Leptin signalling reduces the severity of cardiac dysfunction and remodelling after chronic ischaemic injury

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Received 18 April 2007; revised 17 September 2007; accepted 18 September 2007; online publish-ahead-of-print 20 September 2007

Aims Leptin is elevated under conditions of both obesity and heart failure (HF), and activation of leptin receptor (ObR) signalling is known to increase in vivo cardiac contractility and to have anti-hypertrophic effects on the left ventricle (LV). However, it is unknown whether ObR signalling is altered in cardiomyocytes after myocardial infarction (MI) leading to HF, or if a deficiency in ObR signalling leads to worse HF.

Methods and results In separate experimental protocols, C57BL/6J and leptin-deficient (ob/ob) mice underwent open-chest surgery to induce permanent left coronary artery ligation (CAL) or had a sham operation. Subgroups of ob/ob mice examined were lean (food-restricted), obese (food-ad libitum), and leptin repleted. Four weeks post-surgery, cardiac structure and function was examined by echocardiography, and the activation of cardiac leptin signalling was characterized through quantitative PCR, western blotting, and DNA-binding activities. CAL produced echocardiographic evidence of HF in C57BL/6J mice, elevated circulating leptin, increased cardiomyocyte leptin and ObR expression, and activated myocardial signal transducer and activator of transcription-3 (STAT3). In leptin-deficient ob/ob mice, whether lean or obese, CAL caused increased hypertrophy and dilation, decreased contractility of the LV, and worsened survival relative to wildtype or leptin-repleted mice after CAL. In ob/ob mice, activation of cardiac STAT3 signalling after CAL is enhanced in the presence of leptin and parallels the induction of the STAT3-responsive genes, tissue-inhibitor of metalloproteinase-1 and heat shock protein-70.

Conclusion These data demonstrate that HF increases ObR signalling in cardiomyocytes and that activation of ObR signalling improves functional outcomes in chronic ischaemic injury leading to HF.

1. Introduction

Recent evidence suggests that the predominantly fat-derived, satiety peptide, leptin, can impact on cardiac function.1 The presence of leptin receptor (ObR) in cardiac tissue2 provides a potential signalling pathway through which leptin may modulate cardiac function. Studies in mice demonstrate that the abolition of leptin signalling through leptin-deficiency or ObR-deficiency is associated with age-related ventricular hypertrophy.3,4 This hypertrophy occurs independently of obesity and is abrogated by leptin administration in leptin-deficient mice. In contrast, studies using cultured cardiomyocytes are at variance as to whether leptin induces cellular hypertrophy.5–7 Thus, the modulating effect of leptin in cardiac hypertrophy may differ under in vitro vs. in vivo conditions.

Hypertrophy represents just one of many compensatory and pathological changes that occur in cardiac tissue in response myocardial infarction (MI) and heart failure (HF).8 Based on the above contrast between in vivo and in vitro hypertrophic effects, leptin may potentially improve or worsen cardiac outcomes in HF. However, what few studies are available suggest that leptin may actually play a positive, compensatory role. For example, leptin-resistant db/db mice show greater cardiac hypertrophy, decreased myocardial contractility, and worse survival after ischaemia and reperfusion injury when compared with their wildtype counterparts.9 In vitro studies also show that leptin helps to protect cultured cardiomyocytes...
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from hypoxic insult. Clinically, elevated circulating leptin is found in MI\(^1\) and HF\(^2\) patients, and occurs independent of the presence of obesity. Although the increased circulating leptin characterizing obesity generally results in a state of resistance and down-regulation of ObR signalling in the hypothalamus,\(^3\) it is unknown what impact the hyperleptinemia that occurs after MI and in HF has on cardiac ObR signalling. Moreover, it is unclear whether this association of hyperleptinemia with MI and HF is a non-specific marker of disease, or whether it represents an active compensatory response to myocardial injury.

Elevated leptin levels may potentially play a protective role in HF if ObR signalling is activated in cardiomyocytes. For example, leptin activates signal transducer and activator of transcription-3 (STAT3) through the ObR,\(^4\) and it is known that STAT3 regulates key biochemical elements involved in cardiac remodelling after injury\(^5\) and in HF.\(^6\) Given what is known about ObR signalling through STAT3, it is not unreasonable to postulate that leptin may activate STAT3 and modulate the cardiac specific tissue response to injury after MI. However, the importance of, and mechanisms by which, leptin signalling may influence the response of cardiomyocytes to MI or HF remains incompletely understood.

