean integrated density of the band of interest was determined for comparative groups, and statistical testing (t test) was performed using the integrated density values for each member of the group. For ratios of phosphorylated to total protein, the ratio of the integrated densities of the phosphorylated to total protein of interest within each comparative group was used for statistical testing (t test). For graphical representation of the ratio data, the mean control group ratios (nonfailing or post-VAD) were arbitrarily defined as 1 for increases and 100% for decreases, and the comparative groups (failing and post-VAD) were expressed as a fold or percent change relative to it, respectively.

Immunofluorescence
Cardiac tissue was harvested and fixed in 2% paraformaldehyde and cryoprotected in 30% sucrose, as previously described.6 Short-axis cryotome sections (6 μm) taken at the level of the mid-LV were collected on slides and probed with either anti-leptin (Santa Cruz Biotechnology; catalog No. sc-842) or anti-ObR (Santa Cruz Biotechnology; catalog No. sc-8325) antibodies at a dilution of 1:100. A cy3-linked goat anti-rabbit (Invitrogen; catalog No. A10520) secondary antibody diluted 1:1000 was used for immunofluorescent detection. Both leptin and ObR stained sections were further stained with 488-linked phalloidin to detect sarcomeric actin and DAPI to detect nuclei as described.6 Images were acquired using an Olympus (Melville, NY) Provis AX70 fluorescent microscope at 20× magnification, digitized using a cooled charge-coupled device camera (Optronix Magnifier, East Muskogee, Okla) at 12-bit gray depth, and finally assembled in Adobe Photoshop 8.0 (Adobe Systems Inc, San Jose, Calif).

Statistical Analysis
Data presented are mean ± SEM. Statistical significance of mean changes in LV ejection fraction, body mass index (BMI), and mRNA and protein expressions between nonfailing and failing samples were determined by Student unpaired t test. Student paired t test was used to analyze these same data collected on pre- and post-VAD samples. Mathematical calculations, determination of probability values, and t tests were performed using the computer program Statistical Package for the Social Sciences (SPSS) v16 (SPSS Inc, Chicago, Ill).

The authors had full access to the data and take responsibility for its integrity. All authors have read and agree to the manuscript as written.

Results

Failing Human Hearts Demonstrate Increased BNP Expression
Equal numbers of ischemic (n = 5) and nonischemic (n = 5) patients were included in the failing group, which had an average LV ejection fraction of 15 ± 2% and an average age of 56 ± 4 years old. In contrast, the LV ejection fraction of the nonfailing group was preserved at 63 ± 3% (P < 0.05 versus failing). The BMI (25.7 ± 2.7 versus 26.4 ± 1.8 kg/m²; P > 0.05) and gender distribution (50% men, 50% women) of both nonfailing and failing groups were not significantly different from one another, respectively. Consistent with increased cardiomyocyte stretch and clinical heart failure, the mRNA expression of BNP was increased 4.4 ± 0.4-fold in failing relative to nonfailing hearts (P < 0.05).
Failing Human Hearts Demonstrate Increased Leptin Expression

Relative to nonfailing samples, failing hearts showed 5.4±0.3-fold increased expression of leptin mRNA (Figure 1A). This increased leptin mRNA expression in failing hearts correlated with increased leptin protein expression as determined by western blot using whole tissue homogenates (Figure 1B). Cardiomyocyte-specific leptin expression was confirmed through immunofluorescent staining of sections from nonfailing and failing samples (Figure 1C).

Failing Human Hearts Demonstrate Increased Leptin Receptor Expression

Long form leptin receptor mRNA expression was increased 4.5±0.3-fold in failing relative to nonfailing hearts (Figure 2A). This change in mRNA was paralleled by similar changes in leptin receptor protein as determined by western blot using whole heart homogenates (Figure 2B). Qualitatively, increased leptin receptor protein was localized to the cardiomyocyte in cardiac sections taken from failing hearts (Figure 2C).

Failing Human Hearts Demonstrate Increased Leptin Receptor, STAT-3, and AMPK Phosphorylation

The ratio of phosphorylated (p) ObR to total (t) ObR was increased 1.4±0.1-fold in failing relative to nonfailing hearts (Figure 3A), representing increased receptor activation. Further, the ratio of p/t STAT-3, an established downstream mediator of cardiac leptin signaling, was increased 1.4±0.1-fold, with a 2.3±0.2-fold increase in total STAT-3 protein (Figure 3B). Finally, the ratio of p/t AMP kinase α, which has been linked to leptin signaling and metabolic substrate use in the heart, was also increased 1.9±0.2-fold, with no change in the amount of total AMP kinase α protein present (Figure 3C).

