Leptin Induces Hypertrophy via Endothelin-1–Reactive Oxygen Species Pathway in Cultured Neonatal Rat Cardiomyocytes

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Background—Obesity is a major risk factor for the development of cardiovascular disease. Emerging evidence indicates that leptin, a protein encoded by the obese gene, is linked with cardiac hypertrophy in obese humans and directly induces cardiomyocyte hypertrophy in vitro. However, the mechanisms by which leptin induces cardiomyocyte hypertrophy are poorly understood.

Methods and Results—This study investigated how leptin contributes to cardiomyocyte hypertrophy. Cultured neonatal rat cardiomyocytes were used to evaluate the effects of leptin on hypertrophy. Both endothelin-1 (ET-1) and reactive oxygen species (ROS) levels were elevated in a concentration-dependent manner in cardiomyocytes treated with leptin for 4 hours compared with those cells without leptin treatment. ET-1 stimulated ROS production in a concentration-dependent manner in cardiomyocytes. The augmentation of ROS levels in cardiomyocytes treated with both leptin and ET-1 was reversed by a selective ETA receptor antagonist, ABT-627, and catalase, a hydrogen peroxide–decomposing enzyme. After treatment for 72 hours, leptin or ET-1 concentration-dependently increased total RNA levels, cell surface areas, and protein synthesis in cardiomyocytes, all of which were significantly inhibited by ABT-627 or catalase treatment.

Conclusions—These findings indicate that leptin elevates ET-1 and ROS levels, resulting in hypertrophy of cultured neonatal rat cardiac myocytes. The ET-1–ET A–ROS pathway may be involved in cardiomyocyte hypertrophy induced by leptin. ET A receptor antagonists and antioxidant therapy may provide an effective means of ameliorating cardiac dysfunction in obese humans. (Circulation. 2004;110:1269-1275.)

Key Words: hypertrophy ■ endothelin ■ leptin ■ stress

Obesity is a major risk factor for the development of cardiovascular diseases (CVD), such as hypertension, atherosclerosis, and heart failure.1,2 Leptin, the product of the ob gene, is a plasma protein secreted by adipocytes and other cells and is involved in the regulation of appetite and energy metabolism.3,4 It has also been shown that leptin may contribute to cardiovascular diseases.5,6 Animal studies indicate that long-term administration of leptin results in a sustained increase in arterial pressure.7,8 Clinical evidence has implicated leptin as a potential independent risk factor for coronary heart disease, and increased plasma leptin levels are correlated with cardiac hypertrophy and congestive heart failure.9–11 A recent study supplied evidence that leptin directly induces hypertrophy in rat neonatal cardiomyocytes in vitro.12 However, the mechanism(s) underlying leptin-induced cardiomyocyte hypertrophy is unclear.

It has been well documented that reactive oxygen species (ROS) play an important role in the development of cardiac hypertrophy.13–17 Our laboratory and others have shown that hypertrophic substances such as angiotensin II (Ang II),14,15 norepinephrine (NE),16 and endothelin-1 (ET-1)17 induce cardiomyocyte hypertrophy by stimulating generation of ROS, an effect that can be inhibited by pretreatment with an antioxidant. Several other studies have indicated that leptin is also able to stimulate generation of ROS and ET-1 in endothelial cells.18–20 Therefore, we hypothesized that leptin induces cardiomyocyte hypertrophy via the ET-1–ROS pathway. Our results suggest that leptin stimulates ET-1 production and induces hypertrophy via ET-1–ROS generation in cultured neonatal rat cardiomyocytes. Treatment with ETA receptor antagonist ABT-627 or the antioxidant catalase inhibited leptin-
induced ROS production and hypertrophy in cultured neonatal rat cardiomyocytes.