The purpose of the present study was to test the hypothesis that leptin plays a protective role in MI-induced cardiac dysfunction and HF. Our experimental approach utilized cardiac tissue from infarcted wildtype C57BL/6J mouse hearts to examine post-injury leptin production, ObR expression and activation, and leptin signalling in the myocardium. Further, we determined the physiologic relevance of leptin signalling after chronic myocardial ischaemia by examining post-MI cardiac structure, function, and survival in lean and obese leptin-deficient, as well as leptin-replete, ob/ob mice. Finally, we examined the mRNA expression of candidate genes that are involved in the cardiac specific response to injury after MI and in HF and are potentially regulated in the heart by leptin through STAT3 responsive sites.

2. Methods

2.1 Animal protocols

The investigation conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996) and was approved by the Institutional Care and Use Committee at the University of Pittsburgh. In the first set of experiments, 5- to 6-week-old wildtype male C57BL/6J mice \((n = 18)\) were subjected to baseline echocardiography followed by coronary artery ligation (CAL)/sham surgeries as described previously\(^7\) and detailed in Supplementary material online. In a second set of experiments, 7 groups of 5- to 6-week-old male leptin-deficient ob/ob and wildtype (littermate control) mice were studied after baseline echocardiography. These included three groups of sham animals: (i) lean wildtype food-provided \(ad\) \(libitum\) \((n = 10)\), (ii) obese ob/ob food-provided \(ad\) \(libitum\) \((n = 11)\), and (iii) lean ob/ob food-restricted \((n = 9)\); and four groups of CAL mice: (iv) lean wildtype food-provided \(ad\) \(libitum\) \((n = 16)\), (v) obese ob/ob food-provided \(ad\) \(libitum\) \((n = 18)\), (vi) lean ob/ob food-restricted \((n = 15)\), and (vii) lean ob/ob leptin-treated \((n = 13)\). 0.3 mg/kg/day s.c. by miniosmotic pump starting the day prior to CAL surgery and continuing to 4 weeks, procedural details in Supplementary material online. Repeat echocardiography on surviving animals in both sets of experiments was performed at 2 and 4 weeks post-operatively, followed by body weight measurement, animal sacrifice, and tissue collection.

Left ventricular micro-manometer measurements of maximum developed pressure were performed immediately prior to sacrifice only in the second set of experiments, as detailed previously.\(^8\)

2.2 Echocardiography

Under 2% isoflurane, parasternal short-axis M and B-mode images of the left ventricle (LV) pre- and post-CAL/sham surgeries were obtained using a VisualSonics machine with a 25-MHz linear transducer. Additional details are in Supplementary material online.

2.3 Serum leptin determination

Serum was collected between the hours of 9 - 10 a.m. from wildtype mice at 4 weeks post-CAL/sham surgery and assayed in triplicate by ELISA (Linco, USA). Additional details are in Supplementary material online.

2.4 Quantitative RT–PCR

RNA was isolated from whole tissue homogenates of fat or heart, reverse transcribed, and subjected to real-time RT–PCR using primers and conditions described in Supplementary material online.

2.5 Western blotting

Protein extracts were prepared from whole tissue homogenates of fat or heart, subjected to SDS–PAGE, electro-transferred to PVDF membranes, incubated with primary and secondary antibodies, visualized by enhanced chemiluminescence and X-ray film, and quantified using Image J software (NIH, USA) as detailed in Supplementary material online.

2.6 STAT3 DNA-binding activity

Protein extracts were assayed for STAT3-DNA-binding activity using a STAT3-DNA binding ELISA kit (Active Motif, USA) that is specific for the detection of activated STAT3 according to the manufacturer’s instructions. Specificity of binding was confirmed using competitor and mutant oligonucleotides provided by the manufacturer.

2.7 Histology/immunofluorescence

Mouse cardiac tissues were harvested and fixed in paraformaldehyde/sucrose, as previously described.\(^9\) After 6 \(\mu\m\) cryo-tome sectioning in the short axis at the level of the mid-LV, slides were stained with hematoxalin and eosin (H&E), or used in immunofluorescence for leptin, ObR, or sialic acid and N-acetylglucosaminyl residue (wheat germ agglutinin) staining. Additional details are in Supplementary material online.

