Original Research

Crosstalk between leptin and interleukin-1β abrogates negative inotropic effects in a model of chronic hyperleptinemia

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Abstract

Interleukin 1 beta (IL-1β) is a proinflammatory cytokine with potent cardiosuppressive effects. Previous studies have shown that leptin blunts the negative inotropic effects of IL-1β in isolated adult rat cardiac myocytes. However, the interactions between leptin and IL-1β in the heart have not been examined on a background of chronic hyperleptinemia. To study this interaction, we have chosen the SHHF rat, a model of spontaneous hypertension that ultimately develops congestive heart failure. SHHF that are heterozygous for a null mutation of the leptin receptor (+/*fa*^{cp}, HET) are phenotypically lean but chronically hyperleptinemic and develop heart failure earlier than their normoleptinemic true lean (+/+, LN) littermates. Simultaneous cell shortening and calcium transients were measured in isolated ventricular cardiac myocytes from LN and HET SHHF in response to leptin, IL-1β or IL-1β following one hour pretreatment with leptin. Despite evidence of metabolic leptin resistance, HET myocytes were sensitive to the negative inotropic effect of leptin, similar to LN. Contractility returned to control levels in myocytes from HET that were pretreated with leptin prior to IL-1β, while contractility remained depressed compared with control and similar to leptin alone in LN. Chronic hyperleptinemia resulted in altered JAK/STAT signaling in response to leptin and IL-1β in isolated perfused hearts from HET compared with LN SHHF. Phosphorylated STAT3 (pSTAT3) and STAT5 (pSTAT5) decreased when HET hearts were treated with leptin followed by IL-1β. While decreases in pSTAT3 and pSTAT5 may be associated with abrogation of the acute negative inotropic effects of IL-1β in the presence of leptin in HET, long-term consequences remain to be explored. This study demonstrates that the heart remains sensitive to leptin in a hyperleptinemic state. Crosstalk between leptin and IL-1β can influence cardiac function and cytokine signaling and these interactions are moderated by the presence of long-term hyperleptinemia.

Keywords: leptin, interleukin-1β, cardiac myocytes, signal transducers and activators of transcription, suppressors of cytokine signaling, SHHF rat

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Introduction

Hyperleptinemia accompanies a variety of conditions associated with cardiac functional decline and heart failure, including aging, obesity, type 2 diabetes mellitus and hypertension.[1] Decreased responsiveness to leptin (leptin resistance) is often associated with hyperleptinemia and is primarily selective for metabolic regulatory functions, resulting in impaired appetite suppression, altered thermogenesis and glucose intolerance. However, not all tissues become leptin-resistant and some co-morbidities associated with hyperleptinemia may result not so much from impaired signaling but from continued stimulation by chronically elevated leptin. Leptin has a negative inotropic effect on isolated cardiac myocytes,[2,3] and thus has the potential to suppress cardiac function. However, the role of leptin in cardiovascular disease remains controversial and studies suggest both detrimental and protective effects on the myocardium.[1,4] In reality, alterations in multiple cytokines typically occur in concert with cardiac functional decline in these same hyperleptinemic conditions as a result of low-grade inflammation.[5] While studies have looked at the effects of individual cytokines on cardiomyocyte function, few have examined the impact of multiple cytokine interactions on myocyte contractility or the effect of chronic hyperleptinemia on multiple cytokine interactions. Some of the apparent paradoxical effects of leptin may be the result of interactions with other cytokines whose expressions are simultaneously altered. Interleukin-1 beta (IL-1β) is a proinflammatory cytokine that has been implicated in the development

of cardiosuppression and ventricular dysfunction leading to congestive heart failure.[6,7] IL-1β causes a rapid, profound negative inotropic effect that is mediated through nitric oxide (NO) and sphingolipid signaling pathways.[2,8–10] Although leptin by itself has negative inotropic effects, treatment of isolated adult rat cardiac myocytes with leptin and IL-1β together can abrogate the negative inotropic effects produced by either of these cytokines alone.[2] Thus, crosstalk between IL-1β and leptin appears important in determining the outcome on cardiac contractility.

Leptin and IL-1β share several common signaling pathways, including generation of NO, activation of janus kinase 2 (JAK2), and phosphorylation of signal transducers and activators of transcription (STAT). Overlap of these signaling pathways may mediate crosstalk between cytokines such as leptin and IL-1β and influence the ultimate response to binding of these cytokines to their respective receptors. Binding of leptin to its receptor activates JAK2, resulting in phosphorylation of STAT3 and STAT5.[11] Subsequent expression of suppressor of cytokine signaling-3 (SOCS-3) occurs downstream to activation of STAT3. SOCS-3 is part of a negative feedback loop that blunts leptin signaling through JAK/STAT and is implicated in mediating central leptin resistance.[12,13] However, the inhibitory action of SOCS-3 generated in response to leptin has the potential to extend beyond self-regulation to modulation of signaling by other cytokines such as IL-1β. SOCS-3 prevents IL-1β-induced toxicity in other tissues such as rat pancreatic islet cells.[14–16] Likewise, leptin is protective against effects of IL-1β in pancreatic islet cells[17,18] and cardiomyocytes.[2] The role of this pathway in modulating the inotropic effects of leptin and IL-1β on cardiac myocytes has not been fully explored.

The SHHF/Mcc-*fa*^{cp} (spontaneous hypertensive heart failure) rat is a well characterized, spontaneous model of hypertension and congestive heart failure.[19–21] This strain of rat carries a mutation of the leptin receptor (*fa*^{cp})[19,22] that results in obesity and overt type 2 diabetes mellitus in animals that are homozygous for this mutation. While phenotypically lean and able to produce a functional leptin receptor, SHHF that are heterozygous for the *fa*^{cp} mutation (+/*fa*^{cp}, HET) exhibit traits of chronic leptin resistance, including increased fasting serum leptin, insulin resistance and glucose intolerance compared with their wild-type lean (+/+, LN) littermates. Onset of heart failure and death occurs earlier in HET compared with LN SHHF, suggesting that even mild chronic hyperleptinemia in the absence of obesity contributes to more rapid progression of cardiac functional decline.[20] We tested the hypothesis that cardiac myocytes from chronically hyperleptinemic HET SHHF rats remain sensitive to the cardiosuppressive effects of leptin and that the ability of leptin to modulate the inotropic effects of IL-1β is altered.

