Leptin Attenuates Cardiac Contraction in Rat Ventricular Myocytes
Role of NO

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Abstract—Obesity is commonly associated with impaired myocardial contractile function. However, a direct link between these 2 states has not yet been established. There has been an indication that leptin, the product of the human obesity gene, may play a role in obesity-related metabolic and cardiovascular dysfunctions. The purpose of this study was to determine whether leptin exerts any direct cardiac contractile action that may contribute to altered myocardial function. Ventricular myocytes were isolated from adult male Sprague-Dawley rats. Contractile responses were evaluated by use of video-based edge detection. Contractile properties analyzed in cells electrically stimulated at 0.5 Hz included peak shortening, time to 90% peak shortening, time to 90% relengthening, and fluorescence intensity change. Leptin exhibited a dose-dependent inhibition in myocyte shortening and intracellular Ca$^{2+}$ change, with maximal inhibitions of 22.4% and 26.2%, respectively. Pretreatment with the NO synthase inhibitor $N^\omega$-nitro-L-arginine methyl ester (L-NAME, 100 $\mu$mol/L) blocked leptin-induced inhibition of both peak shortening and fluorescence intensity change. Leptin also stimulated NO synthase activity in a time- and concentration-dependent manner, as reflected in the dose-related increase in NO accumulation in these cells. Addition of an NO donor ($S$-nitroso-$N$-acetyl-penicillamine [SNAP]) to the medium mimicked the effects of leptin administration. In summary, this study demonstrated a direct action of leptin on cardiomyocyte contraction, possibly through an increased NO production. These data suggest that leptin may play a role in obesity-related cardiac contractile dysfunction. (Hypertension. 2000;36:501-505.)

Key Words: hormones ■ myocytes ■ calcium ■ nitric oxide

Obesity is associated with an increased incidence of cardiovascular diseases, such as hypertension, stroke, and congestive heart failure. Although it appears that cardiac function may be normal or enhanced in the early stage of obesity, cardiac hypertrophy and compromised ventricular function develop as a result of the increased cardiac pressure and/or volume overload in obesity. The reduced ventricular function thus leads to impairment of ejection fraction/rate, fractional shortening, and diastolic compliance. The mechanism(s) responsible for the obesity-induced cardiac alteration remains uncertain but may be related to salt sensitivity, insulin resistance, and sympathetic activation.

Leptin, the product of the obesity gene (ob), is a peptide hormone expressed in adipose tissue. Leptin regulates body weight through the inhibition of food intake and promotion of energy expenditure. The leptin receptor has several alternatively spliced variants. One of which, the Ob-Rb variant, is believed to be functional and has been shown to exist in various tissues, including the heart. Recent studies have shown that leptin increases in insulin-resistant states, such as obesity and hypertension. Obesity and hyperinsulinemia are considered the major stimulators of leptin production. Nevertheless, no direct relationship between plasma leptin levels and cardiovascular function has been established. Leptin has been shown to increase heart rate and blood pressure through the stimulation of sympathetic nervous system activity. Furthermore, fasting plasma leptin levels are associated with increased myocardial wall thickness, independent of body composition and blood pressure levels. However, no evidence for a direct effect of leptin on cardiac contractile function has been reported. To address this possibility, we evaluated the effect of leptin on cell shortening, intracellular Ca$^{2+}$, and NO synthase (NOS) activity in myocytes isolated from adult rat ventricles.

