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Leptin Regulates Cardiomyocyte Contractile Function Through Endothelin-1 Receptor–NADPH Oxidase Pathway

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Abstract—Leptin, the obese gene product, plays an important role in the regulation of cardiac function. However, the mechanism behind leptin-induced cardiomyocyte contractile response is poorly understood. This study was designed to examine whether endothelin-1 receptor and NADPH oxidase play any role in leptin-induced cardiac contractile response. Isolated murine cardiomyocytes were exposed to leptin (5, 50, and 100 nmol/L) for 60 minutes in the absence or presence of the ETα receptor antagonist BQ123 (1 μmol/L), the ETβ receptor antagonist BQ788 (1 μmol/L), or the NADPH oxidase inhibitor apocynin (100 μmol/L) before mechanical function was studied. Superoxide levels were measured by dihydroethidium fluorescent dye and the superoxide dismutase–inhibitable reduction of cytochrome c. NADPH oxidase subunit expression (p22phox, p47phox, p67phox, and gp91phox) was evaluated with Western blot. Leptin depressed peak shortening and maximal velocity of shortening/relengthening (±dL/dt), prolonged the duration of relengthening (TR90) without affecting the time-to-peak cell shortening. Consistent with the mechanical characteristics, myocytes treated with leptin displayed a reduced electrically stimulated rise in intracellular Ca2+ (change in fura-2 fluorescence intensity) associated with a prolonged intracellular Ca2+ decay rate. All of the abnormalities were significantly attenuated by apocynin, BQ123, or BQ788. Intracellular superoxide generation was enhanced after leptin treatment, which was partially blocked by apocynin, BQ123, or BQ788. Leptin had no effect on p22phox and gp91phox but upregulated protein expression of p67phox and p47phox, both of which were inhibited by apocynin, BQ123, or BQ788. These results suggest that leptin suppresses cardiac contractile function in ventricular myocytes through the endothelin-1 receptor and NADPH oxidase-mediated pathway. (Hypertension. 2006;47:222-229.)

Key Words: endothelin | cardiac function | obesity | oxidative stress

Obesity is a common medical problem with a multifactorial etiology encompassing genetics, environment, metabolism and lifestyle. Both clinical and experimental data have demonstrated that uncorrected obesity leads to cardiac hypertrophy and compromised ventricular function.1–3 Several mechanisms have been postulated for obesity-induced cardiac dysfunctions with sympathetic activation and endothelial dysfunction being the most important phenotypic traits.4 Nevertheless, the ultimate cause of obesity-induced cardiac dysfunction remains uncertain. Emerging evidence has indicated that the 16-kDa obese gene product leptin, which regulates food intake and energy expenditure, participates in cardiovascular regulation and contributes to altered cardiac function in obesity.5,6 Evidence suggested that obesity is usually associated with increased plasma leptin levels and/or interrupted leptin signaling because of either abnormal expression of leptin and/or the leptin receptor.6 Data from our laboratory revealed that leptin directly depresses cardiomyocyte contraction through mechanism(s) related to NO, Janus kinase/signal transducer and activator of transcription, and p38 mitogen-activated protein kinase signaling pathways.7,8 However, the precise signaling mechanism responsible for leptin-induced cardiac response remains unclear at the receptor and postreceptor levels. Leptin has been indicated to facilitate endothelin-1 (ET-1) generation in endothelial cells9–11 and cardiomyocytes,12 which may contribute to leptin-induced cardiomyocyte hypertrophy through the generation of reactive oxygen species (ROS).12,13 Our earlier study showed that ET-1 significantly enhanced ROS generation via a reduced NADPH oxidase–dependent pathway in human umbilical vein endothelial cells.14 The goal of our present study was to test the hypothesis that ET-1 receptor and NADPH oxidase play a role in leptin-induced cardiomyocyte contractile depression.

Methods

Isolation of Mouse Left Ventricular Myocytes

The experimental procedures described here were approved by our Institutional Animal Use and Care Committee. In brief, adult male C57BL/6J mice (~12 weeks of age) were housed within our animal facility with free access to food and water. Single ventricular myocytes were isolated as described.15 Hearts were removed and perfused with oxygenated (5% CO2–95% O2) Krebs–Henseleit bi

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carbonate (KHB) buffer containing (in mmol/L) 118 NaCl, 4.7 KCl, 1.25 CaCl₂, 1.2 MgSO₄, 1.2 KH₂PO₄, 25 NaHCO₃, 10 HEPES, and 11.1 glucose. Hearts were then perfused with a Ca²⁺/H⁺-free KHB containing Liberase Blendzyme 4 (Hoffmann-La Roche Inc) for 20 minutes. After perfusion, left ventricles were removed and minced to disperse cardiomyocytes in Ca²⁺/H⁺-free KHB buffer. Extracellular Ca²⁺ was added incrementally back to 1.25 mmol/L. Cells with obvious sarcolemmal blebs or spontaneous contractions were not used for mechanical recording.

