Leptin Attenuates the Contractile Function of Adult Rat Cardiomyocytes Involved in Oxidative Stress and Autophagy

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Background: Leptin has been identified as an important protein involved in obesity. As a chronic metabolic disorder, obesity is associated with a high risk of developing cardiovascular and metabolic diseases, including heart failure. The aim of this paper was to investigate the effects and the mechanism of leptin on the contractile function of cardiomyocytes in the adult rat.

Methods: Isolated adult rat cardiomyocytes were exposed to leptin (1, 10, and 100 nmol/L) for 1 hour. The calcium transients and the contraction of adult rat cardiomyocytes were recorded with SoftEdge MyoCam system. Apocynin, tempol and rapamycin were added respectively, and Western blotting was employed to evaluate the expression of LC3B and Beclin-1.

Results: The peak shortening and maximal velocity of shortening/relengthening (dL/dtmax) of cell shortening were significantly decreased, and the time to 50% relengthening was prolonged with leptin perfusion. Leptin also significantly reduced the baseline, peak and time to 50% baseline of calcium transient. Leptin attenuated autophagy as indicated by decreased LC3-II and Beclin-1. All of the abnormalities were significantly attenuated by apocynin, tempol or rapamycin.

Conclusions: Our results indicated that leptin depressed the intracellular free calcium and myocardial systolic function via increasing oxidative stress and inhibiting autophagy.

Key Words: Autophagy • Calcium transient • Cardiac function • Chronic heart failure • Leptin • Oxidative stress
increased nitric oxide production. Our previous study revealed that the endothelin-1-ETA reactive oxygen species (ET-1-ETA-ROS) pathway may be involved in cardiomyocyte hypertrophy induced by leptin. Additionally, leptin regulates cardiomyocyte contractile function through endothelin-1 receptor-nicotinamide adenine dinucleotide phosphate (NADPH) oxidase pathway. It has been shown that leptin suppressed cardiac contractile function in mouse left ventricular myocytes through the endothelin-1 receptor and NADPH oxidase-mediated pathway. A recent study demonstrated that mTOR mediates RhoA-dependent leptin-induced cardiomyocyte hypertrophy, and leptin impaired cardiac contractile function through autophagy dependent mechanism in mouse cardiomyocytes. Nonetheless, the precise mechanism by which leptin reduced myocardial systolic function response has remained unclear at oxidative stress and autophagy. The intent of our present study was to explore the effects and mechanism of leptin on cardiomyocytes contractile function in the adult rat.

**METHODS**

**Isolation of adult rat myocytes**

The experimental programs outlined here were approved by the Institutional Animal Use and Care Committee, Guangzhou Medical University. Primary isolation of adult Sprague-Dawley rat (200 to 225 g) myocytes was performed according to previously established methods. In brief, the rats were sedated with pentobarbital sodium, hearts from adult rats were rapidly excised and perfused with oxygenated (5% CO2-95% O2) Krebs-Henseleit bicarbonate (KHB) buffer containing (in mmol/L) 118 NaCl, 4.8 KCl, 1.8 CaCl2, 1.25 KH2PO4, 1.25 MgSO4·7H2O, 25 NaHCO3, 11.1 glucose and 25 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), pH 7.4. Subsequently, the hearts were perfused with Ca2+-free MEM Joklik’s modified media for 2 or 3 minutes until spontaneous contractions were suspended, and subjected for 30 minutes gradually digesting with Ca2+-free MEM Joklik’s Modified containing 250 U/mL collagenase II (Gibco) and 0.1 mg/mL protease XIV (Sigma-Aldrich) at 37 °C. When time had elapsed, fresh Ca2+-free MEM Joklik’s modified media were subsequently used. Hearts were initially perfused with Ca2+-free MEM Joklik’s modified media to remove residual enzyme, and extracellular Ca2+ was added incrementally back to a level of 1.8 mmol/L. After perfusion, all myocytes that remained were removed, minced, and incubated with a serum-free medium consisting of medium 199 (Gibco) with Earle’s salts containing 25 mmol HEPES and NaHCO3 supplemented with L-carnitine (2 mmol/L), taurine (5 mmol/L), creatine (5 mmol/L), bovine serum albumin (2 mg/mL), penicillin (100 U/mL) and streptomycin (100 mg/mL). Isolated myocytes were filtered through a nylon mesh (187 μm) and collected by natural sedimentation occurring in approximately 2 or 3 minutes. Cells were not selected if they had spontaneous contractions or obvious sarcolemmal blebs.