Methods

Isolation of Ventricular Myocytes
The experimental procedures were approved by the animal investigation committee of the University of North Dakota. Single ventric-
ular myocytes were isolated from adult male Sprague-Dawley rats (200 to 225 g) as described previously. Briefly, the animals were euthanized, and their hearts were rapidly removed and perfused (at 37°C) with oxygenated (5% CO2/95% O2) Krebs-Henseleit bicarbonate (KHB) buffer (mmol: NaCl 118, KCl 4.7, CaCl2 1.25, MgSO4 1.2, KH2PO4 1.2, NaHCO3 25, HEPES 10, and glucose 11.1, pH 7.4). Hearts were subsequently perfused with a nominally Ca2+-free KHB buffer for 2 to 3 minutes until spontaneous contractions ceased, followed by a 20-minute perfusion with Ca2+-free KHB containing 223 U/mL collagenase (Worthington Biochemical Corp) and 0.1 mg/mL hyaluronidase (Sigma Chemical Co). After perfusion, the left ventricle was removed, minced, and incubated with the fresh enzyme solution (Ca2+-free KHB containing 223 U/mL collagenase) for 3 to 5 minutes. The cells were further digested with 0.02 mg/mL trypsin shortening (PS), time to 90% PS (TPS), time to 90% relengthening into an analog voltage signal. Cell shortening and to capture and convert changes in cell length during shortening and of 0.5 Hz, 3 ms in duration. A video-based edge detector was used HEPS 10, at pH 7.4. The cells were field-stimulated at a frequency followed by a 20-minute perfusion with Ca2+ buffer for 2 to 3 minutes until spontaneous contractions ceased, and their hearts were rapidly removed and perfused (at 37°C) with oxygenated (5% CO2/95% O2) Krebs-Henseleit bicarbonate- (KHB) buffer (mmol: NaCl 118, KCl 4.7, CaCl2 1.25, MgSO4 1.2, KH2PO4 1.2, NaHCO3 25, HEPES 10, and glucose 11.1, pH 7.4). Hearts were subsequently perfused with a nominally Ca2+-free KHB buffer for 2 to 3 minutes until spontaneous contractions ceased, followed by a 20-minute perfusion with Ca2+-free KHB containing 223 U/mL collagenase (Worthington Biochemical Corp) and 0.1 mg/mL hyaluronidase (Sigma Chemical Co). After perfusion, the left ventricle was removed, minced, and incubated with the fresh enzyme solution (Ca2+-free KHB containing 223 U/mL collagenase) for 3 to 5 minutes. The cells were further digested with 0.02 mg/mL trypsin shortening (PS) before being filtered through a nylon mesh (300 μm) and collected by centrifugation (60g for 30 seconds). Myocytes were resuspended in a sterilely filtered Ca2+-free KHB buffer containing (mmol) NaCl 131, KCl 4, MgCl2 1, HEPES 10, and glucose 10, supplemented with 2% BSA, with a pH of 7.4 at 37°C. Cells were initially washed with Ca2+-free KHB buffer to remove remnant enzyme, and extracellular Ca2+ was added incrementally back to 1.25 mmol/L. Isolated myocytes were maintained at 37°C in a serum-free medium consisting of medium 199 (Sigma) with Earle’s salts containing 25 mmol HEPES and NaHCO3 supplemented with 2% BSA, with a pH of 7.4 at 37°C. Cells were matin in a serum-free medium of medium 199 (Sigma) with Earle’s salts containing 25 mmol HEPES and NaHCO3 supplemented with BSA (2 mg/mL), L-carnitine (2 mmol/L), creatine (5 mmol/L), taurine (5 mmol/L), glucose (5 mmol/L), insulin (0.1 μmol/L), D-triiodothyronine (0.1 μmol/L), penicillin (100 U/mL), streptomycin (100 μg/mL), and gentamicin (100 μg/mL). Mechanical properties remained relatively stable in myocytes maintained for 12 to 24 hours in the serum-free medium. Cells were not used if they had any obvious sarcolemmal blebs or spontaneous contractions.

Cell Shortening/Relengthening Measurements
Mechanical properties of ventricular myocytes were assessed by using a video-based edge-detection system (IonOptix) as described. Briefly, coverslips with cells attached were placed in a chamber mounted on the stage of an inverted microscope (Olympus X-70) and superfused (~2 mL/min at 25°C) with a buffer containing (mmol/L) NaCl 131, KCl 4, CaCl2 1, MgCl2 1, glucose 10, and HEPES 10, at pH 7.4. The cells were field-stimulated at a frequency of 0.5 Hz, 3 ms in duration. A video-based edge detector was used to capture and convert changes in cell length during shortening and relengthening into an analog voltage signal. Cell shortening and relengthening were assessed by using the following indices: peak shortening (PS), time to 90% PS (TPS), time to 90% relengthening (TR90), and maximal velocities of shortening (+dL/dt) and relengthening (~dL/dt).

Intracellular Ca2+ Transient Measurement
For these experiments, myocytes were loaded with fura 2-AM (0.5 μmol/L) for 10 minutes at 25°C. Fluorescence measurements were recorded with a dual-excitation single-emission fluorescence photomultiplier system (IonOptix). Myocytes were placed on an inverted microscope and imaged through an Olympus Fluor 40x oil objective. Myocytes were exposed to light emitted by a 75-W halogen lamp through either a 360- or 380-nm filter while being stimulated to contract at 0.5 Hz. Fluorescence emissions were detected between 480 to 520 nm by a photomultiplier tube after initial illumination at 360 nm for 0.5 seconds and then at 380 nm for the duration of the recording protocol. The 360-nm excitation scan was repeated at the end of the protocol, and qualitative changes in intracellular Ca2+ concentration ([Ca2+]i) were inferred from the ratio of the fura fluorescence intensity (FFI) at both wavelengths. Fluorescence decay time (τ) was also measured as an indication of the intracellular Ca2+ clearing rate.