Cell Shortening/Relengthening
Mechanical properties of cardiomyocytes were assessed using an IonOptix MyoCam system. In brief, myocytes were placed in a chamber mounted on the stage of an inverted microscope and superfused with a buffer containing (in mmol/L): 131 NaCl, 4 KCl, 1 CaCl₂, 1 MgCl₂, 10 glucose, and 10 HEPES (pH 7.4). The cells were field stimulated with suprathreshold voltage and at a frequency of 0.5 Hz using a pair of platinum wires placed on the opposite sides of the chamber connected to a FHC stimulator. The myocyte being studied was displayed on the computer monitor using an IonOptix MyoCam camera, which rapidly scans the image area at every 8.3 ms such that the amplitude and velocity of shortening/relengthening was recorded with good fidelity. The soft-edge software (IonOptix) was used to capture changes in cell length during contraction.

Intracellular Ca²⁺ Fluorescence Measurement
A separate group of myocytes was loaded with fura-2/AM (0.5 μmol/L) for 15 minutes, and fluorescence intensity was measured with a

**Figure 1.**

Effect of leptin (5, 50, and 100 nmol/L) on myocyte shortening in the absence or presence of BQ123 (1 μmol/L), BQ788 (1 μmol/L), and apocynin (100 μmol/L). (A) PS (normalized to resting cell length); (B) Maximal velocity of shortening and relengthening (+dL/dt); (C) TPS; and (D) TR₁₀. Mean±SEM, n=30 to 50 cells per group; *P<0.05 vs control (no leptin or blockers); #P<0.05 vs 100 nmol/L leptin group.

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dual-excitation fluorescence photomultiplier system (Ionoptix). Myocytes were placed on an inverted Olympus microscope and imaged through a Fluor 11003 oil 40 objective. Cells were exposed to light emitted by a 75-W mercury lamp and passed through either a 360-nm or a 380-nm filter. 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 our 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 fura-2 fluorescence intensity (FFI) at 2 wavelengths.

**Western Blot Analysis of NADPH Oxidase p22\textsuperscript{phox}, p47\textsuperscript{phox}, p67\textsuperscript{phox}, and gp91\textsuperscript{phox}**

Cardiomyocytes were collected and sonicated in a lysis buffer containing 20 mmol/L Tris (pH 7.4), 150 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L EGTA, 1% Triton, 0.1% SDS, and protease inhibitor mixture. Equal amounts of protein lysates (50 μg/lane) were separated on 7% (gp91\textsuperscript{phox}), 10% (p47\textsuperscript{phox} and p67\textsuperscript{phox}), and 15% (p22\textsuperscript{phox}) SDS-polyacrylamide gels in a minigel apparatus (Mini-PROTEAN II, Bio-Rad) and transferred to nitrocellulose membranes (0.2 μm). The membranes were blocked in 5% (weight/vol) nonfat milk in Tris-buffered saline–T buffer, and then incubated with anti-p22\textsuperscript{phox} (1:1000), anti-p47\textsuperscript{phox} (1:1000), anti-p67\textsuperscript{phox} (1:1000), anti-gp91\textsuperscript{phox} (1:1000), and anti-β-actin (1:8000) antibodies at 4°C overnight. NADPH oxidase subunit antibodies were kindly provided by Dr Mark T. Quinn from Montana State University (Bozeman, MT). After incubation with primary antibody, blots were incubated with anti-rabbit IgG horseradish peroxidase–linked antibodies at a dilution of 1:5000 for 100 minutes at room temperature. Immunoreactive bands were detected using the Super Signal West Dura Extended Duration Substrate (Pierce). The intensity of bands was measured with a scanning densitometer (Model GS-800; Bio-Rad) coupled with Bio-Rad PC analysis software. For all of the Western blot analysis experiments, β-actin was used as an internal loading control.

