Abrogated Leptin-Induced Cardiac Contractile Response in Ventricular Myocytes Under Spontaneous Hypertension: Role of JAK/STAT Pathway

Loren E. Wold, David P. Relling, Jinhong Duan, Faye L. Norby, Jun Ren

Abstract—Leptin regulates cardiovascular function. Leptin levels are elevated in obesity and hypertension and may play a role in cardiovascular dysfunctions in these comorbidities. This study was designed to determine the influence of hypertension on the cardiac contractile response of leptin. Mechanical and intracellular Ca\textsuperscript{2+} properties were evaluated using an IonOptix system in ventricular myocytes from spontaneously hypertensive (SHR) and age-matched Wistar Kyoto (WKY) rats. The contractile properties included peak shortening (PS), duration and maximal velocity of shortening/relengthening (TPS/TR\textsubscript{90}, \textpm dL/dt), and fura-fluorescence intensity change (AFFI). NO and nitric oxide synthase (NOS) activity were assessed by the Griess and the \textsuperscript{3}H-arginine/citrulline conversion assays, respectively. The leptin receptor (Ob-R) and the Janus kinase/signal transducer and activator of transcription (JAK/STAT) signaling pathway were evaluated by Western blot analysis. SHR animals displayed significantly elevated blood pressure and plasma leptin levels. Leptin elicited a concentration-dependent inhibition of PS and AFFI in WKY, but not in SHR myocytes. Leptin did not affect TPS, TR\textsubscript{90}, or \textpm dL/dt. The difference in leptin-induced contractile response between the WKY and the SHR groups was abolished by the NOS inhibitor, N\textsuperscript{\textminus}-nitro-L-arginine methyl ester (L-NAME), but not by elevated extracellular Ca\textsuperscript{2+}. Either the JAK2 inhibitor AG-490 or the mitogen-activated protein (MAP) kinase inhibitor SB203580 abrogated the leptin-induced response in the WKY myocytes, whereas AG-490 unmasked a negative response in PS in the SHR myocytes. SHR myocytes displayed similar Ob-R protein abundance and basal NO levels, a blunted leptin-induced increase in NOS activity as well as enhanced basal STAT3 levels compared with the WKY group. These data indicate that the leptin-induced cardiac contractile response is abolished by spontaneous hypertension, possibly because of mechanisms involving altered JAK/STAT, MAP kinase signaling, and NO response. (Hypertension. 2002;39:69-74.)

Key Words: signal transduction ■ hypertension, obesity ■ myocytes ■ cardiac function ■ kinase ■ nitric oxide

Epidemiological evidence revealed that over 60% of US adults are considered overweight.\textsuperscript{1,2} Obesity is shown to predispose the development of various comorbidities such as hypertension, diabetes, and dyslipidemia.\textsuperscript{2-3} Both clinical and experimental evidence has implicated hypertension as the most common comorbidity associated with obesity.\textsuperscript{2} Although hypertension and obesity often exist concurrently, leading to ventricular dysfunction, obesity itself is known to enhance the development of other risk factors, such as hypertension and dyslipidemia.\textsuperscript{4,5}

Leptin, the obesity gene (ob) product, regulates energy metabolism and expenditure. Leptin enhances sympathetic nervous activity,\textsuperscript{6} which may be directly associated with increased body fat composition.\textsuperscript{3-9} Leptin infusion was shown to reduce blood pressure and heart rate, which may be reversed by nitric oxide synthase (NOS) inhibition.\textsuperscript{10,11} Recent evidence indicates that leptin stimulates NO in the cardiovascular system.\textsuperscript{11,12} The signaling pathway underlying the cardiovascular regulation of leptin has not been well defined, although evidence has suggested that leptin may interact with its specific leptin receptors (Ob-Rs)\textsuperscript{13} and act through the Janus kinase (JAK) and signal transducers and activators of transcription (STAT)\textsuperscript{13,14} or mitogen-activated protein (MAP) kinase pathways.\textsuperscript{15}