NO Assay
NOS activity was evaluated by the [1H]arginine to [1H]citrulline conversion assay. Briefly, plated ventricular myocytes (~300 000 per well) were placed in Hanks’ balanced salt solution (HBSS) medium (20 mmol/L HEPES, 1% penicillin-streptomycin, and 0.1% BSA) for 20 minutes at 37°C before being replaced with HBSS containing 1 μCi/mL [1H]arginine (Amersham Pharmacia Biotech, Inc) with Trasylol (0.2 KIU/mL, Mobay Pharmaceuticals) and leptin. The cells were then incubated for 60 minutes before the reaction was terminated by aspiration of the incubation medium and replacement with icod HBSS containing 5 mmol/L l-arginine and 4 mmol/L EDTA. Five minutes later, the termination medium was removed, and cells were lysed with 20 mL Triton (with 5 mmol/L l-arginine and 4 mmol/L EDTA). After sonication, the total lysate was centrifuged (600g at 4°C for 10 minutes). An aliquot of the supernatant was diluted with 1:1 (vol/vol) H2O/Dowex-50W (20-50 pore size, 8% cross-linked), mixed vigorously, and loaded on a polypropylene EconoColumn (BioRad Laboratories, Inc). The gel bed was washed 3 times with 2 mL distilled water, and all effluent was collected. [1H]Citrulline was counted by scintillation.

Experimental Protocols
Myocytes (either fura 2–loaded or nonloaded) were first allowed to contract at a stimulation frequency of 0.5 Hz for 10 minutes to ensure steady state (myocytes with rundown >10% were not studied further) before superfusion with leptin (0.1 to 1000 nmol/L, Sigma) for 15 minutes. Cells were then washed with normal contractile buffer for 5 minutes. In some studies, N-nitro-l-arginine methyl ester (L-NAME, 100 μmol/L, Sigma) was incubated with the myocytes for 15 minutes before leptin addition. The time-dependent response of leptin on NOS activity was determined after the myocytes had been incubated with 100 nmol/L leptin for 15, 30, 45, and 60 minutes. The dose-dependent response of leptin on NOS activity was determined after 60 minutes of incubation of the myocytes with leptin (0.1 to 1000 nmol/L).

Data Analysis
For each experimental series, data are presented as mean ±SEM. Statistical significance (P<0.05) for each variable was estimated by ANOVA or t test, as appropriate.

Results
Effect of Leptin on Myocyte Shortening (PS)
Acute exposure (up to 15 minutes) of leptin did not affect resting myocyte cell length over the range of concentrations tested. A representative trace depicting the effect of leptin (100 nmol/L) on myocyte shortening is shown in Figure 1A. At the end of a 15-minute exposure to this concentration of leptin, myocyte shortening was decreased by ~15%. Leptin exhibited little effect on the duration of shortening (TPS) and relengthening (TR90). Leptin (0.1 to 1000 nmol/L) elicited a concentration-dependent depression of myocyte shortening, with a maximal inhibition of 22.4% obtained at 1000 nmol/L. The concentration at which leptin displayed 50% maximal response (EC50) was 10.1 nmol/L. The depressive effect of leptin on cell shortening was maximal within 8 minutes of exposure and was partially reversible on washout (data not shown). The inhibitory effect of leptin was not associated with any impact on the duration of either TPS or TR90. The ±dL/dt values were not affected, with the exception of +dL/dt at the highest dose of leptin (Table).

Effect of Leptin on Intracellular Ca2+ Transients
To determine whether leptin-induced inhibition of myocyte shortening was due to the reduced availability of intracellular free Ca2+, [Ca2+]i, in response to electrical stimuli in the presence of various concentrations of leptin was examined. Representative traces of intracellular Ca2+ transients shown in
The present study demonstrated that the ob gene product leptin depresses ventricular myocyte shortening and intracel-

**Effect of Leptin on PS and Intracellular Ca^{2+} Transients in the Presence of L-NAME**

Leptin has been shown to increase serum levels of NO, an important regulator in the cardiovascular system. Constitutive NOS and inducible NOS are both present in cardiac myocytes. To examine the potential mechanism of action for leptin, the effect of leptin on PS and intracellular Ca^{2+} transients was reexamined in the presence of the NOS inhibitor L-NAME (100 μmol/L). L-NAME alone had no effect on PS and intracellular Ca^{2+} transients over 30 minutes (data not shown). As shown in Figures 1B and 2B, the leptin-induced decrease in both PS and intracellular Ca^{2+} transients was completely abolished by L-NAME. Leptin (10 nmol/L in the presence of L-NAME even elicited a small but significant positive effect on ΔFFI. These data suggest that leptin may exert its inhibition on PS and intracellular Ca^{2+} transients, at least in part, through NO production.