Materials and methods

The model

All studies were carried out in compliance with The Ohio State University Institutional Animal Care and Use Committee and NIH guidelines for care and use of laboratory animals. SHHF rats were obtained from the colony at The Ohio State University and were housed in a 12-h light/dark cycle with food and water provided *ad libitum*. Zygosity for the *fa*^{cp} gene in phenotypically lean SHHF was determined by amplifying the region of the leptin receptor spanning the mutation, followed by digestion with tru91 and electrophoresis on 5% agarose gel as previously described.[19] Colony records were used to determine differences in age of death due to congestive heart failure between LN ($n = 20$) and HET ($n = 20$) males. For the remaining studies, all SHHF rats were used at five months of age. At this age, hypertension is stable but cardiac functional decline has not yet begun. Following an overnight fast, oral glucose tolerance tests were done in a subset of rats by administering a 75% glucose solution *per os* at a dose of 0.1 mL/100 g body weight.[23] Heparinized plasma was obtained at 0, 30, 60 and 120 min and analyzed for glucose (hexokinase method) and insulin (radioimmunoassay; Linco/Millipore, St Charles, MO, USA). Systolic blood pressure was determined by tail cuff method (Visitech Systems, Apex, NC, USA). A serum sample obtained from the tail vein of fasting rats was used for determination of fasting serum leptin concentration by radioimmunoassay (Rat Leptin RIA; Linco/Millipore).

Isolated cardiac myocytes

Cardiac myocytes were isolated from five-month-old, wild-type LN (+/+) and HET (+/*fa*^{cp}) male SHHF rats ($n = 4$/genotype) as previously described.[2] All media was depyrogenated prior to use by pretreatment with polymyxin-B (10 μg/mL final concentration).[24] Following isolation, myocytes were suspended to a concentration of approximately 200,000 cells/mL in incubation buffer containing (in mmol/L) 4.8 KCl, 118 NaCl, 1.2 $MgSO_4$, 1.2 KH_2PO_4, 0.7 glutamine, 11 glucose, 5 pyruvate, 25 HEPES, 0.05 bathocuproinedisulfonic acid, 0.001 insulin, 1 $CaCl_2$, a complete mixture of amino acids and vitamins, penicillin–streptomycin, at pH 7.4 with 2% bovine serum albumin (BSA). All studies were carried out at room temperature (RT) within 24 h of isolation.

Prior to study, myocytes were incubated with 5 μmol/L fura2-AM and 0.025% Pluronic F-127 (Molecular Probes, Inc, Eugene, OR, USA) for five minutes and then postincubated without fura2 or Pluronic for at least one hour at RT to allow hydrolysis of the ester. Fura2-loaded cells were then allowed to adhere to a laminin-coated microscope glass coverslip (Upstate Biotechnology, Lake Placid, NY, USA) in a flow-through chamber. After adhering, myocytes were superfused at a rate of 1.0 mL/min with buffer containing (in mmol/L) 5 $NaHCO_3$, 20 HEPES, 1.0 $CaCl_2$, 128 NaCl, 3.8 KCl, 11 glucose, 5 pyruvate, 1.3 $MgSO_4$ and 1.2 KH_2PO_4, pH 7.4. Cells were field stimulated at a frequency of 0.2 Hz and 20 ms duration (Cresent Electronics, Sandy, UT, USA), and visible motion of a single myocyte was collected through a low light videocamera (Philips CCD video camera, Andover, MA, USA). Motion was analyzed by a video edge detector (Cresent Electronics) with digital conversion of output for quantitative analysis accomplished (Felix software; Photon Technology International, Monmouth Junction, NJ, USA).[25] Signal averaging was

used to obtain the following parameters for individual cardiomyocytes: cell shortening, maximal velocity of shortening ($+dL/dt$) and re-lengthening ($-dL/dt$), time to peak shortening (TPS), and time to 90% re-lengthening (TR90).

Calcium transients were simultaneously measured with the edge motion measurements. Fluorescence measurements at 510 nm with excitation alternating between 340 and 380 nm were obtained (Photon Technology International Fluorescence System Hardware, Photon Technology International), and data were analyzed with Felix software. Intracellular calcium concentrations ($[Ca^{2+}]$) were inferred as the ratio of fluorescence intensity at wavelengths of 340 and 380 nm. The following parameters were obtained for individual cardiomyocytes: baseline $[Ca^{2+}]$, the change in Ca^{2+} response (the change in fura2 fluorescence ratio amplitude or $\Delta[Ca^{2+}]$), time to 90% decay to baseline $[Ca^{2+}]$ (TR_{Ca90}), and maximal velocities to peak $[Ca^{2+}]$ and to return to baseline $[Ca^{2+}]$ ($+dR/dt$ and $-dR/dt$, respectively, where R is the ratio of the fluorescence intensity at wavelengths of 340 and 380).

Fura2-loaded myocytes were diluted to 50,000–100,000 cells/mL. To assess the interaction of IL-1β and leptin, myocytes were analyzed for electrically stimulated contractility and Ca^{2+} transients following 30-min exposure to rat IL-1β (10 ng/mL; R&D Systems, Minneapolis, MN, USA) with or without a 60-min pretreatment with rat leptin (25 ng/mL; R&D Systems). The effects of leptin alone were studied following a 60-min pretreatment with 25 ng/mL rat leptin. Data were collected from multiple cells during the 30 min following these treatment periods. To assess the role of NO, aliquots of myocytes were incubated with the nitric oxide synthase (NOS) inhibitor, N^{ω}-nitro-l-arginine (50 μmol/L LNA), for 30 min prior to treatment with either leptin or IL-1β.

NOS gene expression and Western protein analysis

To determine whether there were differences in expression of NOS isoforms associated with heterozygosity for the *fa*^{cp} gene, mRNA and protein expressions were examined in the left ventricle from additional LN and HET rats (n = 3–5/genotype). Messenger RNA for NOS isoforms was quantified in the left ventricle from additional LN and HET using realtime polymerase chain reaction as previously described.[26] Primers were as follows: nNOS Nos1 (GenBank no. NM052799) were 5′ to 3′ TTTGCATGGGCTCGATCAT and 3′ to 5′ TGTGCGGACATCTTCTGGC; iNOS Nos2 (NM012611) were 5′ to 3′ AGCGGCTCCATGACTCTCA and 3′ to 5′ TGCACCCAAACACCAAGGT; eNOS Nos3 (XM216065) were 5′ to 3′ TCTCTGCCTCACTCATGGGC and 3′ to 5′ AGAGCCATACAGGATAGTCGCC. For each rat, a threshold cycle for each gene was determined (Ct). Then, a ΔCt was calculated by subtracting the Ct for 18s from Ct of each other gene. The average ΔCt for each gene was calculated for the LN group and the difference between the gene expression in LN and HET was calculated and expressed as the $\Delta\Delta$Ct. The actual fold change in expression between genotypes was calculated as $2^{|\Delta\Delta Ct|}$.