**Methods**

**Cell Culture**

Primary culture of cardiomyocytes was performed according to previous methods. Briefly, hearts from 1-day-old Sprague-Dawley rats were dissected, minced, and placed in a Petri dish. The tissue was trypsinized at 37 °C in a D-Hanks’ balanced salt solution (HBSS; in g/L: 8.00 NaCl, 0.4 KCl, 0.06 KHPO₄, 0.35 NaHCO₃, 0.09 NaH₂PO₄·H₂O, and 0.125% trypsin). After centrifugation (1000 rpm, 15 minutes), cells were collected and suspended in M199 medium (containing 10% FCS, 5 μg/mL insulin, 5 μg/mL transferrin, 100 U/mL penicillin, and 100 μg/mL streptomycin). Cells were then preplated at 37 °C for 1 hour. The cells were diluted to 5 × 10⁶ cells/mL, plated in 96-well, 24-well, or 6-well plates, and cultured for 48 to 64 hours in a medium containing 0.1 mmol/L bromodeoxyuridine to prevent proliferation of nonmyocytes. After being cultured in a nonserum medium for 72 hours, cells were washed twice with HBSS at 37 °C. Washed cells were incubated for 4 hours or for 72 hours in a nonserum medium containing leptin (1 to 1000 ng/mL, R & D Systems), leptin (100 ng/mL) plus ABT-627 (3 × 10⁻⁸ mol/L, Abbott Laboratories), and a selective ET receptor antagonist or leptin (100 ng/mL) plus catalase (200 U/mL, Sigma), an enzyme that specifically decomposes hydrogen peroxide (H₂O₂) to water and molecular oxygen.

**Reactive Oxygen Species**

Intracellular ROS was assessed by the ROS-specific probe 2’,7’-dichlorofluorescein diacetate (DCF-DA, Molecular Probes, Inc.). On culture day 4, the cultured cardiomyocytes were washed with HBSS, and then incubated with DCF-DA (5 μmol/L) in HBSS at 37 °C. After incubation for 1 hour, cardiomyocytes were again washed with HBSS. Fluorescent signals were obtained with a fluorescence conversion microscope (Nikon TE300-ECI) and assayed by its image processing and analysis system. In each case, 5 randomly selected fields in each well were selected for examination.

**RNA Content**

The RNA content was determined by the RNA-sensitive fluorescence probe propidium iodide (PI) after DNase treatment. Cardiomyocytes were treated with diluent (control), leptin (1 to 1000 ng/mL), leptin (100 ng/mL) plus ABT-627 (3 × 10⁻⁸ mol/L), and the amplification of GAPDH mRNA, a standard for RT-PCR analysis. A sense primer 5'-AAGGTGCGGTA-TCACCCCCATTTGGCCGT-3' and antisense primer 5'-CAGTGTGGGATCCCTGAGTGTC-3' were used. Amplification was performed over 23 cycles, each involving 1 minute at 94 °C, 1.5 minutes at 59 °C, and 2 minutes at 72 °C.

**Protein Content**

Cultured cardiomyocytes were treated with leptin, ET-1, ABT-627, antioxidant catalase, or diluent (control) from days 4 to 7 of culture. The cells were washed with PBS and then treated with 5% trichloroacetic acid (TCA; Sigma) at 4 °C for 1 hour to precipitate the protein. The precipitates were dissolved in NaOH (0.1N). The protein content was measured with the Bio-Rad DC protein assay.

**Reverse Transcription–Polymerase Chain Reaction**

Total RNA was isolated from cultured cardiomyocytes using TRIzol reagent (Invitrogen). The atrial natriuretic peptide (ANP) mRNA was analyzed by reverse transcription–polymerase chain reaction (RT-PCR) using primers specific for ANP (sense: 5'-GGGCTCTTCTTCTACACC-3', and antisense: 5'-CTCCAATTCCTGCAATCTCTACC-3'). ANP PCR amplification was performed for 27 cycles at 94 °C for 20 seconds, 55 °C for 15 seconds, and 72 °C for 30 seconds. The amplification of GAPDH mRNA, a constitutively and ubiquitously expressed gene, served as an internal standard for RT-PCR analysis. A sense primer 5'-AAGGTGCGGTA-TCACCCCCATTTGGCCGT-3' and antisense primer 5'-CAGTGTGGGATCCCTGAGTGTC-3' were used. Amplification was performed over 23 cycles, each involving 1 minute at 94 °C, 1.5 minutes at 59 °C, and 2 minutes at 72 °C.

