Impaired cardiac contractile function in ventricular myocytes from leptin-deficient *ob/ob* obese mice

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Abstract

The level of the obese gene product leptin is often positively correlated with body weight, supporting the notion that hyperleptinemia contributes to obesity-associated cardiac dysfunction. However, a link between leptin levels and cardiac function has not been elucidated. This study was designed to examine the role of leptin deficiency (resulting from a point mutation of the leptin gene) in cardiomyocyte contractile function. Mechanical properties and intracellular Ca^{2+} transients were evaluated in ventricular myocytes from lean control and leptin-deficient *ob/ob* obese mice at 12 weeks of age. Cardiac ultrastructure was evaluated using transmission electron microscopy. *ob/ob* mice were overtly obese, hyperinsulinemic, hypertriglycemic, hypoleptinemic and euglycemic. Ultrastructural examination revealed swelling and disorganization of cristae in mitochondria from *ob/ob* mouse ventricular tissues. Cardiomyocytes from *ob/ob* mice displayed reduced expression of the leptin receptor

Ob-R, larger cross-sectional area, decreased peak shortening and maximal velocity of shortening/relengthening, and prolonged relengthening but not shortening duration compared with lean counterparts. Consistent with mechanical characteristics, myocytes from *ob/ob* mice displayed reduced intracellular Ca^{2+} release upon electrical stimulus associated with a slowed intracellular Ca^{2+} decay rate. Interestingly, the contractile aberrations seen in *ob/ob* myocytes were significantly improved by *in vitro* leptin incubation. Contractile dysfunction was not seen in age- and gender-matched high fat-induced obese mice. These results suggested that leptin deficiency contributes to cardiac contractile dysfunction characterized by both systolic and diastolic dysfunction, impaired intracellular Ca^{2+} hemostasis and ultrastructural derangement in ventricular myocytes.

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Introduction

Obesity is a common multifactorial medical problem involving both genetic and lifestyle factors. It is estimated that ~20% of Americans are considered obese and that this is responsible for about 300 000 deaths in the USA each year. Both clinical and experimental data have demonstrated that uncorrected obesity leads to cardiac hypertrophy and compromised ventricular function such as reduced myocardial contractility and diastolic compliance (Sowers 1998, Ren *et al.* 2000a, Eckel *et al.* 2002). Although many comorbidities relating obesity to cardiovascular dysfunction become more severe as body mass index increases in conjunction with body fat distribution, no culprits have been clearly identified to link any mutant gene(s) or lifestyle factors to disrupted cardiovascular function in obesity. It has been postulated that activation of the sympathetic nervous system and impairment of

endothelial function are two pivotal phenotypical traits in obese or over-weight individuals (Kuo *et al.* 2003). However, cellular and molecular mechanisms responsible for sympathetic hyperactivity and/or endothelial dysfunction have not yet been clearly defined. Adding to the complexity, metabolic syndromes such as type 2 diabetes and hypertension are often concurrent with obesity and may bias the sole independent effects of obesity on cardiovascular function. Emerging evidence has indicated that the 16 kDa obese gene (*ob*) product leptin, which regulates food intake and energy expenditure, exerts potent cardiovascular effects and contributes to altered cardiovascular dysfunction under obesity (Barouch *et al.* 2003, Ren 2004, 2005, Minhas *et al.* 2005). Both hypoleptinemia (such as *ob/ob* mice with a point mutation of the leptin gene) and hyperleptinemia (such as *db/db* mice with a leptin receptor mutation) trigger overt obesity (Ross *et al.* 2004). Studies from our laboratory revealed

that leptin directly depresses cardiac contraction in ventricular myocytes (Nickola *et al.* 2000), the effect of which may be blunted by hyperleptinemic conditions such as essential hypertension due to interrupted post-leptin receptor signaling (Wold *et al.* 2002). Uncorrected obesity has been demonstrated to be commonly associated with increased plasma leptin levels and/or interrupted leptin signaling due to a defect in the leptin gene or the leptin receptor. Plasma leptin levels (5–15 ng/ml in lean subjects) have been found to be elevated in all forms of rodent (with the exception of *ob/ob* mice due to leptin gene mutation) and human obesity, whether of genetic, hypothalamic or dietary origin (Ren 2004, 2005), suggesting the state of hyperleptinemic 'leptin resistance' in obesity. However, the role of leptin deficiency in cardiomyocyte function has not been elucidated. Since *ob/ob* mice are a unique model of obesity lacking leptin (Ross *et al.* 2004), the goal of our study was to examine leptin receptor expression, cardiomyocyte contractile function, intracellular Ca^{2+} homeostasis and ultrastructural morphology in ventricular myocytes from leptin-deficient *ob/ob* obese mice heart.