2.8 Determination of infarct area

H&E stained sections of the LV at 4 weeks post-CAL surgery were examined under low power objective for quantification of the area of infarction. This method was validated by direct comparison with Evan’s Blue/triphenyltetrazolium-chloride (TTC) staining in 7 CAL mice. Additionally, echocardiography was used to determine infarct size at 2 and 4 weeks post-CAL and validated against H&E stained images at 4 weeks. Additional details are in Supplementary material online.

2.9 Determination of cardiomyocyte area and width

Wheat germ agglutinin stained sections of the LV at 4 weeks post-sham/CAL surgery were examined under high power (20 \(\times\) ) objective and digitized as described in Supplementary material online. Images were analysed using Image-J software, with > 40 measurements taken per animal in short axis for determination of myocyte area and > 50 measurements taken in long axis per...
animal for determination of myocyte width. Calibration of the software was to a known measured distance at equal objective power in microns.

2.10 Statistics

Mean values for all measured variables are reported as means ± SEM. Differences between means were determined by Student’s independent two-tailed t-test using an established P-value of <0.05 for data in the first set of experiments where the number of comparisons was limited to two means. One-way analysis of variance (ANOVA) was used to determine statistical differences between means for all measured variables in the second set of experiments using wildtype and ob/ob mice. Post hoc comparisons between means were accomplished using the Bonferroni simultaneous tests with an established P-value of <0.05. For validation of methods used to determine infarct size, linear regression was performed comparing H&E to Evan’s Blue/TTC staining, and H&E to echocardiography. Additional details are in Supplementary material online.

3. Results

3.1 Effect of coronary artery ligation on cardiac function and leptin signalling in wildtype C57BL/6J mice

At 4 weeks after CAL/sham surgery in wildtype male C57BL/6J mice, there was no significant difference in body weight or heart rate during echocardiography between groups (Table 1). However, the mean heart weight increased by 46%, the mean wet lung weight increased by 25%, the mean wet/dry lung weight ratio increased by 15%, the mean percentage fractional shortening (%FS) was reduced by 51%, the end diastolic dimension (EDD) increased by 50%, the end systolic dimension increased by 120%, and the average diastolic wall thickness increased by 18% in CAL mice relative to sham mice, demonstrating physiologic and echocardiographic findings consistent with the development of HF in our model of chronic ischaemic injury (Table 1).

At 4 weeks, CAL produced a mean 60% increase in circulating levels of leptin (1.5 ± 0.1 vs. 2.4 ± 0.2 ng/mL; P = 0.01, unpaired t-test) and increased the mRNA expression of leptin in both whole heart and adipose tissue relative to sham mice (Figure 1A). In contrast, mRNA expression and protein levels of the long-form ObR were increased in cardiac but decreased in adipose tissue in response to CAL (Figure 1A–D). Immunofluorescence suggested that the increase in leptin and ObR protein in CAL mice was in the cardiomyocyte, with the receptor predominantly localized to the cell membrane (Figure 2A–B). Relative to sham mice, CAL mice also exhibited elevated cardiac levels of tyrosine-985-phosphorylated (Y985p) ObR and tyrosine-705-phosphorylated (Y705p) STAT3, with a 29% increase in the ratio of Y705p to total STAT3 (Figure 1B and D).

3.2 Effect of leptin deficiency and repletion on survival in mice subjected to coronary artery ligation

In this set of experiments in which animals underwent either sham or CAL surgery, food-restricted and leptin-replete ob/ob mice were weight-matched to lean littermate control wildtype mice, and survival in all three sham groups was 100% at 30 days (Figure 3). In lean wildtype mice, survival
3.3 Effect of leptin deficiency and repletion on cardiac structure and function in mice subjected to coronary artery ligation