Mechanical Unloading of the Failing Human Heart Decreases BNP and Leptin mRNA Expression, and Downregulates Leptin Receptor, STAT-3, and AMPK Activation

Paired heart samples analyzed in this subsequent study were obtained from 8 male patients who received VADs before heart transplant. The LV ejection fraction before VAD support was 13.6±2.1% and the average length of VAD support was 35±12 days. As with the first set of failing hearts, the etiology of heart failure was equally distributed among ischemic (n=4) and nonischemic (n=4) subgroups. The cardiac mRNA expressions of BNP and leptin were decreased to 1.7±0.1% and 3.0±0.2% of their initial values, respectively (Figure 4A and 4B), whereas the cardiac mRNA expression of tumor necrosis factor α was unchanged (Figure 4C). Consistent with a downregulation of cardiac leptin and AMPK signaling, the ratios of p/t ObR (Figure 5A), p/t...
STAT-3 (Figure 5B), and p/t AMPK-α (Figure 5C) were decreased to 45±7%, 75±8%, and 58±8% of pre-VAD values, respectively, with no change in total protein levels, after mechanical unloading.

**Discussion**

Heart failure is characterized by an increase in the production of cytokines that mediate both compensatory and pathological responses. In human heart failure, circulating leptin is increased independent of weight, and previous experiments with leptin-deficient mice demonstrate that leptin plays a protective role in the heart. Specifically, leptin deficiency results in worse survival and greater LV dysfunction in chronic ischemic injury, increased cardiac hypertrophy and apoptosis with aging, and greater mortality in viral myocarditis. In each of these studies, leptin-repletion restored measures of morbidity and mortality to wild-type levels. Although weight was controlled for in the ischemia, hypertrophy, and apoptosis studies, we cannot exclude the possibility that other deleterious consequences of the leptin-deficient state outside obesity contributed to the worse outcomes seen. For example, Ob/Ob mice demonstrate a diabetic phenotype with altered cardiac glucose and fatty acid metabolism that is exacerbated further by their obesity. Therefore, even in lean Ob/Ob mice, diabetes that develops secondary to leptin deficiency may contribute to the worse outcomes seen after MI. Nevertheless, a beneficial cardiac response to leptin in the setting of myocardial injury also likely requires activation of known cardioprotective signaling pathways. Our data demonstrate that cardiac leptin production and leptin receptor, STAT-3, and AMPK activation are increased in human failing hearts and decreased in response to mechanical unloading. These data suggest that the expression of leptin in the failing myocardium is potentially compensatory and is regulated by mechanical signaling and/or wall stress.

In humans, leptin receptor is present in normal and hypertrophied hearts, and the increased systemic leptin levels seen in the setting of MI and heart failure are likely secondary to increased production by fat. However, a major finding in our study was that the failing human heart, independent of BMI, upregulates leptin production and signaling. This is in agreement with our previous study demonstrating an increased cardiac leptin production and signaling after chronic ischemic injury in lean wild-type mice. Studies also demonstrate that systemic leptin deficiency is associated with worse outcomes in the setting of cardiac injury independent of weight, and that restoration of signaling in leptin-deficient mice, or increasing circulating leptin levels in lean wild-type mice, both by administration of exogenous leptin, improves cardiac function at 4 weeks post-MI. Combined, these data suggest that systemic leptin
plays an important role in conserving heart function in the setting of MI, and that exogenous administration may be useful therapy for improving cardiac function in patients with heart failure.

Systemic leptin production is decreased in response to caloric restriction and increased with caloric loading. However, the effect of caloric restriction or loading on cardiac leptin production is unknown. Studies that have examined cardiac leptin production demonstrate increases in response to cardiac injury. It is interesting to note that obesity and its accompanying hyperleptinemia are established risk factors for the development of hypertension, heart failure, and coronary artery disease, yet overweight and obese patients with these cardiovascular diagnoses have more favorable short- and long-term prognoses. Clinically, this “obesity paradox” might be explained by the hyperleptinemia of obesity, and selective resistance to the metabolic actions of leptin centrally, but not in cardiac tissue. Indeed, our data demonstrate increased cardiac leptin production and peripheral activation of leptin signaling in the failing human heart. Thus, both systemic and cardiac production of leptin may contribute to more favorable short- and long-term outcomes in the setting of myocardial injury and heart failure.

The benefits of leptin in heart failure are likely dependent on the activation of cardiac leptin signaling. On the basis of this, we hypothesized that the mechanism of improvement likely involves the activation of beneficial leptin signaling pathways in the heart, including STAT-3 and AMPK. Indeed, leptin...

Figure 3. ObR signaling is upregulated in the failing human heart. Data for bar graphs are expressed as fold changes in mean values ± SEM of failing (n=10) relative to nonfailing (n=6) samples, which were arbitrarily assigned a value of 1 after statistical calculations were performed. *P<0.05 relative to nonfailing samples. A, Mean fold change in the ratio of phosphorylated (p) to total (t) ObR protein (left graph) with representative Western blot (right panel) and molecular weight markers are shown. B, As in A, except that data for p/t STAT-3 are shown. C, As in A, except that data for p/t AMPK are shown.