Protein expression of NOS was done by Western analysis as previously described.[26] Following incubation with primary antibody (eNOS 1:1000, iNOS 1:10000, nNOS 1:500; BD Biosciences, San Jose, CA, USA), membranes were incubated with secondary antibody (goat anti-rabbit IgG 1:2000; Upstate Cell Signaling Solutions, Charlottesville, VA, USA) in blocking buffer. Membranes were then developed using Lumiglo Chemiluminescent Substrate (Upstate Cell Signaling Solutions) and Hyperfilm ECL (GE Healthcare, Piscataway, NJ, USA). Bands corresponding to 140 kDa for eNOS and 155 kDa for nNOS were subjected to densitometry. A positive control sample was loaded onto each gel and expression was normalized for all samples on different blots by comparison to the positive control. The average of densitometric readings of the LN was arbitrarily set at 1.0, and HET were calculated and statistically analyzed compared with this baseline group. Uniformity of protein loading was confirmed by Ponceau S staining.

STAT and SOCS-3 signaling

For studies of STAT and SOCS signaling in response to IL-1β and leptin, isolated heart preparations were studied using additional LN and HET SHHF. Rats were injected intraperitoneally with 65 mg pentobarbital. An abdominal incision was made and heparin (0.7 mg) was injected into the inferior vena cava. The thorax was opened and hearts were rapidly excised and placed in ice-cold modified Krebs–Henseleit buffer containing (in mmol/L) 118 NaCl, 4.8 KC1, 1.2 $MgSO_4$, 1.2 KH_2PO_4, 25 $NaHCO_3$, 1.0 $CaC1_2$, 0.68 glutamine, 11 glucose, 5 sodium pyruvate, 0.001 insulin, 0.5 bathocuproinedisulfonic acid, an amino acid and vitamin supplement (50× MEM and BME, 10 mL/L each; Gibco Invitrogen, Carlsbad, CA, USA) and penicillin–streptomycin. Hearts were cannulated via the aorta and perfused retrograde in a non-recirculating Langendorff mode at 10 mL/min with Krebs–Henseleit buffer saturated with 95% O_2/5% CO_2 to achieve a pH of 7.4 at 37°C. After a 30-min equilibration period, hearts were perfused for 15 min of buffer followed by 15 min with IL-1β (10 ng/mL), leptin (150 ng/mL) for 30 min, or leptin for 30 min with addition of IL-1β during the last 15 min. Hearts were rapidly frozen in liquid nitrogen and stored at −80°C.

Frozen samples of left ventricle were ground and solubilized in 1× Cell Lysis buffer (Cell Signaling Technology, Danvers, MA, USA) containing 20 mmol/L Tris, 150 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L EGTA, 1% Triton X-100, 2.5 nmol/L Na pyrophosphate, 1 nmol/L β-glycerolphosphate, 1 mmol/L Na_3VO_4, 1 μg/mL leupeptin and 1 mmol/L phenylmethylsulfonyl fluoride, pH 7.4. Protein concentration was assayed using the Micro BCA Protein Assay (Thermo Scientific, Waltham, MA, USA). Total and phosphorylated STAT3 and STAT 5 were determined using commercially available kits according to the manufacturer's instructions (PhosphoPlus® Stat3 [Tyr705] Antibody Kit #9130 and PhosphoPlus® Stat5 [Tyr694] Antibody Kit #9350, Cell Signaling Technology).

For SOCS-3, 75 μg protein was loaded on a 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) separating gel with a 4% stacking gel.

Electrophoresis was run in a buffer containing 25 mmol/L Tris, 192 mmol/L glycine, and 0.1% SDS, pH 8.3 at 150 V for 70 min (Bio-Rad Criterion Cell, Hercules, CA, USA), using HeLa cells + interferon-gamma cell lysate as a positive control (SC2222; Santa Cruz Biotechnology, Santa Cruz, CA, USA). Proteins were then transferred on to a nitrocellulose membrane (Sigma, St Louis, MO, USA) for 45 min at 15 V using transfer buffer (TBS) containing 25 mmol/L Tris base, 192 mmol/L glycine and 20% methanol, pH 8.3 (Bio-Rad). After washing for five minutes in TBS, the blots were incubated for one hour at RT in 25 mL blocking buffer containing 5% non-fat dry milk in TBS with 0.1% Tween-20 (TBS-Tween). After three washes, blots were incubated with primary antibody (1:500, SOCS3 #SC9023; Santa Cruz Biotechology Inc) in TBS-Tween with 5% BSA overnight at 4°C. Blots were subsequently washed three times in TBS-Tween at RT and incubated with secondary antibody (1:2000, Goat anti Rabbit IgG-HRP #SC2030; Santa Cruz Biotechnology) in blocking buffer for one hour. Blots were developed using Amersham ECL Plus Western Blotting Detection System (GE Healthcare), and bands quantified using a Typhoon 9410 Variable Mode Imager (Amersham Biosciences, Piscataway, NJ, USA).

Statistics

Data were analyzed using GraphPad Prism 5 (GraphPad Software, Inc, La Jolla, CA, USA) and given as mean ± SEM. Characteristics of the genotypes were compared using Student's *t*-tests or analysis of variance (ANOVA). The Kaplan–Meier survival test was used to compare age of death from congestive heart failure of LN and HET. Development of end-stage heart failure was equated with survival. The Wilcoxan Mann–Whitney non-parametric test was used to analyze plasma leptin concentrations. Effects of genotype and cytokine treatment on myocyte contractility, myocyte calcium transients, and STAT and SOCS Westerns were compared using two-way ANOVA.

Results

Characteristics of HET and LN male SHHF

HET develop end-stage congestive heart failure significantly earlier than LN littermates (median survival of 18 and 22 months, respectively; Figure 1). At five months of age, hypertension is comparable in LN and HET (Table 1). Fasting serum leptin concentrations are significantly increased in the absence of weight gain in HET compared with LN SHHF. HET exhibit metabolic characteristics associated with leptin resistance, including insulin resistance and glucose intolerance compared with age-matched LN SHHF (Figure 2).

Inotropic effects of IL-1β and leptin in isolated cardiac myocytes

Resting cardiomyocyte cell length was similar between LN (104.4 ± 2.8 μm) and HET (109.7 ± 2.8 μm) as was baseline cell contractility and calcium transient parameters (Figures 3 and 4). Incubation of myocytes with leptin produced a negative inotropic effect that was similar in magnitude between LN and HET (Figure 3), indicating that HET myocytes remain sensitive to the cardiosuppressive effect of leptin. Incubation with leptin resulted in significant negative lusitropic effects in the HET myocytes characterized by decreases in the velocities of shortening and re-lengthening in myocytes from HET compared with control. Myocytes from LN did not show significant changes in $\pm dL/dt$ in response to leptin. Incubation of myocytes with LNA did not affect baseline contractility (Figure 5). The suppression of cell shortening by leptin (as demonstrated in Figure 3) was inhibited by incubation with LNA in both LN and HET, suggesting that the negative inotropic effects were mediated by generation of NO (Figure 5).