**Effect of Leptin on NOS Activity**

To further ensure the potential involvement of NO in leptin-induced cardiac contractile action, the effect of leptin on NOS activity was measured directly. Data presented in Figure 3 indicate that leptin elicited a concentration- and time-dependent increase in NOS activity in ventricular myocytes (Figure 3). Furthermore, incubation of myocytes with the NO donor S-nitroso-N-acetyl-penicillamine (SNAP, 10 to 100 μmol/L) for 15 minutes elicited a depression of myocyte shortening, indicating that elevated NO production is associated with the depression of cardiac contraction, as reported previously (Figure 4).

**Discussion**

The present study demonstrated that the ob gene product leptin...
Compromised cardiac systolic function has been reported in Zucker obese rat hearts\textsuperscript{27} and, recently, at the isolated ventricular myocyte level in the same animal model.\textsuperscript{28} It has been suggested that insulin resistance and decreased adrenergic responsiveness, including attenuated receptor density and postreceptor mechanisms,\textsuperscript{2,25,29} may contribute to the depressed cardiac contractile function in obesity. Evidence has suggested that the satiety factor secreted by adipose tissue, leptin, may be a link between adiposity and insulin resistance,\textsuperscript{25} inasmuch as there is a close association between hyperleptinemia and hyperinsulinemia.

Leptin has been demonstrated to induce proliferation, differentiation, and functional activation of hemopoietic and embryonic cells.\textsuperscript{30–32} Therefore, one could hypothesize that leptin might also play a role in the functional activation of the cell at the myocardial level. Administration of leptin has been shown to increase renal, adrenal, and lumbar sympathetic nerve activity.\textsuperscript{33} However, this generalized sympathoexcitatory activity is not always followed by an increase in arterial pressure. This has been credited, to a certain extent, to the possibility that the leptin-induced release of NO may contribute to the homeostasis of the cardiovascular system.\textsuperscript{20} The fact that L-NAME inhibition unmasked a positive response of leptin (100 nmol/L) on intracellular Ca\textsuperscript{2+} transients, observed in the present study, may support the notion of a direct sympathetic effect by leptin. Ambient NO levels have been shown to modulate cardiac contractile function. Constitutive and inducible NOS are present in cardiac myocytes.\textsuperscript{21,22} The data in the present study indicate that leptin increases NOS activity in cardiac myocytes. This may lead directly to depression of cardiac cell contraction. Further study is warranted to determine the involvement of specific isoforms of constitutive NOS (neuronal or endothelial NOS) in leptin-induced cardiac response.

One important defect of cardiac contraction in obesity is the decrease in diastolic compliance and prolonged relaxation.\textsuperscript{4,26,34} This prolongation may be related to the ventricular hypertrophy–induced reduction of sarcoplasmic Ca\textsuperscript{2+} uptake.\textsuperscript{4} Results from the present study revealed that acute leptin exposure did not affect the duration of shortening and relengthening. This indicates that other factors may contribute to the obesity-induced cardiac dysfunction, although whether long-term exposure of leptin affects the intracellular Ca\textsuperscript{2+} clearing mechanisms, such as sarcoplasmic Ca\textsuperscript{2+}-ATPase and the Na\textsuperscript{+}-Ca\textsuperscript{2+} exchanger, remains to be determined.

There has been some debate regarding the role of leptin in cardiac disease. Serum leptin levels may be different according to the clinical stage of the heart problem, ie, an early- to mid-stage increase and an end-stage decline, specifically in patients with cachexia. Multiple factors have been implicated in the regulation of leptin, many of which are subject to endocrine and metabolic influences themselves. The increased leptin levels in early-stage cardiac problems and decreased levels in cachectic end-stage problems may be related to the predominant decline of muscle mass during the initial phases of the disease, with subsequent reduction of the lean/fat ratio. The reduction in this ratio directly promotes leptin production. The decline in leptin levels that occurs in
cachexia with advanced disease may be due to an additional decline in adipose tissue mass accompanied by the loss of body weight. Whether abnormal leptin or its receptor is implicated in the pathogenic process of cardiac diseases or, more likely, is a result of the cardiac and metabolic derangement needs to be further clarified.

In conclusion, the present study demonstrates, for the first time, the cardiac depressive action of leptin. Although these data provide a small step toward elucidating the role of leptin in obesity-related cardiac dysfunction, the role of leptin in mediating the autonomic, cardiovascular, renal, and endocrine changes associated with increased adiposity is still unclear and deserves further study.

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References