**Incorporation of [3H]Leucine**

[3H]leucine incorporation was measured as described previously. Cultured cardiomyocytes were treated with leptin, ET-1, ABT-627, antioxidant catalase, or diluent (control) and coincubated with [3H]leucine (1.5 μCi/mL, 50 Ci/mmol, Amersham) from days 4 to 7 of culture. The medium was aspirated, and the cells were washed with PBS and then treated with 5% TCA at 4 °C for 1 hour to precipitate the protein. After 2 washings with cold 95% ethanol, radioactivity incorporated into the TCA-precipitable material was determined by liquid scintillation counting after solubilization in 0.1N NaOH.

**ET-1 Immunoassay**

ET-1 levels in cardiomyocytes were determined with a chemiluminescence-based immunoassay with a commercial kit (R & D Systems Inc.). Homogenates from the cultured cardiomyocytes were centrifuged at 20,000g for 30 minutes at 4 °C, and the supernatant was assayed for ET-1 content.

**Statistical Analysis**

All results are expressed as mean ± SEM. One-way ANOVA was used for multiple comparisons. A value of P < 0.05 was considered significant.

**Results**

**Effect of Leptin on Hypertrophy in Cultured Cardiomyocytes**

After 72 hours of treatment, leptin (1 to 1000 ng/mL) increased total RNA levels, [3H]leucine incorporation, and cell surface area in cultured cardiomyocytes in a concentration-dependent manner. Treatment with the ETA receptor antagonist ABT-627 (3 × 10⁻⁸ mol/L) or catalase (200 U/mL) significantly inhibited the effect of leptin on hypertrophy (Figure 1, B–F). Leptin (100 ng/mL) also enhanced ANP mRNA levels in cultured cardiomyocytes, an effect that was inhibited by ABT-627 (3 × 10⁻⁸ mol/L) or catalase (200 U/mL) treatment (Figure 1A).

**Effect of Leptin on ET-1 Generation in Cultured Cardiomyocytes**

To study the potential effect of leptin on ET-1 levels, cultured cardiomyocytes were subjected to different concentrations of leptin (1 to 1000 ng/mL) treatment for 4 hours. Leptin treatment induced augmentation of ET-1 levels in a concentration-dependent manner in cultured cardiomyocytes compared with those cells without leptin treatment (Figure 2).
Effect of Leptin on Intracellular ROS Production in Cultured Cardiomyocytes

After treatment for 4 hours, leptin (1 to 1000 ng/mL) increased ROS levels of cultured cardiomyocytes in a concentration-dependent manner, an effect that was mitigated by treatment with the ET<sub>A</sub> receptor antagonist ABT-627 (3 × 10<sup>-5</sup> mol/L) or catalase (200 U/mL) (Figure 3, A and B).
Effect of ET-1 on Hypertrophy in Cultured Cardiomyocytes
Total RNA levels, cell surface area, [3H]leucine incorporation, and protein content of cultured cardiomyocytes were significantly enhanced in a concentration-dependent manner after treatment with ET-1 (10^{-10} to 10^{-8} mol/L) for 72 hours. Treatment with ET_A receptor antagonist ABT-627 (3×10^{-8} mol/L) or catalase (200 U/mL) markedly inhibited the effect of ET-1 on hypertrophy in cultured cardiomyocytes (Figure 4, A–D).