Materials and Methods

Experimental animals and plasma measurement

The experimental procedures described in this study were approved by the University of Wyoming (Laramie, WY, USA) and the University of North Dakota (Grand Forks, ND, USA) Animal Use and Care Committees. In brief, homozygous B6·V-lep^{ob}/J male mice were obtained from the Jackson Laboratory (Bar Harbor, ME, USA) at 3 weeks of age and were housed within the School of Pharmacy Animal Facility at the University of Wyoming with free access to food and water until 12 weeks of age. Age- and weight-matched wild-type C57BL/6J mice were used as lean controls. Blood glucose was monitored with a glucometer (Accu-ChekII, model 792; Boehringer Mannheim Diagnostics, Indianapolis, IN, USA). Systolic and diastolic blood pressures were examined with a semi-automated, amplified tail cuff device (IITC, Inc., Woodland Hills, CA, USA). Plasma leptin, insulin and triglycerides levels were measured by RIA (Linco Research, St Charles, MO, USA). A separate cohort of 3-week-old male C57BL/6J mice was randomly divided into two groups and fed with either a control diet containing 10% kcal from fat (no. D12450B, designated as low fat; Research Diet Inc., New Brunswick, NJ, USA) or a high fat diet containing 45% of kcal from fat (no. D12451; Research Diet Inc.) for 9 weeks. The high fat diet was calorically rich (high fat diet = 4.83 kcal/g vs low fat diet = 3.91 kcal/g) because of the higher fat composition. However, the two diets possessed similar nutrient composition (Li *et al.* 2005).

Western blot analysis of leptin receptor Ob-R

Total protein was prepared as described previously (Duan *et al.* 2003). In brief, left ventricles were rapidly removed and homogenized in a lysis buffer containing 20 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton, 0.1% SDS and 1% protease inhibitor cocktail. Samples were then sonicated for 15 s and centrifuged at 12 000 g for 20 min at 4 °C. The protein concentration of the supernatant was evaluated using protein assay reagent (Bio-Rad, Hercules, CA, USA). Equal amounts (50 µg protein/lane) of protein and pre-stained molecular weight marker (Gibco-BRL, Gaithersburg, MD, USA) were loaded onto 7% SDS-polyacrylamide gels in a minigel apparatus (Mini-PROTEAN II; Bio-Rad), before being separated and transferred to nitrocellulose membranes (0.2 µm pore size; Bio-Rad). Membranes were incubated for 1 h in a blocking solution containing 5% non-fat milk in Tris-buffered saline (TBS), washed in TBS and incubated overnight at 4 °C with an anti-Ob-R (1:200) monoclonal antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA). After incubation with the primary antibody, blots were incubated with an anti-mouse IgG horseradish peroxidase-linked antibody at a dilution of 1:5000 for 60 min at room temperature. Immunoreactive bands were detected using the Super Signal West Dura extended duration substrate (Pierce, Milwaukee, WI, USA). The intensity of the bands was measured with a scanning densitometer (model GS-800; Bio-Rad) coupled with Bio-Rad PC analysis software.

Transmission electron microscopy

Tissue of the left ventricles of two randomly selected mice from each group was fixed with 2.5% glutaraldehyde/1.2% acrolein in fixative buffer (0.1 M cacodylate and 0.1 M sucrose, pH 7.4), postfixed with 1% osmium tetroxide, followed by 1% uranyl acetate, dehydrated through a graded series of ethanol concentrations, and embedded in LX112 resin (LADD Research Industries, Burlington VT, USA). Ultra thin sections (~50 nm) were cut on the ultramicrotome, stained with uranyl acetate, followed by lead citrate, and viewed on a Hitachi H-7000 transmission electron microscope equipped with a 4 k × 4 k cooled charge coupled device (CCD) digital camera.