Figure 2. Effect of leptin (100 nmol/L) on intracellular Ca\(^{2+}\) transients in the absence or presence of BQ123 (1 μmol/L), BQ788 (1 μmol/L), and leptin+apocynin groups; (B) Resting FFI; (C) electrically stimulated rise in FFI (ΔFFI); and (D) Intracellular Ca\(^{2+}\) transient decay rate. Mean±SEM, n=30 to 50 cells per group; *P<0.05 vs control (no leptin or blockers); #P<0.05 vs 100 nmol/L leptin group.
Intracellular Fluorescence Measurement of Superoxide

Intracellular superoxide ($O_2^-$) was monitored by changes in fluorescence intensity resulting from intracellular probe oxidation according a method described previously. After a 60-minute leptin (5, 50, and 100 nmol/L) treatment with or without pharmacological inhibitors, myocytes were loaded with 5 μmol/L dihydroethidium (DHE; Molecular Probes) for 30 minutes at 37°C and washed with PBS 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). Fluorescence was calibrated with InSpeck microspheres (Molecular Probes). More than 150 cells per group were evaluated using the grid crossing method for cell selection in >15 visual fields per experiment.

Quantitation of $O_2^-$ Anion Release by Cytochrome c Reduction

$O_2^-$ anion was also quantitated by the SOD–inhibitable reduction of cytochrome c. In brief, 35 μL of cytochrome c (40 μmol/L) was mixed with 140 μL of samples containing the test sample. The reduction of cytochrome c was measured by enzyme-linked immunosorbent assay (ELISA) in a microplate reader (Bio-Rad Laboratories). The absorbance at 550 nm was measured, and the amount of reduced cytochrome c was calculated based on the standard curve.
Experiment Protocols
Cardiomyocytes were incubated with leptin (5, 50, and 100 nmol/L) for 60 minutes in a serum-free defined medium consisting of Medium199 (Sigma) with Earle’s salts. The concentration range of leptin was chosen based on the pathophysiological levels of the hormone and relevant earlier studies. In some studies, myocytes were incubated with the ETα receptor antagonist BQ123 (1 μmol/L), the ETβ receptor antagonist BQ788 (1 μmol/L), the NADPH oxidase inhibitor apocynin (100 μmol/L), and the O2•− scavenger tempol (1 mmol/L) for 30 minutes before leptin addition. Cardiac myocyte shortening and intracellular Ca2⁺ transients (30 to 50 cells per group) were evaluated. Intracellular O2•− in cardiomyocytes before and after leptin treatment (in the absence or presence of the ET-1 receptor antagonists and the NADPH oxidase inhibitor apocynin) were measured.

Statistical Analyses
Differences were evaluated by ANOVA with repeated measures. A Tukey test was used as a post hoc analysis. Statistical significance was considered to be P<0.05.

Results
Effect of Leptin on Myocyte Shortening in the Absence or Presence of BQ123, BQ788, and Apocynin
The average resting cell length for ventricular myocytes used in this study was 109.2±0.9 μm (n=582). Cardiomyocytes treated with leptin (50 and 100 nmol/L) displayed reduced peak shortening (PS) and maximal velocity of shortening/dt, and prolonged TR90, and normal time-to-PS (TPS) compared with myocytes without leptin treatment. The leptin-induced depression in PS and dL/dt and prolongation of TR90 were significantly attenuated by apocynin, BQ123, and BQ788 except that BQ788 failed to affect prolonged TR90 elicited by leptin. Lower concentration of leptin (5 nmol/L) did not affect cardiomyocyte mechanics (Figure 1).

Effect of Leptin on Intracellular Ca2⁺ Transients in the Absence or Presence of BQ123, BQ788, and Apocynin
Consistent with mechanical characteristics, myocytes treated with leptin displayed reduced FFI change (ΔFFI), an indication of electrically stimulated increase in intracellular Ca2⁺, and slowed intracellular Ca2⁺ decay rate, an indication of intracellular Ca2⁺ extrusion associated with unchanged resting intracellular Ca2⁺ level (resting FFI). Apocynin, BQ123, and BQ788 significantly attenuated leptin-induced alteration in intracellular Ca2⁺ handling with the exception that BQ788 failed to elicit any statistically significant effect on leptin-induced prolongation of intracellular Ca2⁺ decay (Figure 2).