Effect of ET-1 on Intracellular ROS Production in Cultured Cardiomyocytes
ROS levels of cultured cardiomyocytes treated with ET-1 (10^{-10} to 10^{-8} mol/L) increased in a concentration-dependent manner. Pretreatment with ABT-627 (3×10^{-8} mol/L), a selective ET_A receptor antagonist, significantly reversed the effect of ET-1 on ROS production (Figure 5, A and B). ROS generation induced by ET-1 in cultured cardiomyocytes was also decreased by treatment with antioxidant catalase (200 U/mL) (Figure 5, A and B).

Discussion
The present study demonstrates for the first time that (1) leptin stimulates ET-1 production and (2) leptin concentration-dependently induces ROS generation and hypertrophy, both of which were significantly inhibited by pretreatment with ABT-627, a selective ET_A receptor antagonist, and catalase, an enzyme that specifically decomposes hydrogen peroxide to water and molecular oxygen in the cultured neonatal rat cardiomyocytes. Collectively, these data suggest that ET-1 and oxidative stress are involved in leptin-induced hypertrophy in cultured neonatal rat cardiomyocytes.

An opposing observation from a recent study indicated that leptin exerts an antihypertrophic effect on the hearts of
mice. In that study, left ventricular hypertrophy occurred in \textit{ob/ob} mice that lack leptin, whereas leptin infusion completely reversed such increases in left ventricular wall thickness. The exact reasons for this discrepancy in the role of leptin in cardiac hypertrophy are unclear. Possible explanations for this discrepancy may be that these 2 studies used different models (rats versus mice) and different treatment (in vivo versus in vitro).

\textbf{ET-1 and Leptin-Associated Cardiac Hypertrophy}

The growing evidence from both clinical and animal studies shows that ET-1 plays an important role in the development of cardiac hypertrophy and heart failure. Recently, several studies reported that ET-1 levels in obese subjects are increased, which suggests the participation of ET-1 in the pathogenesis of obesity-associated cardiovascular disease. In

\textbf{Figure 4.} Effect of ET-1 on total RNA levels (A), cell surface area (B), protein synthesis (C), and protein content (D) in cultured neonatal rat cardiomyocytes. Cultured neonatal rat cardiomyocytes were incubated with different concentrations of ET-1 ($10^{-10}$ to $10^{-8}$ mol/L) for 72 hours. Total RNA levels were assayed by RNA-sensitive fluorescence probe PI after DNase treatment. Cell surface area was analyzed by a digital camera fixed to a microscope and Leica image processing and analysis system. Protein synthesis was evaluated by $[^3H]$leucine incorporation. Protein content was determined by Bio-Rad DC protein assay. Values were reported as mean±SEM for $n=4$. *$P<0.05$ vs control, #$P<0.05$ vs ET-1 $10^{-8}$ mol/L group.