Isolation of mouse left ventricular myocytes

Single ventricular myocytes were enzymatically isolated as described previously (Duan *et al.* 2003). Briefly, hearts were removed and perfused (at 37 °C) with oxygenated (5% CO₂–95% O₂) Krebs–Henseleit bicarbonate (KHB) buffer containing (in mM) 118 NaCl, 4.7 KCl, 1.25 CaCl₂, 1.2 MgSO₄, 1.2 KH₂PO₄, 25 NaHCO₃, 10 HEPES and 11.1 glucose. Hearts were subsequently perfused with a Ca²⁺-free KHB buffer containing

223 U/ml collagenase D (Worthington Biochemical, Freehold, NJ, USA) for 20 min. After perfusion, left ventricles were removed and minced to disperse the individual ventricular myocytes in Ca^{2+} -free KHB buffer. Extracellular Ca^{2+} was added incrementally back to 1.25 mM. Myocytes with obvious sarcolemmal blebs or spontaneous contractions were not used. Only rod-shaped myocytes with clear edges were selected for recording of mechanical properties or intracellular Ca^{2+} transients. A cohort of ventricular myocytes was incubated *in vitro* (at 37 °C) with various concentrations of leptin (0.1, 0.5 and 1.0 nM) for 1 h before cell mechanics were examined.

Cell shortening/relengthening

Mechanical properties of ventricular myocytes were assessed using an IonOptix MyoCam system (IonOptix Corp., Milton, MA, USA) (Duan *et al.* 2003). In brief, myocytes were placed in a chamber mounted on the stage of an inverted microscope and superfused with a buffer containing (in mM) 131 NaCl, 4 KCl, 1 CaCl_2 , 1 MgCl_2 , 10 glucose and 10 HEPES, at pH 7.4. The cells were field stimulated with a suprathreshold voltage and at a frequency of 0.5 Hz (3 ms duration) with the use of a pair of platinum wires placed on opposite sides of the chamber connected to an FHC Inc. (Frederick Haer & Co.) stimulator (Brunswick, NE, USA). The polarity of stimulatory electrodes was reversed frequently to avoid possible build up of the electrolyte by-products. The myocyte being studied was displayed on the computer monitor with the use of an IonOptix MyoCam camera, which rapidly scans the image area every 8.3 ms such that the amplitude and velocity of shortening/relengthening is recorded with good fidelity. The soft-edge software (IonOptix) was used to capture changes in cell length during contraction. The cross-sectional area was calculated using the product of cell length and average cell width.

Intracellular Ca^{2+} fluorescence measurement

A separate cohort of myocytes was loaded with fura-2/AM (0.5 μM) for 15 min, and fluorescence intensity was measured with a dual-excitation fluorescence photomultiplier system (IonOptix) (Duan *et al.* 2003). Myocytes were placed on an inverted Olympus microscope and imaged through a Fluor 40 $\times$ oil objective. Cells were exposed to light emitted by a 75W mercury lamp and passed through either a 360 nm or a 380 nm filter. The myocytes were stimulated to contract at 0.5 Hz. Fluorescence emissions were detected between 480 nm and 520 nm by a photomultiplier tube after cells were first illuminated at 360 nm for 0.5 s and then at 380 nm for the duration of the recording protocol (333 Hz sampling rate). The 360 nm excitation scan was repeated at the end

of the protocol, and qualitative changes in intracellular Ca^{2+} concentration were inferred from the ratio of the fluorescence intensity at two wavelengths.

Intracellular fluorescence measurement of superoxide (O_2^-)

The intracellular O_2^- level was monitored by changes in fluorescence intensity resulted from intracellular probe oxidation according to a previously described method (McPherson & Yao 2001). Following 60 min of leptin (0.1–1.0 nM) treatment, isolated cardiac myocytes were loaded with 5 μM dihydroethidium (DHE) (Molecular Probes, Eugene, OR, USA) for 30 min at 37 °C and washed with phosphate-buffered saline buffer. Cells were sampled randomly using an Olympus BX-51 microscope equipped with an Olympus MagnaFire SP digital camera and ImagePro image analysis software (Media Cybernetics, Silver Spring, MD, USA). Fluorescence was calibrated with InSpeck microspheres (Molecular Probes). More than 150 cells per treatment group were evaluated using the grid-crossing method for cell selection in more than 15 visual fields per experiment.