Effect of Leptin on p22phox, p47phox, p67phox, and gp91phox Expression in the Absence or Presence of BQ123, BQ788, Apocynin, and Tempol
To confirm the involvement of NADPH oxidase in leptin-induced cardiomyocyte contractile dysfunction, we evaluated the expression of the NADPH oxidase subunit (p22phox, p47phox, p67phox, and gp91phox) using Western blot. Figure 3 showed that leptin had little effect on p22phox and gp91phox, however, it upregulated protein expression of p47phox and p67phox. Consistent with the mechanical data, the leptin-induced upregulation of p47phox and p67phox was significantly attenuated by all 3 of the antagonists. We also evaluated the effect of the O2•− anion scavenger tempol (1 mmol/L) on leptin and apocynin-induced alteration in p47phox and p67phox expression. Tempol failed to elicit any effect on basal and leptin-induced elevation in p47phox and p67phox expression. Furthermore, tempol did not affect apocynin-elicited inhibition on leptin-induced upregulation of p47phox and p67phox.

Effect of Leptin on Intracellular O2•− Generation
Activation of NADPH oxidase is believed to promote O2•− generation, and our data shown in Figure 4 demonstrated that leptin at 50 and 100 nmol/L facilitated O2•− generation using either DHE fluorescent dye or the SOD-inhibitable reduction of cytochrome c technique. The leptin-induced elevation in intracellular O2•− was significantly attenuated by the ETα receptor antagonist BQ123, the ETβ receptor antagonist BQ788, or the NADPH oxidase inhibitor apocynin.

Discussion
The major finding of this study is that both ET-1 receptors and NADPH oxidase are possibly involved in leptin-elicited cardiomyocyte contractile depression. Our results revealed that leptin induced mechanical abnormalities of cardiomyocyte, including depressed PS, reduced ±dL/dt, and prolonged TR90. In addition, intracellular Ca2⁺ fluorescence measurement demonstrated decreased ΔFFI and slowed intracellular Ca2⁺ decay after leptin exposure, suggesting abnormality in intracellular Ca2⁺ handling. Interestingly, decreased PS, ±dL/dt, and ΔFFI were significantly attenuated by all 3 of the antagonists, whereas the prolonged TR90 and intracellular Ca2⁺ decay rates were significantly attenuated by apocynin and BQ123 (BQ788 showed a trend of attenuation that failed to reach statistical significance). These findings, in conjunction with Western blot analysis and O2•− generation using DHE fluorescent dye and the SOD-inhibitable cytochrome c reduction techniques, implied that leptin suppresses cardiac contractile function in ventricular myocytes through ET-1 receptor and NADPH oxidase-mediated pathways. We reported that leptin (0.1 to 1000 nmol/L) exhibits concentration-dependent negative-inotropic effects on myocyte contraction and intracellular Ca2⁺ release via a NO-dependent pathway. Our recent observation suggested involvement of Janus kinase/signal transducer and activator of transcription and p38 mitogen-activated protein kinase pathways in leptin-induced negative cardiac contractile response. It was also demonstrated that leptin activates fatty acid oxidation, decreases triglyceride content, and alters adenylyl cyclase function, which may regulate cardiac contractile function. Nevertheless, the mechanism underlying leptin-induced cardiomyocyte contractile depression is far from clear.

Our data suggested that intracellular O2•− generation and NADPH oxidase subunit expression (p67phox and p47phox) were
enhanced by leptin, the effect of which was cancelled off by the ET<sub>A</sub> and ET<sub>B</sub> receptor antagonists BQ123 and BQ788 but not the O<sub>2</sub><sup>-</sup> scavenger tempol. These data indicate that the ET-1 receptor is likely upstream of NADPH oxidase in leptin-induced cardiac contractile response. NADPH oxidase is composed mainly of 4 subunits; 2 of them are localized in the membrane (gp91<sub>phox</sub> and p22<sub>phox</sub>), and the others are in the cytosol (p47<sub>phox</sub> and p67<sub>phox</sub>).<sup>25</sup> Our study showed that leptin increased intracellular O<sub>2</sub><sup>-</sup> level and protein expression of p47<sub>phox</sub> and p67<sub>phox</sub> but not p22<sub>phox</sub> and gp91<sub>phox</sub>, suggesting the involvement of cytosolic subunits of NADPH oxidase in leptin-elicited activation of NADPH oxidase. Our data revealed that p47<sub>phox</sub> and p67<sub>phox</sub> were upregulated by leptin within a short time frame (60 minutes). Although this acute effect on protein expression is somewhat surprising, several studies have indicated that induction or upregulation of p47<sub>phox</sub> and p67<sub>phox</sub> protein may occur within the same time frame, as short as 15 minutes.<sup>26,27</sup> O<sub>2</sub><sup>-</sup> and ROS are known to cause myocyte dysfunction.<sup>28–30</sup> It should be mentioned that the ET<sub>A</sub> and ET<sub>B</sub> receptor antagonists significantly and independently attenuated leptin-induced O<sub>2</sub><sup>-</sup> generation, NADPH oxidase subunit (p47<sub>phox</sub> and p67<sub>phox</sub>) activation, and cardiac contractile response, indicating a permissive role of the 2 ET-1 receptors in leptin-induced cardiac effects. Additional study is warranted to elucidate the interaction between the ET<sub>A</sub> and ET<sub>B</sub> receptor in the cardiac function regulation of leptin.