\textbf{Figure 5.} Effect of ET-1 on ROS production in cultured neonatal rat cardiomyocytes. Cultured neonatal rat cardiomyocytes were incubated with different concentrations of ET-1 ($10^{-10}$ to $10^{-8}$ mol/L). After 4 hours of incubation, ROS levels were measured by ROS-specific probe $2',7'$-dichlorofluorescein diacetate. A, In situ detection of ROS with fluorescence microscopy. Fluorescent signal intensity reflects ROS levels. B, Concentration-dependent effects of ET-1 on ROS. Values were reported as mean±SEM for $n=4$. *$P<0.05$ vs control, #$P<0.05$ vs ET-1 $10^{-8}$ mol/L group.
addition, a recent study demonstrated that leptin is able to upregulate ET-1 production in human umbilical vein endothe-
lium cells.20 Therefore, these data indicate that leptin may affect cardiac function via ET-1. There are at least 2 cardiac ET-1 receptors, ET<sub>A</sub> and ET<sub>B</sub>.27,32,33 ET-1 exerts its inotropic and hypertrophic effects mainly through the activation of the G protein–coupled ET<sub>A</sub> receptors on cardiomyocytes.32,33 In contrast, ET<sub>B</sub> receptors only mediate inotropic effects without having any effect on hypertrophy.32,33 In our present study, the results showed that leptin-induced ET-1 generation and treatment with ET<sub>A</sub> receptor antagonist ABT-627 significantly inhibited leptin-induced hypertrophy in cultured neonatal rat cardiomyocytes. These data suggest that the ET-1–ET<sub>A</sub> pathway mediates leptin-induced hypertrophic effects in cultured neonatal rat cardiomyocytes. However, it is important to note that blockade of ET<sub>A</sub> receptors by ABT-627 cannot completely abolish leptin-induced ROS production and hence the hypertrophic effect in the cultured neonatal rat cardiomyocytes. These results suggest that an ET-1–ET<sub>A</sub> pathway is one of the contributing factors in leptin-induced cardiomyocyte hypertrophy. Furthermore, the possible influence of ET<sub>B</sub> receptors on leptin-induced ROS generation and hypertrophy in cardiomyocytes is not clear and is currently being investigated. Finally, it is also of interest to note that leptin may induce hypertrophy through other pathways, including the activation of adenylate cyclase,34 which is a main effector of β-adrenergic receptors and mediates β-adrenergic receptor agonist-induced cardiac hypertrophy, and PPAR-α, which can be activated by its endogenous activator leptin.35,36 Overexpression of PPAR-α in mice results in cardiac hypertrophy.37 Activation of the JAK/STAT pathway may be another mechanism by which leptin induces hypertrophy, because activation of the JAK/STAT pathway is an important signal involved in the hypertrophic effect induced by hsp56 and angiotensin II in rat cardiomyocytes,38 and leptin can stimulate this pathway.39

Oxidative Stress and Leptin-Associated Cardiac Hypertrophy

Recent evidence has shown that obesity causes a state of chronic oxidative stress and inflammation.2,40 It is also well known that oxidative stress contributes to cardiac hypertrophy, because hypertrophic substances such as ET-1, norepinephrine, angiotensin II, and cytokines such as TNF-α induce cardiomyocyte hypertrophy via ROS generation.14–17 In addition, clinical and animal studies have demonstrated that the failing heart is subjected to increased oxidative stress, and antioxidant therapy has been shown to preserve left ventricular function during the development of chronic heart failure.41–43 However, it is unknown whether oxidative stress contributes to cardiac hypertrophy in the obese state. Our results show that leptin induces ET-1–ET<sub>A</sub>–mediated ROS generation and hypertrophy, which can be reversed by treatment with antioxidant catalase and the ET<sub>A</sub> receptor antagonist ABT-627 in cultured neonatal rat cardiomyocytes. Thus, the results suggest that elevated leptin levels in obese subjects may increase cardiac oxidative stress via the ET-1–ET<sub>A</sub> pathway, resulting in cardiac hypertrophy. Therefore, antioxidant therapy aimed at reducing oxidative stress induced by leptin may provide an effective means to ameliorate cardiac dysfunction in obese patients.

In summary, the present study demonstrates for the first time that leptin induces hypertrophy through the ET-1–ET<sub>A</sub>–ROS pathway, which can be reversed by treatment with the ET<sub>A</sub> receptor antagonist ABT-627 and antioxidative catalase in cultured neonatal rat cardiomyocytes. ET<sub>A</sub> receptor antagonists and antioxidant therapy strategies may provide an effective means in ameliorating cardiac dysfunction in obese individuals.

Acknowledgments

This study was supported by grants from the Guangzhou Education Committee and the Education Ministry of the People’s Republic of China. The authors would like to thank Alicia De Marco (Michigan State University, East Lansing) for her editorial assistance with the manuscript.

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Circulation. 2004;110:1269-1275; originally published online August 16, 2004;
doi: 10.1161/01.CIR.0000140766.52771.6D
Circulation is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2004 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7322. Online ISSN: 1524-4539

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