**Intracellular Ca2+ transient measurement**

Myocardial cells were loaded with Fura-2/AM (2 μmol/L) for 30 minutes at 37 °C. The intensity of fluorescence was measured with a dual-excitation fluorescence photomultiplier system (IonOptix, Westwood, MA, USA). Myocytes were placed on an inverted Nikon microscope (TE2000, Nikon, Tokyo, Japan) and imaged through a Fluor 40 objective. Cells were exposed to light emitted by a 75-W halogen lamp and passed through either a 360- or 380-nm filter while being stimulated to contract at 1 Hz. Fluorescence emissions were detected between 480 nm and 520 nm by a photomultiplier tube after initial illumination at 360 nm for 0.5 s, and then at 380 nm for the duration of the recording protocol. It could be reflected that the change of intracellular free Ca2+ concentration through the ratio of two wavelength fluorescence intensity (F360/F380). IonOptix Video Power was employed to record the changes of intracellular free calcium at 1000 Hz speed during myocardial contraction. Intracellular Ca2+ transient were evaluated by several indexes: baseline (bl), peak, time to 50% baseline (TB50%) and time to 50% peak (TP50%).

**Cell shortening/relengthening measurements**

We used a video-based edge-detection system (IonOptix) as described to assess contractile properties of ventricular myocytes. Briefly, cells were placed in a chamber mounted on an inverted microscope and incubated with medium 199. The cells were
field stimulated with suprathreshold voltage and at a frequency of 1 Hz using a pair of platinum electrodes placed on the opposite sides of the chamber connected to an electrical stimulator (FHC Inc, Brunswick, NE, USA). The myocyte being designated was displayed on the computer monitor using an IonOptix MyoCam camera, which rapidly scanned the image area every 8.3 ms such that the amplitude and velocity of shortening/relengthening was recorded. A video-based edge detector was used to capture changes in cell length during shortening and relengthening. Myocyte shortening and relengthening was assessed by using the following indexes: peak shortening (PS), time to 50% PS (TPS 50%), time to 50% relengthening (TR50%), and maximal velocity of shortening (+dL/dtmax) and relengthening (-dL/dtmax).

**Reactive oxygen species**

Intracellular superoxide was assessed by the superoxide-specific probe dihydroethidium (DHE; Sigma-Aldrich, St. Louis, MO, USA). The fresh isolated adult cardiomyocytes were washed with Hanks’ Balanced Salt Solution (HBSS) and then incubated with DHE (10 μmol/L) in HBSS at 37°C. After incubation for 30 minutes, cardiomyocytes were washed again with HBSS. Fluorescence images were acquired with Nikon confocal laser-scanning microscope A1R under uniform settings and assayed by its image processing and analysis system. The experiments were repeated at least 5 times.

**Protein immunoblotting**

Cardiomyocytes were collected and sonicated in a lysis buffer containing 1× cell lysis buffer (Cell Signaling, Danvers, MA, USA), 30 mmol/L diithiothreitol, 100 mmol/L PMSF, 200× aprotinin, 200× leupeptin and 100× phosphatase inhibitor. 60 μg of proteins were separated on 10% and 15% SDS-polyacrylamide gels in a minigel apparatus (Bio-Rad, Hercules, CA, USA), and were then transferred electrophoretically to polyvinylidene difluoride membranes. The membranes were blocked with 5% nonfat milk for an hour, and then incubated with antibodies against LC3B, Beclin-1 and β-actin at 4°C overnight. After incubation with primary antibody, blots were incubated with anti-rabbit IgG goat peroxidase – linked antibodies at a dilution of 1:5000 for an hour at room temperature. Antigens for proteins of target were detected by the luminescence method. The intensity of bands was measured with a scanning densitometer (Bio-Rad) and Bio-Rad PC analysis software. For all analysis experiments using Western blotting, β-actin was used as control.