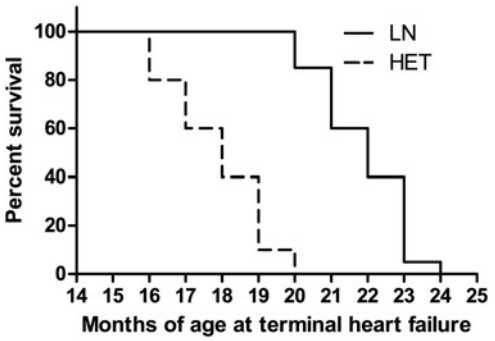

Figure 1 Kaplan–Meier survival curves for LN and HET rats ($n = 20$/genotype). Spontaneous development of end-stage heart failure was equated with mortality. $P < 0.05$; survival was significantly decreased by four months in HET compared with LN male SHHF. LN, lean wild-type SHHF; HET, heterozygous SHHF

Incubation with IL-1β for 30 min resulted in marked suppression of cell shortening as well as decreased maximal velocities of shortening and re-lengthening ($\pm dL/dt$) in both LN and HET (Figure 3). In LN, IL-1β significantly slowed $-dR/dt$ and prolonged both TR90 and TR_{Ca90}, suggesting a sluggish re-uptake of calcium in LN myocytes in response to IL-1β. These effects of IL-1β were less pronounced in HET. As with leptin, incubation with LNA abolished cardiosuppression in LN and HET, suggesting that the effects of IL-1β were mediated by production of NO (Figure 5).

When myocytes were preincubated with leptin, the marked cardiosuppressive effects of IL-1β were not observed (Figures 3 and 4). In LN, effects on contractility and Ca transients were similar between myocytes treated with leptin alone or leptin followed by IL-1β. In contrast, incubating HET myocytes with IL-1β following treatment with leptin not only failed to produce further

Table 1 Characteristics of five-month-old LN and HET SHHF rats

Parameter	LN (n = 12–14)	HET (n = 14–20)
Body weight (g)	387.0 ± 3.8	372.8 ± 3.9*
Systolic blood pressure (mmHg)	171 ± 7	174 ± 5
Fasting serum leptin (ng/mL)	1.4 (0.6–2.2)	2.0* (1.0–5.2)

LN, lean wild-type SHHF; HET, heterozygous SHHF
Values are mean ± SEM except for leptin which is median and (range)
*$P < 0.05$ between LN and HET

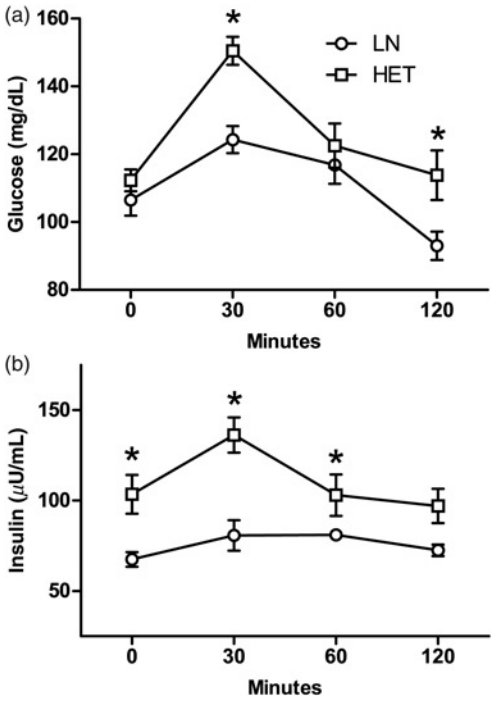

Figure 2 Plasma glucose (a) and insulin (b) responses following administration of an oral glucose load of 0.1 mL of 75% glucose solution/100 g body weight in five-month-old LN and HET SHHF rats ($n = 4$/genotype). $^{*}P < 0.05$ between genotypes at indicated time point. LN, lean wild-type SHHF; HET, heterozygous SHHF

cardiosuppression but abrogated the negative inotropic effects of either leptin or IL-1β. Cell shortening was normalized and was significantly greater in HET compared with LN. This was associated with an increase in $\Delta[Ca^{2+}]$ and a more rapid calcium uptake ($-dR/dt$), compared with control HET cells and compared with LN cells treated with leptin + IL-1β.

NOS gene and protein expression

Baseline expression of eNOS protein was significantly increased in HET compared with LN while expression of eNOS mRNA was not significant ($P = 0.0571$) (Figure 6). While nNOS gene expression was increased in HET compared with LN, this was not accompanied by a significant increase in protein expression. iNOS mRNA expression was not significantly different between LN and HET, and protein was not detectable on Western analysis.

STAT and SOC-3 signaling

Baseline levels of total STAT3, pSTAT3 and SOCS-3 were similar between LN and HET, while baseline levels of total STAT5 and pSTAT5 were 2.9- and 2-fold greater, respectively, in HET compared with LN (data not shown). Perfusion of LN hearts with leptin resulted in a 2.7-fold increase in pSTAT5 and mild but non-significant increases in pSTAT3 (Figure 7). In contrast, perfusion of HET hearts with leptin resulted in a significant decrease in pSTAT3 and a failure to induce additional phosphoryation of STAT5. SOCS-3 showed slight induction in response to leptin in both LN and HET.

LN showed no significant change in pSTAT3 or pSTAT5 and a 35% increase in SOCS-3 in response to treatment with IL-1β. In contrast, IL-1β resulted in a 48% increase in pSTAT3 and a nearly three-fold induction of SOCS-3 in HET. Perfusion of HET hearts with IL-1β resulted in a significant decrease in pSTAT5.

When LN hearts were perfused with leptin followed by IL-1β, there was no increase in pSTAT5. In HET, pretreatment with leptin prevented the IL-1β-induced increase in pSTAT3 and SOCS-3 while phosphorylation of STAT5 was decreased similar to IL-1β alone.

Discussion

Heterozygosity for a defect in the leptin receptor significantly affects the development of congestive heart failure in hypertensive, heart failure-prone SHHF rats. Even though the increases in fasting leptin are mild, this and previous studies have shown that HET die of heart failure significantly earlier than wild-type LN SHHF.[20] Similarly, chronic hyperleptinemia has been associated with the development of cardiovascular disease in humans.[1,4] However, mechanisms underlying the action of leptin on cardiac function and the sometimes paradoxical observations of the effects of leptin on the cardiovascular system are poorly understood. These conflicting observations may arise, in part, from the fact that leptin does not operate in isolation in the whole animal, and outcome likely depends on the simultaneous interactions of leptin with other cytokines within cardiac tissue.