Statistical analyses

Data are presented as means $\pm$ S.E.M. Statistical significance ($P < 0.05$) for each variable was estimated by ANOVA or Student's *t*-test where appropriate. A Dunnett's test was used for *post hoc* analysis.

Results

General features of experimental animals and expression of the leptin receptor Ob-R

The general features of 12-week-old male lean and *ob/ob* obese mice are compared in Table 1. The *ob/ob* mice were overtly obese compared with their lean controls. The body, heart and liver weights were all significantly heavier than those from the age-matched lean control group. The heart-to-body weight ratio was decreased in the *ob/ob* groups as a result of the significantly elevated body weight. The plasma leptin levels were significantly lower in *ob/ob* mice compared with the lean control group. Plasma levels of insulin and triglycerides were significantly higher in *ob/ob* mice compared with those from lean controls (Table 1). Following an acute intraperitoneal glucose (2 g/kg) injection, the post-challenge glucose level remained elevated up to 120 min in *ob/ob* mice compared with that from the lean controls (data not shown), indicating glucose intolerance and insulin resistance in *ob/ob* obese mice. Using Western blot analysis, we revealed that the expression of the long-form leptin receptor Ob-R was significantly reduced in myocardium

Table 1 General features of 12-week-old male lean control and *ob/ob* mice. Data are expressed as means ± S.E.M.

Parameter	Lean control (n=9)	<i>ob/ob</i> obese (n=8)
Body weight (g)	28.3 ± 0.3	56.6 ± 1.4*
Heart weight (g)	0.20 ± 0.01	0.33 ± 0.01*
HW/BW (mg/g)	7.1 ± 0.2	5.8 ± 0.1*
Liver weight (g)	1.45 ± 0.04	3.55 ± 0.26*
Kidney weight (g)	0.41 ± 0.01	0.47 ± 0.02
Blood glucose (mg/dl)	94.3 ± 2.2	98.9 ± 2.4
Systolic pressure (mmHg)	104.5 ± 4.0	112.5 ± 4.4
Diastolic pressure (mmHg)	71.8 ± 2.8	76.0 ± 3.1
Leptin (ng/ml)	6.47 ± 0.73	1.42 ± 0.42*
Insulin (ng/ml)	0.59 ± 0.09	5.33 ± 0.86*
Triglycerides (mg/dl)	53.8 ± 6.1	120.2 ± 13.3*

HW=heart weight; BW=body weight.
P<0.05 vs lean controls.

from *ob/ob* mice compared with that of the lean controls (Fig. 1).

Electron microscopic characteristics of hearts from lean control and ob/ob obese groups

Leptin-deficient obesity triggered extensive focal damage in myocardial tissue. This was evidenced by cytoarchitectural damage of mitochondria and abundance of lipid droplets in cardiac myocytes from *ob/ob* obese mouse hearts. The damage to mitochondria included swelling, disorganization of cristae and loss of integrity. No overt

ultrastructural abnormality was observed in ventricular samples from lean control mice (Fig. 2).

Cell shortening and relengthening of cardiac myocytes from lean control and ob/ob mice: effect of in vitro leptin replenishment