Leptin levels have been shown to be elevated in hypertension, suggesting a potential link between hyperleptinemia and cardiovascular dysfunction in hypertension.\textsuperscript{16,17} However, current knowledge of the relationship between leptin and hypertension has been primarily limited to the vascular system. The aim of this study was to examine the influence of hypertension on leptin-induced cardiac contractile response and the potential signaling mechanisms in isolated left ventricular myocytes from spontaneously hypertensive rats (SHR) and age-matched normotensive Wistar-Kyoto (WKY) rats.
Effect of Leptin on Baseline Mechanical Characteristics in Cells From Adult WKY and SHR Rat Hearts

<table>
<thead>
<tr>
<th>Cell Group</th>
<th>TPS (ms)</th>
<th>TR 0 (ms)</th>
<th>+dL/dt (μm/s)</th>
<th>−dL/dt (μm/s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WKY, baseline</td>
<td>231.0±20.9</td>
<td>322.2±25.0</td>
<td>99±14</td>
<td>−78±10</td>
</tr>
<tr>
<td>Leptin, 1 nmol/L</td>
<td>254.4±19.3</td>
<td>349.3±25.0</td>
<td>97±15</td>
<td>−91±11</td>
</tr>
<tr>
<td>Leptin, 10 nmol/L</td>
<td>250.4±22.7</td>
<td>345.2±34.5</td>
<td>78±8</td>
<td>−77±10</td>
</tr>
<tr>
<td>Leptin, 100 nmol/L</td>
<td>231.5±17.4</td>
<td>282.3±37.0</td>
<td>70±7</td>
<td>−75±8</td>
</tr>
<tr>
<td>Leptin, 500 nmol/L</td>
<td>254.4±21.9</td>
<td>317.1±34.5</td>
<td>77±9</td>
<td>−67±8</td>
</tr>
<tr>
<td>Leptin, 1000 nmol/L</td>
<td>224.2±24.0</td>
<td>318.5±31.4</td>
<td>63±9</td>
<td>−56±7</td>
</tr>
<tr>
<td>SHR, baseline</td>
<td>140.8±10.8</td>
<td>267.2±26.8</td>
<td>88±16</td>
<td>−104±18</td>
</tr>
<tr>
<td>Leptin, 1 nmol/L</td>
<td>163.4±9.6</td>
<td>304.8±35.6</td>
<td>88±20</td>
<td>−88±15</td>
</tr>
<tr>
<td>Leptin, 10 nmol/L</td>
<td>169.5±11.7</td>
<td>298.2±32.9</td>
<td>105±23</td>
<td>−102±18</td>
</tr>
<tr>
<td>Leptin, 100 nmol/L</td>
<td>163.7±13.5</td>
<td>270.7±32.9</td>
<td>84±17</td>
<td>−102±17</td>
</tr>
<tr>
<td>Leptin, 500 nmol/L</td>
<td>159.0±10.6</td>
<td>294.9±43.0</td>
<td>114±29</td>
<td>−110±18</td>
</tr>
<tr>
<td>Leptin, 1000 nmol/L</td>
<td>141.5±11.6</td>
<td>280.5±43.8</td>
<td>91±17</td>
<td>−115±20</td>
</tr>
</tbody>
</table>

TPS indicates time-to-90% PS; TR 0 , time-to-90% relengthening; +dL/dt, maximal velocities of shortening and relengthening. Data represent mean±SEM. n=27 for both WKY and SHR groups.

*P<0.05 vs WKY group.

Methods

Experimental Animals and Leptin Assay

The experimental procedures were approved by the Animal Investigation Committee of the University of North Dakota. WKY and SHR rats, 20 to 30 weeks old, were obtained from Harlan Laboratories (Indianapolis, IN) and maintained on a 12/12-hour light/dark illumination cycle and allowed food and water ad libitum. Blood pressure was obtained by the tail-cuff method (ITTC Inc). Plasma was collected when the animal was euthanized, and a leptin radioimmunoassay kit (Linco Research) was used to determine plasma leptin levels.