Figure 4. Mechanical unloading of the failing human heart downregulates the expression of BNP and leptin, but not tumor necrosis factor α mRNAs. Data for bar graph are expressed as percent changes in mean values ± SEM of post-VAD (n=8) relative to paired pre-VAD (n=8) samples, which were arbitrarily assigned a value of 100% after statistical calculations were performed. *P<0.05 relative to failing, pre-VAD samples. A, Percent change in BNP mRNA. B, As in A, except that leptin mRNA was measured. C, As in A, except that tumor necrosis factor α mRNA was measured.
mitochondrial metabolic dysfunction,33 improves baseline contractility of the LV,34 decreases cardiomyocyte hypertrophy35 and apoptosis,36 and increases VEGF and other angiogenic growth factors.37 VAD support also results in a reduction in the expression of genes that are important markers of cardiac remodeling, including BNP.38 Consistent with this, in our series of pre- and post-VAD patients, cardiac production of BNP decreased with mechanical unloading. Similarly, cardiac mRNA levels of leptin decreased post-VAD, and decreased levels of leptin receptor, STAT-3, and AMPK phosphorylation were seen. In contrast, the expression of TNF-α remained unchanged, suggesting that our observed decrease in leptin production and signaling was not a nonspecific response to mechanical unloading. It is possible, however, that a mild degree of ischemia that accompanies decompensated heart failure, regardless of etiology, might be contributory to the changes we see in p/t AMPK ratios. Nevertheless, our finding of decreased STAT-3 and AMPK activation with mechanical unloading suggests that the cardiac production of leptin may influence the response of the failing heart to alterations in workload.

Although our data suggest a beneficial role for leptin in failing human hearts, there are several limitations to our study that deserve comment. First, our analysis was limited to the activation state of STAT-3 and AMPK. There are other known cardiac leptin signaling pathways that may mediate beneficial responses in heart failure that were not examined here, including Akt39 and phosphatidylinositol-3-kinase.40 Second, because this study was conducted with acutely collected human tissue, it is largely observational in its findings, and we are unable to assess responses in the failing human heart to leptin-specific agonist or antagonist challenge. Third, heart failure is a complex process involving the production of many cytokines and the activation of numerous intracellular signaling pathways. For example, in addition to leptin, the activation of STAT-3 and AMPK in the failing heart could also arise from leptin-independent pathways, including interleukin-641 and adiponectin,42 respectively. In addition, we acknowledge that diverse changes in cardiac STAT-3 phosphorylation have been reported in the failing human heart,43–45 and that it remains unclear whether leptin can activate AMPK in both skeletal46 and cardiac striated muscle.47 However, our observation of increased leptin production in the failing human heart is consistent with activation of downstream components of known leptin signaling pathways, including STAT-3 and AMPK. Finally, we did not measure serum leptin or changes in wall stress or workload in the failing or mechanically unloaded human heart, although changes in these measures are anticipated based on previous reports.4,8,34,48,49

In conclusion, our data shows increased leptin production and signaling in the failing human heart, with altered expression in response to changes in cardiac wall stress or workload. These observations complement studies reporting increased circulating leptin in human heart failure,8 as well as acutely after MI,7 independent of weight. In vitro studies using cultured cardiomyocytes50,51 and results from experiments using animal models of leptin deficiency subjected to cardiac injury5,6 suggest that leptin is a cardioprotective cytokine. The beneficial effect of STAT-3 and AMPK activation in the
failing heart has been reported in multiple studies and provides a plausible pathway by which physiological increases in leptin signaling may be beneficial to the injured heart.

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Disclosures

None.

References


**CLINICAL PERSPECTIVE**

The obesity paradox refers to an increase risk of developing hypertension, coronary artery disease, and heart failure with increasing body mass index but paradoxically overweight and obese patients with these cardiovascular diagnoses demonstrate more favorable short- and long-term prognoses versus patients with a more normal body mass index. A key advance in our understanding of obesity was the discovery of leptin, a circulating, predominantly fat-derived cytokine, which regulates metabolism and limits appetite through centrally mediated pathways. As more fat accumulates, more leptin is produced. However, a condition of “central leptin resistance” may develop in the presence of chronically increased circulating leptin, in which leptin fails to suppress appetite, setting the stage for clinical obesity. Aside from obesity, circulating leptin is increased independent of body mass index after myocardial infarction and in heart failure. In this study, we examined cardiac leptin production and signaling in failing and mechanically unloaded human hearts from overweight (body mass index $\geq 26$ kg/m$^2$) but not obese patients. Relative to nonfailing hearts, failing cardiac tissue demonstrated increased cardiac leptin production and signaling through its receptor, as well as activation of cardioprotective signal transducer and activator of transcription-3 and AMP-activated protein kinase proteins. Mechanical unloading with a ventricular assist device reversed these changes. Taken together, these findings suggest that the hyperleptinemia seen in obesity, cardiac ischemia, and heart failure might contribute to the obesity paradox by mediating the activation of cardioprotective peripheral (ie, cardiac) leptin signaling pathways to improve outcomes in the presence of central leptin resistance.
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