Cardiomyocytes from *ob/ob* mice displayed a larger resting cell length ($122.8 \pm 2.6 \mu\text{m}$ vs lean control $115.3 \pm 3.1 \mu\text{m}$, $P < 0.05$, $n = 71\text{--}79$ cells per group) and cross-sectional area, decreased peak shortening (PS) and maximal velocity of shortening/relengthening ($\pm \text{dL/dt}$), prolonged time-to-90% relengthening (TR_{90}) and normal time-to-peak shortening (TPS) compared with myocytes from the lean control mice (Fig. 3). Consistent with the mechanical characteristics, myocytes from *ob/ob* mouse hearts displayed reduced fura-fluorescence intensity change (ΔFFI), an indication of an electrically stimulated increase in intracellular Ca^{2+} , associated with a slowed intracellular Ca^{2+} decay rate and normal resting intracellular Ca^{2+} level (basal fura-2 fluorescence intensity) (Fig. 4). Interestingly, leptin deficiency-induced mechanical and intracellular Ca^{2+} aberrations in *ob/ob* mouse myocytes were significantly improved with a 1-h *in vitro* incubation of *ob/ob* mouse cardiomyocytes with various concentrations of leptin (0.1–1.0 nM). Reduced PS, $\pm \text{dL/dt}$ and ΔFFI , as well as prolonged TR_{90} and intracellular Ca^{2+} decay rate in *ob/ob* mouse cardiomyocytes were completely restored to the value of the lean controls following all concentrations of leptin incubation. However, the enlarged cross-sectional area of *ob/ob* mouse myocytes (compared with the lean controls) was not affected by leptin at the concentrations tested. Leptin itself significantly shortened TR_{90} (at 0.1 and 0.5 nM) and increased resting intracellular Ca^{2+} levels (Figs 3 and 4). Leptin did not significantly affect cell mechanics in ventricular myocytes from lean control mice (data not shown), similar to findings from our other independent study (F Dong and J Ren, unpublished data).

Figure 1 Western blot analysis of leptin receptor Ob-R in left ventricles from lean control and *ob/ob* obese mice. Values are means ± S.E.M., $n = 4$. * $P < 0.05$ vs the lean control group. The top section shows a representative Western blot using anti-Ob-R antibody.

Generation of O_2^- in cardiac myocytes from lean control and ob/ob mice: effect of in vitro leptin replenishment

To explore the potential mechanism underlying the compromised cardiac contractile function in *ob/ob* mouse myocytes, we evaluated O_2^- levels in cardiomyocytes using the DHE fluorescence probe. Our data shown in Fig. 5 revealed that *ob/ob* mouse myocytes possessed significantly higher O_2^- level compared with myocytes from the lean controls. Consistent with our mechanical observation shown in Figs 3 and 4, the 1-h *in vitro* incubation of leptin completely ablated the enhanced O_2^- levels in *ob/ob* mouse myocytes at all concentrations of peptide tested. Leptin treatment itself did not

Figure 2 Transmission electron microscopic micrographs exhibiting ultrastructural changes in left ventricular myocytes from lean control and *ob/ob* obese mouse hearts. (A) ($\times 4000$) and (C) ($\times 20\,000$) show the regular structure and the size of mitochondria of left ventricular myocytes from the lean control group; (B) ($\times 4000$) and (D) ($\times 20\,000$) show the changed mitochondria of left ventricular myocytes from *ob/ob* obese mouse hearts. Arrows indicate swelling and disorganization of cristae in mitochondria. Some of them have vacuoles. The arrowheads indicate accumulated lipid droplets.

significantly affect DHE fluorescence intensity in lean control myocytes (data not shown).

Cell shortening and relengthening of cardiac myocytes from high fat diet-induced obese mice

To understand whether cardiomyocyte dysfunction in *ob/ob* obese mice was due to leptin deficiency or indirectly through obesity, we used the high fat diet-induced obesity model (Li *et al.* 2005) to produce age- (12 week) and gender-matched obese C57BL/6J mice. Following a 9-week feeding regimen starting at 3 weeks of age, lean C57BL/6J mice fed with our high fat diet (45% fat vs 10%

fat in the low fat diet) displayed significantly elevated body weight and heart weight, although the heart-to-body weight ratio remained unchanged. Somewhat surprisingly, cardiomyocytes from the high fat-fed mice displayed normal cross-sectional area and contractile function (measured by PS, $\pm$ dL/dt, TPS and TR₉₀) compared with myocytes obtained from age-matched low fat diet-fed mice (Fig. 6).