**Experimental protocols**

Cardiomyocytes (loaded with Fura-2/AM) were first allowed to contract at a stimulation frequency of 1 Hz for 10 minutes to ensure a steady state before perfusion with leptin (1 to 100 nmol/L, R&D Systems, Minneapolis, MN, USA) for 1 hour. In some studies, cardiomyocytes were incubated with 100 μM apocynin (NADPH oxidase inhibitor), 100 μM tempol (superoxide scavenger) or 100 nM rapamycin (mTOR inhibitor) for 30 minutes before leptin perfusion.

**Statistical analyses**

Values are expressed as mean ± standard error of the mean (SEM). Differences were compared by analysis of variance (ANOVA) or t test, as appropriate. p < 0.05 was considered as the level of significance. All statistical tests were analyzed using GraphPad Prism software version 5.0 (GraphPad Software, La Jolla, CA, USA).

**RESULTS**

**Effect of leptin on cardiomyocyte shortening and calcium transient**

Acute exposure (up to 1 hour) of leptin in different concentrations did not impact the resting myocardial cell length. Representative traces of cardiomyocyte shortening were shown in Figure 1A. Leptin (1 to 100 nmol/L) exhibited a concentration-dependent depression of cardiomyocyte contractility. Compared with the control, leptin perfusion (1 to 100 nmol/L) demonstrated attenuated PS and ±dL/dtmax, and a prolonged time to TR50% at the highest dose of leptin. However, leptin elicited little effect on TPS50% (Figure 1). Acute exposure of leptin attenuated peak of calcium transients in cardiomyocytes. Leptin (10 and 100 nmol/L) significantly depressed the baseline of calcium transient (Figure 2).
Effect of leptin on cardiomyocyte shortening, intracellular calcium transients in the presence of apocynin, tempol and rapamycin

The leptin-induced suppression of PS and $\pm dL/dt_{\text{max}}$ were significantly reversed by apocynin, tempol and rapamycin (Figure 3). Consistent with cardiomyocyte shortening, apocynin, tempol and rapamycin significantly attenuated leptin-induced alteration in intracellular Ca$^{2+}$, such as $\text{bl}$, peak and TB50% (Figure 4).

Effect of leptin on superoxide production in the absence or presence of apocynin, tempol and rapamycin

The intracellular reactive oxygen species (ROS) level in the cardiomyocytes of adult rat was significantly increased in the group treated with leptin (10 nmol/L) compared with the control group. Additionally, treatment with apocynin (100 μM), tempol (100 μM) or rapamycin (100 nM) markedly inhibited ROS production induced by leptin (Figure 5).

Effect of leptin on LC3B and Beclin-1 expression in the absence or presence of apocynin, tempol and rapamycin

To confirm the possible relationship of oxidative stress and autophagy in leptin-induced cardiomyocyte contractile dysfunction, expression of LC3B and Beclin-1 were determined. Our results suggested that leptin perfusion significantly decreased expression of LC3B-II and Beclin-1. All of the abnormalities were significantly attenuated by apocynin, tempol, or rapamycin (Figure 6-8). These results suggested that oxidative stress and...
autophagy were involved in the myocardial anomalies induced by leptin.

DISCUSSION

Leptin is a 16-kDa peptide hormone produced by adipocytes, and regulates energy metabolism to stabilize fat levels. Plasma leptin levels correlate with BMI and have been found to be three to four times higher in obese and diabetic patients. Leptin is directly involved in cardiovascular systolic dysfunction in obesity. Clinically chronic heart failure was related to hyperleptinaemia, and it has been reported that leptin may be implicated as participating in pathophysiologic catabolism of heart failure. Hyperleptinaemia apparently restrained cardiac contraction and produced negative cardiac effect. Laboratory evidence has demonstrated that leptin perfusion may damage systolic function and decrease intracellular Ca²⁺ transients in the ventricular myocytes isolated from adult rat hearts. Meanwhile, leptin also acted on myocyte adenylate cyclase and depressed the response of β-receptor agonist in heart.