Figure 4. Effect of leptin on O<sub>2</sub><sup>-</sup> generation using dihydroethidium fluorescent probe and the SOD-inhibitable reduction of cytochrome c in the absence or presence of BQ123, BQ788, and apocynin. (A) Control myocytes; (B) Leptin (50 nmol/L); (C) Leptin (100 nmol/L); (D) Leptin (100 nmol/L)+apocynin (100 μmol/L); (E) Leptin (100 nmol/L)+BQ123 (1 μmol/L); (F) Leptin (100 nmol/L)+BQ788 (1 μmol/L); (G) Apocynin (100 μmol/L); (H) BQ123 (1 μmol/L); and (I) BQ788 (1 μmol/L). J shows the summary of experiments of DHE fluorescent probe. K shows the summary of production of O<sub>2</sub><sup>-</sup> measured as the SOD-inhibitable reduction of cytochrome c. Mean±SEM, n=15 to 20 fields (total 150 to 200 cells) per group for J and n=4 assays for K; *P<0.05 vs control group; #P<0.05 vs 100 nmol/L leptin group.
the NADPH oxidase subunit, thus preventing upregulation of p47phox and p67phox. Nevertheless, additional study is warranted to elucidate the effect of leptin and ET-1, if any, on NADPH oxidase assembly and activation. Another interesting point is that apocynin may initiate a negative feedback on NADPH oxidase through stimulation (rather than inhibition) of ROS production. However, our finding that tempol failed to alter apocynin/leptin-elicited effect on p47phox and p67phox expression does not favor this feedback scenario. It was also indicated that apocynin is required to be converted into another compound by peroxidase and hydrogen peroxide to perform its inhibition on $O_2^{-}$ production. Therefore, the apocynin-elicited effects on NADPH oxidase and $O_2^{-}$ production may result from structurally different compounds. Last but not least, although the mechanism by which apocynin prevented leptin-induced upregulation of p47phox and p67phox is unclear, the finding from our current study was consistent with our earlier observation that the NADPH inhibitor was able to antagonize upregulation of p47phox as a result of prolonged high-glucose incubation (24 hours).19

**Perspectives**

Obesity leads to cardiac hypertrophy, ventricular dysfunction, reduced diastolic compliance, and a cluster of metabolic syndromes, including diabetes, hypertension, insulin resistance, and hyperlipidemia. To date, several theories have been postulated for obesity-associated cardiac dysfunctions, such as gene mutation, salt sensitivity, insulin sensitivity, sympathetic activation, and lifestyle factors, although none of these has been fully validated as the ultimate culprit for cardiac abnormalities in sustained obesity. Recent clinical and experimental evidence suggested overtly elevated plasma leptin levels in overweight or obese individuals. Although hyperleptinemia per se has been speculated to independently contribute to obesity-related ventricular dysfunction, the mechanism of action behind hyperleptinemia-induced cardiac response is essentially unknown. The findings from our current study have depicted that leptin suppresses cardiac contractile function and facilitates ROS generation in ventricular myocytes through ET-1 receptor and NADPH oxidase–mediated pathway(s). These observations are consistent with the notion that the adipokine stimulates local ET-1 production from a number of cell types, including cardiomyocytes and endothelial cells, ET-1 is known to affect cardiac function, and stimulate myocyte growth and myofibrillogenesis through ROS and activation of stress signaling–mediated mechanisms. Although it is premature to credit the detrimental cardiac effects of hyperleptinemia to ET-1 and its membrane receptors at this time, our findings that leptin may work its way through the ET-1 receptor and NADPH oxidase–dependent mechanisms to depress cardiomyocyte contractile function should shed some light on new therapeutic strategy against hyperleptinemia-associated cardiac dysfunction in obesity and metabolic syndrome.

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**References**


18. Zhao and Karissa LaCour for their assistance in data analysis.