Discussion

The major finding from our study revealed mechanical, intracellular Ca²⁺ homeostasis and morphological

Figure 3 Contractile properties of myocytes from C57BL/6J lean control and *ob/ob* mouse hearts as well as *ob/ob* mouse myocytes treated with various concentrations of leptin (0.1–1.0 nM) for 1 h. (A) Representative traces depicting myocyte shortening of cells from lean control, *ob/ob* and *ob/ob* cells treated with 0.5 nM leptin, (B) cross-sectional area, (C) PS (normalized to cell length), (D) maximal velocity of shortening (+dL/dt) and relengthening (-dL/dt), (E) TPS and (F) TR₉₀. Values are means $\pm$ S.E.M., $n=79$ and 71 cells for control and *ob/ob* groups respectively, $n=28$ –33 cells for the leptin treatment groups. * $P<0.05$ vs the lean control group, # $P<0.05$ vs the *ob/ob* group.

Figure 4 Intracellular Ca^{2+} transient properties of ventricular myocytes from lean control and *ob/ob* obese mouse hearts as well as *ob/ob* mouse myocytes treated with various concentrations of leptin (0.1–1.0 nM) for 1 h. (A) Representative intracellular Ca^{2+} transients in ventricular myocytes from lean control, *ob/ob* obese mice and *ob/ob* cells treated with 0.5 nM leptin, (B) resting FFI – indication of resting intracellular Ca^{2+} level, (C) ΔFFI – indication of electrically stimulated intracellular Ca^{2+} release and (D) intracellular Ca^{2+} transient decay rate. Values are means $\pm$ S.E.M., $n=49$ and 40 cells for control and *ob/ob* groups respectively, $n=23$ –30 cells for the leptin treatment groups. * $P<0.05$ vs the lean control group, # $P<0.05$ vs the *ob/ob* group.

aberrations in leptin-deficient *ob/ob* obese mouse hearts at the isolated ventricular myocyte level. Our results revealed major mechanical abnormalities including depressed peak cell shortening, reduced maximal velocities of shortening/relengthening and prolonged duration of relengthening in cardiac myocytes from leptin-deficient *ob/ob* obese mice. Furthermore, our intracellular Ca^{2+} fluorescence measurement demonstrated decreased ΔFFI associated with slowed intracellular Ca^{2+} decay rate in myocytes

from *ob/ob* mice, indicative of compromised electrically stimulated intracellular Ca^{2+} release and extrusion. These mechanical deficits were accompanied by a 27% reduction in the expression of leptin receptor Ob-R in *ob/ob* myocardium. Interestingly, the mechanical dysfunction in *ob/ob* mouse myocytes was ablated by *in vitro* treatment of recombinant leptin in *ob/ob* mouse myocytes. This was consistent with the observation that leptin treatment antagonized the elevated O_2^- generation in *ob/ob*

Figure 5 O_2^- production measured by a DHE fluorescence probe in myocytes from (A) C57BL/6J lean control, (B) *ob/ob* mice and (C) *ob/ob* mouse myocytes treated with various concentrations of leptin (0.1–1.0 nM) for 1 h (C–E). (F) Summarized data from 29–33 measurements. Values are means $\pm$ S.E.M. * $P < 0.05$ vs the lean control group, # $P < 0.05$ vs the *ob/ob* group.

Figure 6 Contractile properties of myocytes from 9-week-old low fat- or high fat-fed C57BL/6J mice. (A) Body and heart weights and heart-to-body weight ratio, (B) cross-sectional area, (C) PS (normalized to cell length), (D) maximal velocity of shortening (+dL/dt) and relengthening (−dL/dt), (E) TPS and (F) TR₉₀. Values are means ± S.E.M., $n=53$ cells per group. * $P<0.05$ vs the low fat group.

mouse myocytes, suggesting a likely role of superoxide anion in leptin deficiency-induced cardiomyocyte mechanical dysfunction.

Although human obesity is commonly associated with excessive leptin (Ren 2004), both hypo- and hyperleptinemia (leptin resistance) have been demonstrated to induce obesity due to interrupted leptin signaling in energy expenditure (Ross *et al.* 2004). In our current experimental setting, the leptin-deficient *ob/ob* mice at 12 weeks of age were euglycemic and normotensive, thus excluding possible contributions from diabetes and/or hypertension to the cardiac anomalies reported in the *ob/ob* mice in our study. However, these *ob/ob* mice