In those animals that survived for 4 weeks after CAL, leptin deficiency, either in the presence or absence of obesity, worsened cardiac function, whereas repleting leptin improved cardiac function. At both 2 and 4 weeks post-sham surgeries, echocardiography on leptin-deficient ob/ob mice demonstrated that the %FS (~50%), EDD (~3 mm), and average wall thickness (~1.0 mm) were comparable to that seen in the wildtype sham group (Tables 2 and 3; representative echocardiographic images in Supplementary material online). At 2 weeks post-CAL, all wildtype and ob/ob mice (whether lean, obese, or repleted with leptin) demonstrated comparable reductions in %FS (~50% reduction), similar infarct areas (~6.1 mm²), and near equal increases in EDD (~50% increase), and average wall thickness (~20% increase), relative to wildtype sham mice (Table 2). These changes after CAL remained relatively stable at 4 weeks in the wildtype CAL group, as the mean %FS was ~24%, the mean EDD was ~4.4 mm, the mean infarct area was ~6.2 mm², and the average wall thickness was ~1.20 mm (Table 3). However, in leptin-deficient ob/ob mice, whether lean (food-restricted) or obese (food-ad libitum), CAL at 4 weeks caused additional decrements in contractility (FS = 15–17%), increased LV dilation (EDD of ~5.4 mm), and increased hypertrophy (average wall thickness of ~1.26 mm) relative to wildtype CAL mice at 2 or 4 weeks, all in the presence of stable infarct area (Table 3). This contrasts with the %FS, EDD, and average wall thickness in the ob/ob leptin-replete group, all of which remained at levels comparable to lean wildtype mice at 2 and 4 weeks post-CAL (Tables 2 and 3). Similar to the changes seen in the echocardiographic assessment of contractility, the dp/dt max after CAL in both lean and obese ob/ob mice was reduced relative to either lean wildtype mice or lean ob/ob mice with leptin replacement (Table 3).

3.4 Effect of leptin deficiency and repletion on histologic determination of cardiac hypertrophy and infarct area in mice subjected to coronary artery ligation

Compared with sham mice, average myocyte width (Figure 4A) and cross-sectional area (Figure 4B) were significantly increased in non-infarcted myocardium from wildtype animals subjected to CAL, consistent with the development of hypertrophy. Additional increases in myocyte width and cross-sectional area were seen in leptin-deficient mice compared with wildtype mice subjected to CAL, independent of body weight (Figure 4). In contrast, CAL in ob/ob mice that were repleted with leptin resulted in myocyte width and cross-sectional areas comparable to wildtype mice subjected to CAL (Figure 4). The mean histologic infarct area (representative images in Supplementary material online) was comparable in all CAL groups at 4 weeks post-surgery (wildtype 6.17 ± 0.10 mm²; ob/ob ad libitum 6.19 ± 0.32 mm²; ob/ob food-restricted 6.22 ± 0.18 mm²; ob/ob leptin replete 6.25 ± 0.24 mm²) and demonstrated a strong correlation with both the 4-week post-CAL echocardiographic (r = 0.95, P < 0.001) and Evan’s Blue/TTC staining (r = 0.98, P < 0.001) determined area when linear...
regression analysis was applied (regression curves and representative images in Supplementary material online).

3.5 Effect of coronary artery ligation on cardiac STAT3 signal transduction in leptin-deficient and leptin-replete mice

At 4 weeks post-CAL, hearts from both wildtype and leptin-repleted ob/ob mice demonstrated increases in total and Y705p-STAT3, with a 30% increase in the ratio of Y705p-STAT3/total STAT3, relative to leptin-deficient ob/ob mice (food-restricted and food-ad libitum) post-CAL (Figure 5A and B). This observed increase in the ratio of Y705p-STAT3/total STAT3 in the hearts of wildtype and leptin-repleted ob/ob mice at 4 weeks post-CAL was accompanied by ~2-fold increase in the DNA-binding activity of STAT3, relative to leptin-deficient ob/ob mice (food-restricted and food-ad libitum) post-CAL (Figure 5C). Consistent with this finding, the cardiac mRNA levels of the STAT3 responsive genes, heat shock protein-70 (hsp70),18 and tissue inhibitor of matrix metalloproteinase-1 (TIMP1)19 were significantly increased after CAL in wildtype and ob/ob mice repleted with leptin, relative to leptin-deficient ob/ob mice (food-restricted and food-ad libitum) post-CAL (Figure 5D and E). This is in contrast to the cardiac mRNA levels of other genes induced in HF, which are not known to be STAT3 responsive, including TIMP2 (Figure 5F), as well as hsp27, TNFα, and IL-1β (data not shown), all of which failed to show any significant differences between the four CAL groups.