Previous studies have shown that leptin, when applied to isolated adult rat cardiac myocytes, is cardiosuppressive.[2,3] In this study, myocytes from HET and LN exhibited a similar negative inotropic response to leptin. Thus, cardiomyocytes from HET remain responsive to inotropic effects of leptin despite evidence of leptin resistance including hyperleptinemia, insulin resistance and glucose intolerance. Conditions associated with naturally developing hyperleptinemia such as aging and weight gain are often associated with impaired leptin signaling that is selective to pathways controlling energy metabolism.[4] This results in attenuated thermogenic and satiety responses, favoring development of metabolic abnormalities. However, other leptin-induced responses such as the ability to stimulate the sympathetic nervous system[27–29] and up-regulate endothelin[26] remain intact. Ongoing responsiveness of cardiomyocytes to the inotropic effects of leptin in the face of hyperleptinemia may contribute to the development of cardiac dysfunction in hyperleptinemic states.

Low-grade inflammation often accompanies hyperleptinemic states,[4] resulting in increased production of proinflammatory cytokines, many of which can adversely affect cardiac function.[30] One such proinflammatory cytokine is IL-1β, a potent negative inotrope that has been linked to cardiac functional impairment and development of congestive heart failure.[6,7,30] Several studies have suggested that leptin and circulating leptin concentrations may modulate

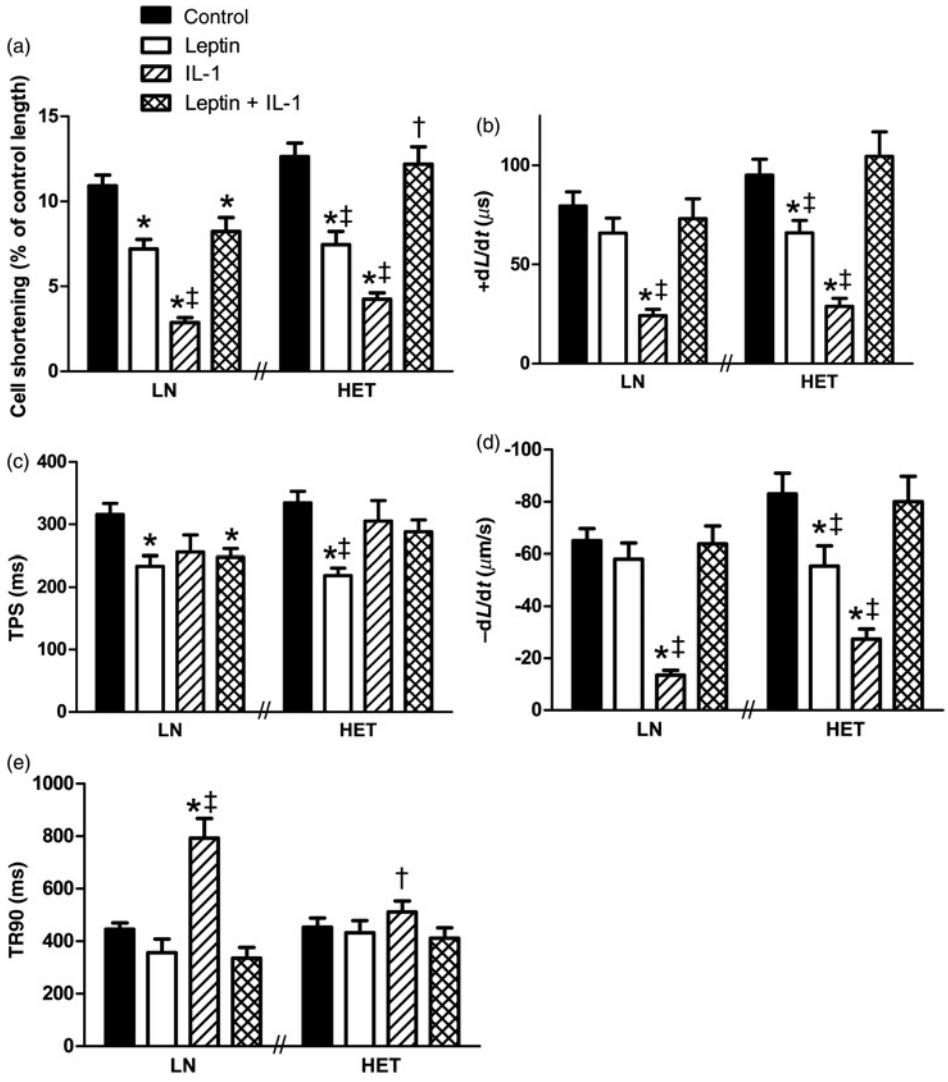

Figure 3 Contractility of isolated cardiac myocytes from LN and HET SHHF rats that were treated with leptin (25 ng/mL), interleukin (IL)-1β (10 ng/mL) or leptin followed by IL-1β. Contractility was measured by video edge detection and the effects of these cytokines on (a) cell shortening, (b) maximal velocity of shortening ($+dL/dt$), (c) time to peak shortening (TPS), (d) maximal velocity of re-lengthening ($-dL/dt$) and (e) time to 90% re-lengthening (TR90) were determined. $^{*}P < 0.05$ significantly different from control within a genotype, $^{\ddagger}P < 0.05$ IL-1β or leptin treatment significantly different from Leptin + IL-1β within a genotype. $^{\dagger}P < 0.05$ HET significantly different from LN for a given cytokine treatment. Bars represent mean $\pm$ SEM for 12–32 cells/group. LN, lean wild-type SHHF; HET, heterozygous SHHF

the cardiovascular effects of IL-1β. *Ob/ob* mice that do not produce leptin[31] or mice with low serum leptin due to starvation[32] are more sensitive to the lethal cardiovascular effects of endotoxin which are mediated, in part, by IL-1β. Mortality was decreased by treatment with leptin. In isolated adult cardiac myocytes from Sprague–Dawley rats, pretreatment with leptin prevented the acute negative inotropic effects of IL-1β.[2] In those models, circulating leptin levels were either low (*ob/ob* mice or fasting mice) or normal (Sprague-Dawley rats). Whether leptin modulates the cardiosuppressive effects of IL-1β in hyperleptinemic states has not been previously explored. LN SHHF rats are normoleptinemic, with circulating leptin concentrations similar to that reported for Sprague–Dawley rats,[2] while HET have mild but chronically elevated fasted serum leptin. IL-1β causes rapid, profound suppression of contractility in isolated myocytes from both HET and LN rats. A sluggish re-uptake of calcium by LN myocytes in response to IL-1β was similar to previous reports.[2,9] The effects of IL-1β on calcium transients were less pronounced in HET, suggesting possible altered responses of HET cardiomyocytes to IL-1β.