were hyperinsulinemic, hypertriglyceridemic and glucose intolerant, indicating the presence of metabolic syndrome. Obesity is usually associated with type 2 diabetes, hypertension and metabolic syndrome (Sowers 1998, Eckel *et al.* 2002). Leptin may reduce insulin release and enhance insulin sensitivity, consistent with elevated insulin levels in *ob/ob* obese mice (Table 1). However, long-term hyperleptinemia commonly seen in human obesity may trigger leptin resistance, suppress insulin sensitivity and ultimately induce insulin resistance (Ren 2004). Intracerebroventricular administration of leptin increases arterial blood pressure (Matsumura *et al.* 2000), with the pressor effect proportional to the cerebrospinal level of leptin (Shirasaka

et al. 2003). This was consistent with the elevation of blood pressure in *ob/ob* mice following leptin reconstitution despite reduction in food intake and body weight (Aizawa-Abe *et al.* 2000, Mark & Sivitz 2002). Beginning at around puberty (between 4 and 6 weeks of age), moderate hyperglycemia develops in *ob/ob* mice. This hyperglycemia is transient and blood glucose levels are expected to drop back within the normal range after 12 weeks of age (Coleman 1978), consistent with the euglycemic status found in our study. The role of leptin in lipid (triglyceride) metabolism may participate in the cardiac regulation of the hormone. Leptin oxidizes excessive long-chain fatty acids to benefit cardiac function. The leptin-induced fatty acid oxidation is believed to be defective in *ob/ob* mice, allowing accumulation of triglycerides and subsequent non-oxidative cellular injury. The hearts of *ob/ob* mice were found to be metabolically inefficient and unable to modulate substrate utilization in response to increased fatty acid supply (Mazumder *et al.* 2004). This is supported by a recent finding that leptin repletion to *ob/ob* mice reconciled elevated insulin and triglycerides levels as well as abnormal cardiomyocyte contractile function (Minhas *et al.* 2005), confirming the key role of leptin in fatty acid metabolism and cardiac function regulation.

In our study, the 12-week-old *ob/ob* mice exhibited significantly heavier body and heart weights compared with the age-matched lean control group. Marked cardiac hypertrophy has been reported in *ob/ob* mice at 6 months of age; this can be reversed by leptin infusion but not caloric restriction (Barouch *et al.* 2003). The inability of caloric restriction (although with equal body weight loss to leptin infusion) to correct cardiac hypertrophy (wall sickness and myocyte size) in *ob/ob* mice (Barouch *et al.* 2003) indicates a unique role of leptin in cardiac architecture. We observed enlargement in cross-sectional areas of *ob/ob* mouse myocytes in our current study; this was not affected by short-term leptin treatment, indicating that cardiac hypertrophy due to interrupted leptin signaling is a chronic process. Cardiac remodeling is a critical factor in the transition from compensated to de-compensated states and contributes to compromised cardiac function and morphology. The ultrastructural feature in cardiomyocytes from *ob/ob* obese mice has not been well characterized previously, other than an indication of enhanced formation of lipid droplets (Giacomelli & Wiener 1979). Our study demonstrated significant morphological changes, especially swelling and disorganization of cristae in mitochondria from *ob/ob* mouse ventricular tissues. These ultrastructural changes in leptin-deficient *ob/ob* obese mice may contribute to the sub-optimal contractile function of ventricular myocytes, although further study is warranted.

Perhaps the most important finding of our study was that *in vitro* leptin treatment reconciles leptin deficiency-induced cardiomyocyte dysfunction. Depressed cardiomyocyte