4. Discussion

Our results demonstrate that leptin has protective and beneficial effects in the setting of myocardial injury leading to cardiac dysfunction and eventual HF. Studies to date have reported an association between hyperleptinemia and HF12,20 but have not demonstrated a role for leptin in the progression or amelioration of the disease. Here, we show
up-regulated expression and activation of leptin and its receptor in mice subjected to chronic ischaemic cardiac injury resulting from permanent ligation of the left anterior descending coronary artery. We demonstrate the functional significance of leptin signalling in HF by showing increased hypertrophy and dilation, decreased contractility of the LV, and worse survival in leptin-deficient ob/ob mice after experimentally induced MI. These worse functional and survival outcomes in leptin-deficient mice subjected to CAL occurred independent of changes in body mass and were associated with a decrease in STAT3 activation and reduced expression of STAT3-responsive genes in the heart. Furthermore, repleting leptin in the ob/ob mouse subjected to CAL increased cardiac STAT3 activation, and restored the wildtype HF and survival phenotypes. These data demonstrate a direct cause and effect relationship between the anti-obesity cytokine leptin and improved cardiac outcomes and survival in HF.

A major finding of the present study was that experimentally induced HF in mice results in an up-regulation of leptin and ObR expression in cardiac tissue. Specifically, our immunofluorescence data show that cardiac expression of leptin and ObR is largely confined to the cardiomyocyte (Figure 2A and B), suggesting that changes in mRNA and protein levels as determined using whole heart homogenates (Figure 1A-D) predominantly reflect cardiomyocyte rather than non-cardiomyocyte production. Although the production of leptin outside of adipose tissue is not a novel finding, the increased production of leptin by the failing cardiomyocyte has not previously been reported and suggests that leptin may act in an autocrine or paracrine fashion in response to cardiac injury. Despite this increased production of leptin by failing cardiomyocytes, adipose tissue likely remains the major contributor to the overall elevation in circulating leptin levels in HF, as the relative level of leptin transcripts in fat is much higher than in cardiac tissue.
from the same CAL animal (data not shown). Nevertheless, the importance of elevated circulating leptin levels and increased production of leptin in the failing heart is dependent on its ability to activate leptin signalling in the myocardium.

HF increased cardiomyocyte ObR expression (Figure 1), despite the presence of elevated circulating leptin that may be expected to down-regulate ObR levels. We found that adipose ObR mRNA and protein expression was markedly down-regulated in CAL mice relative to sham mice (Figure 1). Under the classical condition of hyperleptinemia due to obesity, ObR expression in fat or brain has been reported as either down-regulated\(^{21}\) or unchanged,\(^{22}\) relative to normal weight controls. Down-regulation of receptor expression in the face of hyperleptinemia has been shown to limit leptin signalling, at least in the brain,\(^{13}\) and may contribute to the development of leptin resistance. In contrast, our observation of increased ObR expression in cardiac, but not adipose, tissue in the presence of elevated circulating leptin was an unexpected finding and suggests a potential compensatory role for ObR signalling in cardiomyocytes in response to HF. Indeed, we demonstrate an increase in phosphorylation of the ObR and STAT3 in CAL relative to sham mouse hearts, consistent with activation of leptin signalling in failing cardiac tissue. Localization of the long-form ObR to the cardiomyocyte, with qualitatively greater ObR staining demonstrated at the cell membrane in the failing relative to the non-failing heart (Figure 2B), further suggests that the increases observed in long-form ObR transcript and protein (Figure 1) might translate into functionally significant changes in receptor activity. This observation of a large number of leptin receptors on the cell membrane in failing relative to non-failing hearts is especially important to note since only 5–25% of the total cellular ObR pool is located in an active form at the cell surface under physiologic conditions; the remaining percentage being inactive.
in intracellular pools. Thus, experimental MI leading to HF causes increases in leptin and ObR expression in cardiac tissue that activates downstream leptin signalling. Impaired leptin signalling adversely impacted on mortality within the first 10 days post-MI (Figure 3). Moreover, in surviving animals, impaired leptin signalling exacerbated cardiac morbidity between 2 and 4 weeks post-MI (Tables 2 and 3). Specifically, our data demonstrate changes in LV structure and function that are consistent with the development of HF at 2 weeks post-CAL (Table 2). At 4 weeks post-CAL, we observed no additional change in LV structure or function only in wildtype and leptin-repleted ob/ob mice (Table 3). In leptin-deficient ob/ob mice, whether lean or obese, significantly worse echocardiographic evidence of HF was present by 4 weeks post-CAL (Table 3). This stabilization of echocardiographic parameters of HF by 2 weeks post-CAL in wildtype and leptin-replete ob/ob mice, with minimal additional change in LV structure and function at 4 weeks, is consistent with what has been published previously in this model of chronic CAL induced cardiac dysfunction. Our finding of significant additional dilation of the LV, and depression of cardiac contractility at 4 weeks in lean and obese leptin-deficient ob/ob mice, in the absence of an increase in infarct area, suggests that leptin plays a role in mitigating against adverse cardiac remodelling between 2 and 4 weeks post-CAL. These improvements in cardiac morbidity in animals with intact vs. impaired leptin signalling were associated with activation of the STAT3 pathway.