Isolation of Ventricular Myocytes and Cell Shortening/Relengthening Measurements

Ventricular myocytes were isolated as described and the mechanical properties of myocytes were assessed using a IonOptix MyoCam system. Cell mechanics were assessed using the following indices: shortening (PS), time-to-90% PS (TPS) and time-to-90% relengthening (TR0), maximal velocities of shortening (+dL/dt) and relengthening (−dL/dt).

Intracellular Ca2+ Transient Measurement

Myocytes were loaded with fura-2-AM (0.5 μmol/L) for 10 minutes at 25°C and fluorescence was measured with a dual-excitation single-emission fluorescence photomultiplier system (IonOptix) as described previously.12 Fluorescence decay time (τ) was calculated as an indicator of the intracellular Ca2+ clearing rate.12

NO and NOS Activity

Basal NO levels were determined by the Griess assay.18 NOS activity was evaluated by the [3H]-arginine to [3H]-citrulline conversion assay as described.12

Western Blot for Ob-R and STAT3

Monoclonal antibody to Ob-R and polyclonal antibody STAT3 were purchased from Santa Cruz Biotechnology, Inc. Western blot analysis was performed on ventricular myocytes treated for 15 minutes with or without leptin (500 nmol/L) as previously described.19

Data Analysis

Data are mean±SEM. Statistical significance (P<0.05) for each variable was estimated by t test or two-way ANOVA followed by a Dunnett’s post hoc analysis.

Results

Experimental Animals and Plasma Leptin Level

There was no significant difference in body weight (WKY, 400±8 g versus SHR, 404±10 g, n=12 per group, P>0.05) or heart-to-body weight ratio (WKY, 4.41±0.21 mg/g versus SHR, 4.32±0.15 mg/g, n=12 per group, P>0.05) between groups. The SHR rats exhibited significantly elevated systolic blood pressure (WKY, 116±2 mm Hg versus SHR, 184±3 mm Hg, n=12 per group, P<0.05) and plasma leptin levels (WKY, 3.15±0.09 ng/mL versus SHR, 6.65±0.30 ng/mL, n=12 per group, P<0.05) associated with reduced liver-to-body weight ratio (WKY, 33.1±1.0 mg/g versus SHR, 26.6±0.3 mg/g, n=12 per group, P<0.05) compared with the WKY counterparts. There was no difference in other biometric measures between the WKY and SHR rats.

Baseline Mechanical and Fluorescence Properties of WKY and SHR Myocytes

The average resting cell length (CL) for myocytes from WKY and SHR groups was 102.8±3.8 μm (n=120) and 131.8±6.7 μm (n=115), respectively (P<0.001). The peak cell shortening (PS) was significantly depressed in SHR myocytes compared with the WKY group (3.3±0.4% versus 6.0±0.8% of CL, respectively, P<0.001). SHR myocytes displayed a significantly shorter TPS compared with the WKY group.

Effect of Leptin on Myocyte PS

Acute exposure (up to 15 minutes) of leptin did not affect CL. A representative trace depicting the effect of leptin on myocyte shortening is shown in Figure 1A and 1B. Leptin
decreased PS by 23.4±8.6% in WKY, but not in SHR myocytes. Figure 1C shows that leptin induced a concentration-dependent depression of PS in the WKY group with a maximal inhibition of 35.7%. The effect of leptin on PS was maximal within 8 minutes and was reversible on washout (data not shown). However, the leptin-induced depression in PS was blunted in SHR. TPS, TR90, and dL/dt were not affected by leptin in either myocyte group (Table).