That sustained hyperleptinemia influences acute cardiomyocyte responsiveness to IL-1β is supported by the differences in contractility observed when myocytes from HET and LN were pretreated with leptin prior to addition of IL-1β. IL-1β in the presence of leptin no longer produced marked suppression of cardiomyocyte contractility. In the LN, cell shortening was suppressed to a level comparable to that observed when myocytes were treated with leptin alone. Additional suppression by IL-1β was blocked, but cell shortening remained depressed when compared with control LN myocytes. In contrast, cell shortening was similar to control when HET myocytes were pretreated with leptin followed by IL-1β. The amplitude of the calcium transient was significantly increased and the

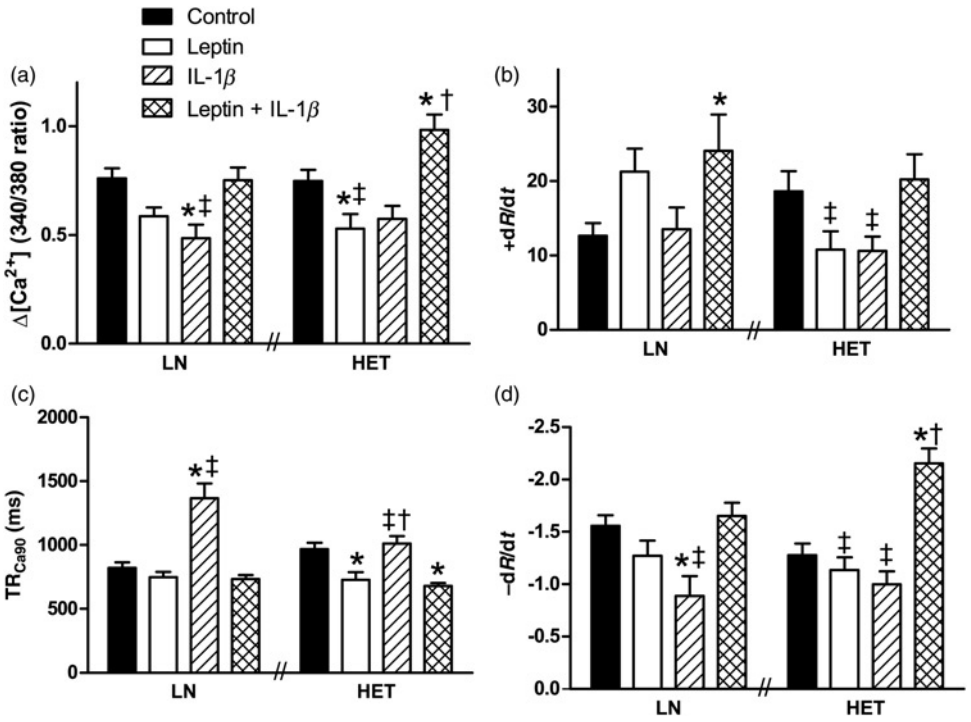

Figure 4 Calcium transients in isolated cardiac myocytes from LN and HET SHHF rats that were treated with leptin (25 ng/mL), IL-1β (10 ng/mL) or leptin followed by IL-1β (Leptin + IL-1β). Calcium transients were measured and the effects of these cytokines on (a) the change in Ca^{2+} response ($\Delta[Ca^{2+}]$), (b) maximal velocity to peak $[Ca^{2+}]$ ($+dR/dt$), (c) time to 90% decay to baseline $[Ca^{2+}]$ (TR_{Ca90}) and (d) the maximal velocity to return to baseline $[Ca^{2+}]$ ($-dR/dt$) were determined. $^{*}P < 0.05$ significantly different from control within a genotype, $^{\ddagger}P < 0.05$ IL-1β or leptin treatment significantly different from Leptin + IL-1β within a genotype. $^{\dagger}P < 0.05$ HET significantly different from LN for a given cytokine treatment. Bars represent mean ± SEM for 12–32 cells/group. LN, lean wild-type SHHF; HET, heterozygous SHHF

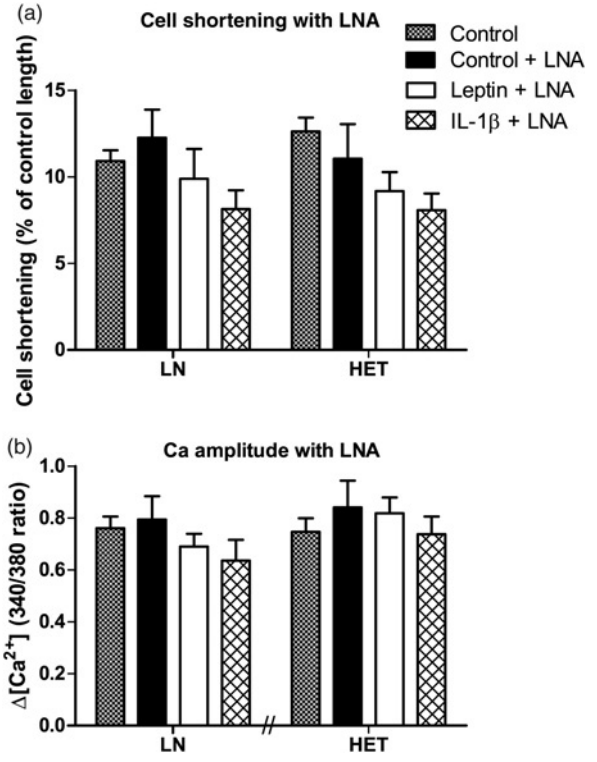

Figure 5 Effect of LNA (N^{ω}-nitro-L-arginine) on (a) cell shortening and (b) the change in Ca^{2+} response ($\Delta[Ca^{2+}]$) in isolated cardiac myocytes from LN and HET SHHF rats. There was no significant difference between treatments or genotypes. Bars represent mean ± SEM for 8–12 cells per group. LN, lean wild-type SHHF; HET, heterozygous SHHF

return to baseline calcium concentrations was more rapid compared with HET control, HET treated with leptin alone or similarly treated LN myocytes. These findings suggest a synergistic effect of leptin and IL-1β that restored contractility in HET myocytes. Such crosstalk between leptin and IL-1β may have important ramifications on cardiac function during inflammatory responses in individuals who are hyperleptinemic.