Our observation of cardiac STAT3 induction, phosphorylation, and increased DNA-binding activity in response to HF is consistent with activation of leptin signalling and potentially plays a broader role in the failing heart by regulating the expression of genes that impact HF outcomes and response to chronic ischaemic injury in a cardioprotective manner (Figure 6). Interestingly, both the TIMP1 and hsp70 promoters are reported to have functional STAT3 elements that mediate transcriptional activation. It is known that TIMP1 plays an important role in limiting HF development after MI, since TIMP1-deficient mice develop significantly worse heart function than wildtype mice in response to CAL. Also, it has been shown that hsp70 protects mitochondrial function and is associated with the preservation of ventricular performance after ischaemic injury. Further, it has been suggested that induction of hsp70 serves a protective role in the setting of cardiac injury and HF by facilitating the repair of damaged ion channels, restoring redox balance, inhibiting pro-inflammatory cytokines, and preventing activation of apoptotic pathways. Thus, our observed lack of TIMP1 and hsp70...
induction in leptin-deficient ob/ob mice subjected to CAL may arise from a relative decrease in STAT3 activation. Also, the lack of a leptin-dependent change in the expression of non-STAT3 responsive genes in the myocardium 4 weeks post-CAL, including hsp27, TIMP2, TNFα, and IL-1β (data not shown), suggests that the cardiac induction of TIMP1 and hsp70 mRNA in response to CAL is a leptin-, and most likely STAT3-mediated, effect that may serve to limit adverse cardiac remodelling in HF.

In addition to the activation of leptin signalling after MI, our data at 4 weeks post-CAL show that leptin-deficiency results in a greater amount of cardiac hypertrophy relative to wildtype and leptin-replete ob/ob groups, as determined by echocardiographic and histologic measurements. Our data are also consistent with previous in vivo studies demonstrating increased cardiomyocyte size in leptin-deficient and leptin-resistant animals in response to aging3,4 or cardiac injury.9 The hypertrophic effects associated with leptin-deficiency reported in the aging study occurred independent of the presence of obesity. Comparably, in our study both lean and obese leptin-deficient mice exhibited similar morbidity and mortality, which were significantly worse than either lean wildtype or leptin-replete ob/ob mice after MI (Figure 3 and Table 3). However, we cannot exclude the possibility that the alterations in cellular metabolism that characterize the leptin-deficient state, including changes in energy substrate metabolism, hyperglycemia, and insulin resistance which are present in the ob/ob mouse even before the onset of obesity,31 contributed to the cardiac morbidity and mortality associated with MI.

Obesity is a recognized risk factor for cardiovascular events.32 However, an ‘obesity paradox’ has been described in patients with established HF. Specifically, those HF patients with higher BMI have a lower overall mortality risk relative to those HF patients with normal BMI.33 It is interesting to speculate that an obesity-associated increase in circulating leptin, coupled with increased activation of leptin signalling in cardiac tissue, may provide a mechanism underlying this apparent ‘paradox.’ Further defining the role of leptin signalling in the heart and other organs after MI may provide insight into the co-morbidity that exists between obesity and HF and potentially result in therapeutic interventions for this growing, highly vulnerable, segment of the population.

**Supplementary material**

Supplementary material is available at *Cardiovascular Research* online.
Acknowledgements

We thank David Schwartzman for his comments; Greg Gibson and Ben Burkhead for their technical assistance; and the Center for Biologic Imaging at the University of Pittsburgh.

Funding

National Institutes of Health (1-K08 HL087009-01 to K.R.M); American Heart Association (0675024N to K.R.M., 0535642U to K.R.M.).

Conflict of interest: none declared.

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