Effect of Leptin on PS in the Presence of L-NAME and Elevated Extracellular Ca²⁺
Constitutive NOS (cNOS) and inducible NOS (iNOS) are present in cardiac myocytes. We have shown that leptin increased NOS activity in ventricular myocytes. The NOS inhibitor Nω-nitro-arginine methyl ester (L-NAME, 100 μmol/L) alone had no effect on PS (data not shown). As shown in Figure 2A, the leptin-induced decrease in PS was abolished by preincubating cells with L-NAME for 15 minutes in myocytes from the WKY group, suggesting that leptin may exert its inhibition on PS, at least in part, through NO production. Furthermore, incubation of the myocyte with the NO donor DETA NONOate (100 μmol/L) for 15 minutes elicited a depression of myocyte shortening (11.5±6.6% versus 18.0±6.9% in WKY and SHR myocytes, respectively, n=24 cells/group), indicating elevated NO production is associated with depression of cardiac contraction as reported previously. To evaluate the role of enhanced Ca²⁺ influx on leptin-induced cardiac contractile depression, extracellular Ca²⁺ was increased from 1.0 mmol/L to 2.7 mmol/L and the leptin response on cell shortening was re-examined. Figure 2B shows that the attenuated response to leptin in hypertension is not affected by an increase in extracellular Ca²⁺ concentration.

Effect of Leptin on Intracellular Ca²⁺ Transients
To determine whether leptin-induced inhibition of myocyte shortening was due to reduced availability of intracellular free Ca²⁺ ([Ca²⁺]), [Ca²⁺], was examined in response to electrical stimuli in the presence of leptin (1 to 1000 nmol/L). Representative traces of intracellular Ca²⁺ transients shown in Figure 3A show that 100 nmol/L leptin had no significant effect on FFI in SHR myocytes. Leptin elicited a concentration-dependent inhibition of FFI, with a maximal inhibition of 16.04% in WKY myocytes; however, there was no effect in the concentration range tested of leptin on SHR myocytes (Figure 3B). Neither resting FFI nor was affected by leptin in either rat group (data not shown).

Effect of Leptin on NO and NOS Activity
Leptin elicited a concentration-dependent increase in NOS activity in myocytes from the WKY group. However, the response was blunted in the SHR group compared with that of the WKY group (P<0.05 at 500 nmol/L) (Figure 4). There was no difference in basal NO levels between WKY (1.10±0.66 nmol/μg protein, n=6 samples) and SHR (0.78±0.17 nmol/μg protein, n=6 samples) myocytes.

Effect of Leptin on PS in the Presence of AG-490 and SB203580
Both JAK/STAT and MAP kinase pathways have been shown to be involved in the signaling of leptin. To determine whether JAK2 and MAP kinase were involved in the leptin-induced cardiac contractile response, the effect of leptin on PS was re-examined by preincubating the cells with the JAK2 inhibitor AG-490 (20 μmol/L) or the MAP kinase inhibitor SB203580 (20 μmol/L) for 15 minutes. Neither inhibitor alone had any effect on PS. Interestingly, the
leptin-induced decrease in PS in WKY myocytes was completely abolished by either inhibitor, suggesting that leptin may exert its inhibition on PS through JAK2 and/or MAP kinase pathways. However, preincubation of SHR myocytes with AG-490 unmasked a significant depressive response to leptin. Inhibition of MAP kinase pathway with SB203580 unmasked a negative response in PS at 500 nmol/L leptin, although not at 1000 nmol/L (Figure 5).

Figure 3. Effect of leptin on intracellular Ca\(^{2+}\) transient changes. A, Typical experiment showing the effect of leptin (100 nmol/L) on intracellular Ca\(^{2+}\) transients from SHR ventricular myocytes. Solid and dashed traces show Ca\(^{2+}\) transients before and 10 minutes after leptin exposure. B, Concentration-dependent response of leptin (1 to 1000 nmol/L) to intracellular Ca\(^{2+}\) transient changes (ΔFFI). ΔFFI is expressed as the percent change of the respective baseline value. Values are mean±SEM. *P<0.05 vs baseline value.

Figure 4. Concentration-dependent response of leptin (1 to 1000 nmol/L) on NOS activity in myocytes from adult WKY and SHR rat hearts. The incubation time was 20 minutes. Values are mean±SEM. n=10 assays. #P<0.05 vs WKY group.