Several mechanisms are likely to mediate the interaction of leptin and IL-1β. Activation of JAK2/STAT3 with subsequent activation of NOS and generation of NO has been implicated in cytokine-induced cardiosuppression. IL-6, a member of the same gp130 cytokine family as leptin, produces a rapid negative inotropic response in adult rat ventricular myocytes that is mediated by phosphorylation of STAT3 and production of NO.[33] IL-6-associated decreases in contractility were prevented by blockade of STAT3 phosphorylation.[33] Increased activation of STAT3 and production of NO also is observed in donor human hearts with severe ventricular dysfunction.[34] Generation of NO has been demonstrated to mediate cardiosuppression by both leptin and IL-1β,[3,10] and suppression of cell shortening by either leptin or IL-1β in the current study was prevented by treatment with LNA, a NOS inhibitor. Suppression of pSTAT3 could contribute to the restoration of contractility observed when HET myocytes are treated with leptin and IL-1β together.

A potential pathway whereby crosstalk may occur between leptin and IL-1β is through JAK2/STAT3-mediated generation of SOCS-3. Both leptin and

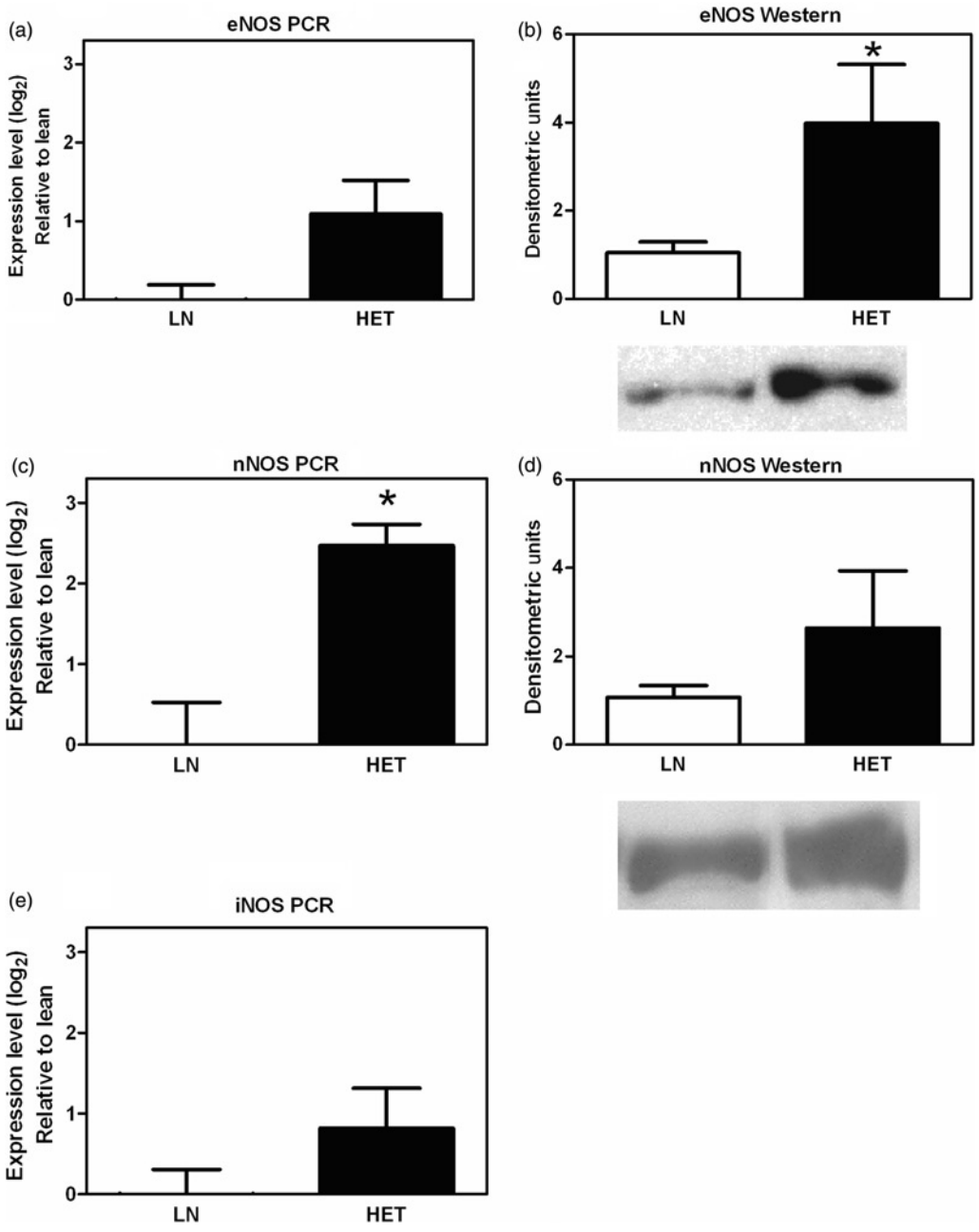

Figure 6 Expression of mRNA and protein for the nitric oxide synthase (NOS) isoforms in left ventricles from five-month-old LN and HET SHHF rats. $^{*}P < 0.05$, HET significantly different from LN. Bars represent mean $\pm$ SEM for 3–5 rats per group. Representative Western blots are shown under their respective groups. LN, lean wild-type SHHF; HET, heterozygous SHHF; PCR, polymerase chain reaction

IL-1β can increase SOCS-3 expression through JAK2/STAT3 signaling.[12,13,35,36] SOCS-3 binds to the SH-2 domain of the leptin receptor and directly to JAK2 to inhibit signaling.[36] In hyperleptinemic states, increased SOCS-3 expression occurs in tissues such as the brain and has been implicated in the development of central leptin resistance through negative feedback on the leptin receptor to down-regulate signaling.[36] The generation of SOCS-3 may have effects beyond self-regulation of leptin signaling. SOCS-3 has been shown to modulate IL-1β signaling and has proven effective in abrogating the effects of IL-1β in non-cardiac tissues such as the pancreas.[14–16] This negative feedback pathway may provide a mechanism whereby signaling by IL-1β in the heart is modified by leptin and where differences between LN and HET may develop as a result of sustained hyperleptinemia in the HET.