contractile function has been demonstrated in Zucker obese rats with concomitant hypertension and diabetes (Ren *et al.* 2000a,b). Similarly, cardiac mechanical dysfunction was observed in high fat diet-induced obesity (Dobrian *et al.* 2000, 2001). This similarity in mechanical dysfunction suggests a comparable mechanism in cardiac dysfunction between certain genetically predisposed and diet-induced obesity states. However, we did not observe any notable mechanical dysfunction in high fat diet-induced obesity. The discrepancy in cardiac function between the current study and those reported earlier (Dobrian *et al.* 2000, 2001) may be underscored by the species (mouse vs rat) and age (3-week-old vs adulthood) of the experimental animals. Nevertheless, the negative finding in cardiac contractile function in our age-matched diet-induced obese C57BL/6J mice suggests a unique role of leptin status rather than body weight gain in cardiac dysfunction at this stage of life. Our earlier study (Nickola *et al.* 2000) indicated that the satiety hormone leptin inhibits myocyte contraction and intracellular Ca^{2+} release, which may be blunted in hyperleptinemic leptin-resistant states (Wold *et al.* 2002). With this physiological role of leptin in mind, our current finding of impaired cardiomyocyte contractile function under leptin deficiency appears paradoxical. Our result of diminished leptin receptor Ob-R and a recent report from Minhas *et al.* (2005) shed some light on this leptin paradox. Minhas *et al.* (2005) demonstrated that sarcomere shortening and Ca^{2+} transients following β -adrenergic stimulation were impaired, accompanied by depressed activation of protein kinase A (PKA) in cardiomyocytes from *ob/ob* mice; this may be restored by recombinant leptin repletion. Their finding is in support of our observation that *in vitro* leptin treatment reconciles cardiomyocyte contractile function in *ob/ob* mouse myocytes. The fact that we used isolated cardiomyocyte (compared with *in vivo* leptin repletion) indicates that such an effect of leptin is cardiomyocyte specific although an indirect effect from non-myocyte components such as the β -adrenergic system may also play a role. Desensitized cardiac β -adrenergic responsiveness is a hallmark for heart failure and cardiac complications in diabetes and obesity (Choi & Rockman 1999). The improved β -adrenergic responsiveness in cardiomyocytes from *ob/ob* mice following leptin repletion is associated with improved protein expression of the stimulatory guanine nucleotide-binding protein α subunit ($\text{Gs}\alpha$), enhanced PKA activity and enhanced phosphorylation of the sarco(endo)plasmic reticulum Ca^{2+} -ATPase 'lock'-phospholamban (Minhas *et al.* 2005). These data provided a convincing link between adequate leptin signaling and cardiac function and suggested a mechanism by which leptin deficiency may lead to cardiac dysfunction (Ren 2004, 2005). It is worth mentioning that a fall in leptin not only impairs cardiac function but also leads to immune deficiency (e.g. lymphoid atrophy and T-lymphocyte dysfunction),

which may be restored by leptin repletion (Mancuso *et al.* 2002). Depressed immune defense may itself contribute to compromised cardiac function. In addition, defects in leptin-activated cell signaling pathways such as nitric oxide or changes in other adipokines such as visfatin in obesity may also contribute to cardiac contractile dysfunction in hyper- or hypoleptinemia-associated cardiac dysfunction (Winters *et al.* 2000, Sethi & Vidal-Puig 2005).

Experimental limitations

Although we have isolated the effects of leptin deficiency from confounding factors such as hypertension, hyperglycemia and possibly obesity in our study, hyperinsulinemia is present in *ob/ob* mice due to a compensatory hyperplasia and hypertrophy of the pancreatic β -islet cells. The insulin and leptin hormonal signaling cascades are closely interrelated (Ren 2004, 2005). The notion that leptin plays a discrete role in obesity simply through regulation of insulin signaling may not be excluded at this point. In addition, high fat diet-induced obesity did not produce similar body weight gain to that of leptin-deficient *ob/ob* mice and this may dampen the contribution of obesity in cardiac dysfunction in *ob/ob* mice. Moreover, the fat and lean body mass may be completely different between diet-induced obesity and *ob/ob* obesity because of the catabolic effect of leptin.

Conclusion

Our current study has demonstrated compromised cardiomyocyte contractile function associated with abnormal cardiac ultrastructure and diminished leptin receptor expression in the left ventricle from leptin-deficient *ob/ob* obese mice. The contractile aberrations in *ob/ob* myocytes were paralleled by superoxide anion generation and were significantly improved by *in vitro* leptin incubation. Contractile dysfunction was not seen in age- and gender-matched high fat-induced obese mice. These results should provide a solid rationale for the role of leptin status in cardiac function. These findings have prompted the speculation that leptin may serve as a physiological regulator of cardiac function whereas abnormal plasma leptin levels including hyper- or hypoleptinemia may act as a pathophysiological trigger and/or marker for cardiac diseases. The understanding of the effect of leptin on cardiomyocyte contractile function should provide insights into the new treatment plans for obesity-associated cardiovascular complications.

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