Leptin Receptor and Leptin-Induced STAT3 Tyrosine Phosphorylation
As shown in Figure 6, Western blot analysis revealed that the Ob-R protein abundance was similar between the WKY and SHR myocytes, suggesting the attenuated leptin-induced cardiac contractile response was unlikely caused by the loss of Ob-R in hypertension. Interaction between leptin and Ob-R has been shown to specifically activate members of JAK family of cytoplasmic tyrosine kinase and phosphorylate: STAT1, STAT3, STAT5, and STAT6.22,23 Our results indicate that leptin activated STAT3 in both WKY and SHR myocytes. However, basal STAT3 was substantially higher in the SHR myocytes than in the WKY myocytes.

Discussion
This study demonstrated that the leptin-induced depression of PS and intracellular Ca\(^{2+}\) transients in ventricular myocytes was abrogated by hypertension. The leptin-induced inhibition in myocyte contraction was prevented by pretreatment with the nonselective NOS inhibitor L-NAME, the JAK2 inhibitor AG-490, and the MAP kinase inhibitor SB203580, but unaffected by elevated extracellular Ca\(^{2+}\). Although basal NO levels were comparable between the two groups, the leptin-induced increase of NOS activity was blunted by hypertension. In addition, the Ob-R levels were not different between
Our data revealed that the leptin-induced cardiac contractile response is blunted by spontaneous hypertension. The comparable Ob-R protein levels between the WKY and the SHR myocytes favor a post-receptor mechanism responsible for the disparate response to leptin, although altered receptor binding may not be ruled out. Ob-R is a protein containing a single transmembrane domain similar to the Class I cytokine receptors. The long-tail intracellular peptide motifs in Ob-R may interact with a specific kinase, such as tyrosine kinase, to promote transcription through the STAT pathway. Our study revealed, for the first time, that leptin induces activation of STAT3 in the cardiac myocytes similar to its action in the neuroendocrine system. The observation that the JAK2 inhibitor AG-490 prevented the leptin-induced depression in myocyte contraction further supports the role of JAK/STAT signaling in cardiac actions of leptin. The enhanced basal STAT3 phosphorylation in SHR myocytes may be essential in that it masked the leptin response in hypertension (thus additional activation failed to work). In addition, MAP kinase and phosphatidylinositol-3 (PI-3) kinase pathways have also been implicated in the signaling of leptin action. The observation that the MAP kinase inhibitor SB203580 blocked leptin-induced cardiac response lends proof to this notion, although its relevance to the JAK/STAT signaling pathway remains to be determined.

In this study, leptin-induced cardiac contractile response was abolished by the NOS inhibitor L-NAME, which is consistent with the ability of leptin to stimulate NOS activity. Interestingly, the capacity for leptin to stimulate NOS activity was blunted in SHR myocytes. In genetic hypertension (SHR), NOS activity is enhanced, although the basal NO levels may be unaltered, as indicated in this study. The increased NOS may shadow the leptin action, or may not be effectively sensed by the cardiovascular system, largely because of the increased deactivation of NO. Activation of JAK/STAT pathway has been shown to stimulate NOS activity, which is consistent with the enhanced basal JAK/STAT activity and enhanced NOS activity in SHR myocytes. Also, PI-3 kinase, an upstream signaling molecule to NO, may be affected by hypertension and play a role in the abrogated leptin response in the SHR group. The PI-3 pathway has been implicated in leptin signaling. Finally, hyperleptinemia and impaired receptor function have been confirmed in obesity, although its role in the cardiovascular dysfunction in hypertension is not clear.

The loss of leptin-induced cardiac response in spontaneous hypertension may have significant pathophysiological relevance. It may be a compensatory mechanism to prevent further ventricular function impairment in sustained hypertension or hyperleptinemia. Although the present study sheds some light on the role of hyperleptinemia in cardiovascular function in hypertension, the mechanism underlying the pathophysiological role of leptin in hypertension still deserves further study. Although hyperleptinemia appears to be associated often with cardiac hypertrophy, further study is warranted to determine whether hyperleptinemia itself is a cause of the change in the structure of the heart.

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