In the LN, leptin induced pSTAT3 and SOCS-3 in the heart, similar to previous reports.[37–39] Surprisingly, pSTAT3 consistently decreased in response to leptin in the HET. The cause for this paradoxical decline in pSTAT3 in response to leptin is uncertain. There was no difference in baseline SOCS-3 between LN and HET. The mild increase in SOCS-3 in HET following leptin treatment potentially could contribute to feedback suppression of JAK2/STAT3

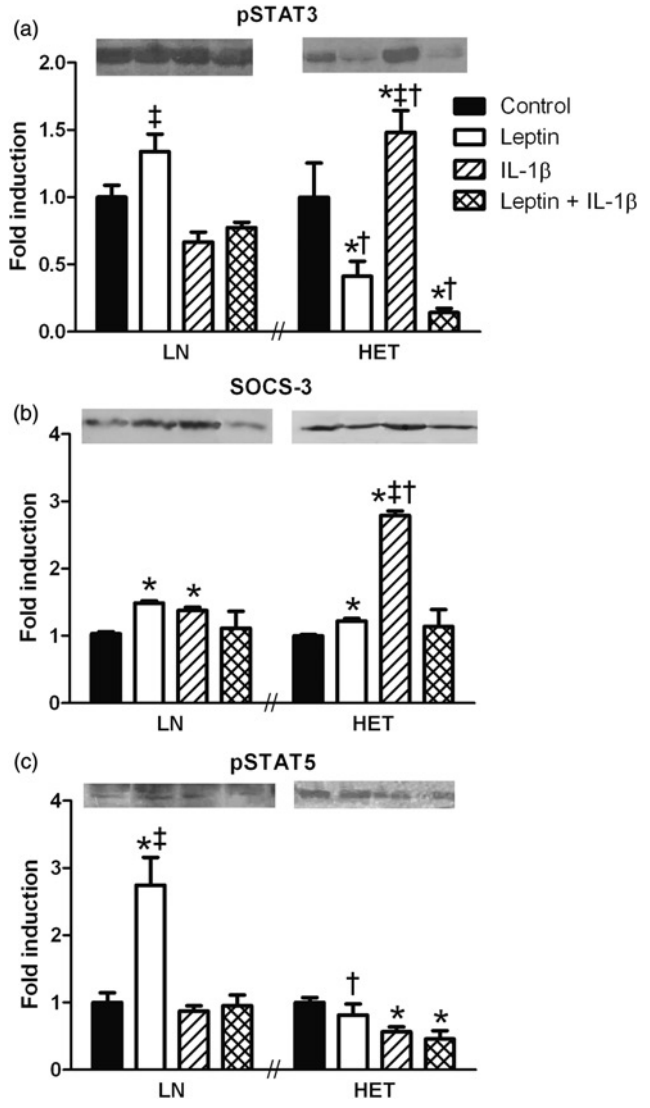

Figure 7 Induction of (a) phosphorylated STAT3 (pSTAT3), (b) expression of SOCS-3 protein and (c) phosphorylated STAT5 (pSTAT5) in response to leptin, interleukin (IL)-1β or leptin followed by IL-1β (Leptin + IL-1β) in the left ventricle of isolated perfused hearts from five-month-old LN and HET SHHF rats. $^{*}P < 0.05$ significantly different from control within a genotype. $^{\ddagger}P < 0.05$ IL-1β or leptin treatment significantly different from Leptin + IL-1β within a genotype. $^{\dagger}P < 0.05$ HET significantly different from LN for a given cytokine treatment. Bars represent mean ± SEM for 4–5 rats/group. Representative Western blots are shown above their respective groups. LN, lean wild-type SHHF; HET, heterozygous SHHF

signaling. The rapidity of the decline in pSTAT3 suggests that activity of phosphatase pathways may be up-regulated in the chronically hyperleptinemic HET. Protein tyrosine phosphatase 1B (PTP1B), which targets JAK2 signaling, is activated in response to leptin signaling[40] and is a potential candidate for mediating the effects on JAK2/STAT3 signaling observed in the HET. PTP1B is increased in relationship to zygosity of the leptin receptor defect gene in d*b*/d*b* mice[41] and in Zucker rats.[42] However, PTP1B targets JAK2 and pSTAT5; therefore effects on STAT3 phosphorylation would be indirect[43] and other phosphatases that directly target pSTAT3 should be further investigated in the heart. Differences between LN and HET in JAK2/STAT3 signaling in response to IL-1β also were apparent. In the HET, IL-1β significantly induced both pSTAT3 and SOCS3, and this response was prevented by pretreatment with leptin.

Leptin induces phosphorylation of STAT5 in the brain, and pSTAT5 appears to play a role in central regulation of energy balance and body weight by leptin;[11,44] however, leptin-induced STAT5 signaling in the heart is largely unexplored. In the LN, leptin induced a 2.7-fold increase in pSTAT5, suggesting that STAT5 signaling may predominate in the heart in response to leptin. This is consistent with the finding that baseline total STAT5 and pSTAT5 were increased in HET. Failure of leptin to further induce pSTAT5 in the HET may be the consequence of continuous exposure to higher concentrations of endogenous leptin, precluding additional phosphorylation in response to leptin receptor binding. Another contributing factor may be increased activity of phosphatases as a result of hyperleptinemia such as PTP1B which specifically dephosphorylates pSTAT5.[43] In the HET, IL-1β with or without pretreatment with leptin consistently decreased pSTAT5 compared with control, while in the LN, leptin-induced phosphorylation of STAT5 was suppressed by IL-1β. This suggests that IL-1β may stimulate dephosphorylation of pSTAT5. NO induces a rapid and reversible cGMP-mediated STAT5 dephosphorylation in bronchial epithelial cells and immune cells.[45–48] A similar mechanism may occur in the heart in response to NO generation by IL-1β and warrants further investigation.

In conclusion, our study shows that cardiac myocytes remain responsive to leptin in the face of chronic hyperleptinemia and selective leptin resistance. Crosstalk between leptin and IL-1β with modulation of JAK/STAT signaling pathways may benefit short-term cardiac function by abrogating acute negative inotropic effects of IL-1β. Interactions between leptin and cardiosuppressive inflammatory cytokines such as IL-1β may help explain the seemingly paradoxic findings that obese patients who are hyperleptinemic have better survival than normoleptinemic or hypoleptinemic individuals following septic shock or myocardial infarction.[1,49] At the same time, long-term consequences of hyperleptinemia and crosstalk with inflammatory cytokines may not be favorable. Low-grade, chronic inflammation with increased production of inflammatory cytokines, including IL-1β, accompanies hyperleptinemic states such as aging and obesity and has been implicated in development of cardiovascular disease associated with these conditions.[50] Both pSTAT3 and pSTAT5 appear to play a role in protective preconditioning in ischemia,[51–53] and activation of STAT3 has been suggested to be cardioprotective against the effects of inflammation.[54] Suppression of pSTAT3 and pSTAT5 signaling observed when IL-1β and leptin interact on a background of hyperleptinemia may result in loss of these cardioprotective effects and ultimately contribute to cardiac functional decline.

Author contributions: All authors participated in the design, interpretation of the studies and analysis of the data and review of the manuscript. MJR, BJH and RAA conducted the experiments. SAM provided critical animals. MJR and BJH wrote the manuscript with assistance from SAM and RAA.

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