Our experiment has used various available leptin con-
centrations to perfuse cardiomyocytes isolated from adult Sprague-Dawley rats. IonOptix was used to record the index of cardiomyocyte shortening and intracellular Ca\textsuperscript{2+} transients. Our results demonstrated that leptin has a depressing effect on intracellular free calcium and myocardial systolic function. Because leptin could suppress myocardial systolic function and intracellular free calcium, it may impel heart failure onset and progression. Therefore, understanding the mechanisms by which leptin induces contractile function depression has become increasingly important.

Present compelling evidence has shown that leptin was a result of vascular endothelial cell dysfunction, migration of smooth muscle hyperplasia and myocardial hypertrophy that was correlated with histocytic oxidative stress in cardiovascular system. This observation was supported by recent in vitro studies that leptin could not only improve active oxygen content in endothelial cells, activate activator protein-1 (AP-1) and NF-\kappa\B, but also increase adhesion molecule expression. These effects could be blocked by ROS scavenger Genistein.\textsuperscript{21} The experiment implied that this ROS scavenger cleared active oxygen by inhibiting the activity of NADPH oxidase, and decreased the roles of active oxygen in the field of anti-platelet aggregation activity and cell adhesion. Besides, physiologic leptin concentration resulted in inflammatory injury and promoted atherosclerosis in aortic endothelial cells by increasing fatty acid oxidation via protein kinase A.\textsuperscript{22} Furthermore, growing information and evidence from both clinical and animal studies has shown that autophagy existed in myocytes from the failing heart, together with apoptosis and necrosis.\textsuperscript{23} Recently, several studies reported that autophagy inhibitor bafilomycin A1 could result in cardiac functional depression in limoseric mice but not affect the normal one. On the other hand, mTOR inhibitor rapamycin could decrease serum leptin levels in mice, inactivation of leptin and the leptin receptor system gene led to increasing autophagy in cardiac tissue.\textsuperscript{24} However, there has been debate regarding the role of autophagy in heart failure,

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**Figure 6.** Effect of apocynin on leptin inhibited autophagy. Cells isolated from adult rat hearts were treated with leptin (10 nmol/L) for an hour in absence and in presence of NADPH oxidase inhibitor apocynin (100 \mu\textit{M}), (A) Representative gel blots. Remainings for statistical graphs. (B) LC3B; (C) Beclin-1. Mean ± SEM, \textit{n} = 5 independent experiments.

**Figure 7.** Effect of tempol on leptin inhibited autophagy. Cells isolated from adult rat hearts were treated with leptin (10 nmol/L) for an hour in the absence and in the presence of superoxide scavenger tempol (100 \mu\textit{M}). (A) Representative gel blots. (B) LC3B; (C) Beclin-1. Mean ± SEM, \textit{n} = 5 independent experiments.

**Figure 8.** Effect of rapamycin on leptin inhibited autophagy. Cells isolated from adult rat hearts were treated with leptin (10 nmol/L) in the absence and in the presence mTOR inhibitor rapamycin (100 nM). (A) Representative gel blots. Remaining for statistical graphs. (B) LC3B; (C) Beclin-1. Mean ± SEM, \textit{n} = 5 independent experiments.
and the extent to which it could both promote disease progression and provide protection as well. Therefore, in the present study, we observed whether NADPH oxidase inhibitor apocynin, superoxide scavenger tempol and mTOR inhibitor rapamycin could prevent leptin-induced cardiomyocyte contractile dysfunction. Our results indicated that the inhibition of PS and \( \pm dL/dt_{\text{max}} \) were significantly attenuated by apocynin, tempol and rapamycin. Meanwhile, apocynin, tempol and rapamycin significantly suppressed leptin-induced alteration in intracellular Ca\(^{2+} \) performed by bl, peak and TB50%. Leptin inhibited autophagy as indicated by decreased LC3-II and Beclin-1. All of the abnormalities were significantly attenuated by apocynin, tempol and rapamycin. Collectively, these data suggested that leptin decreased concentration of intracellular free calcium and myocyte contractility. This information provided evidence that oxidative stress and autophagy were involved in leptin-induced myocyte contractile defect. Although the sequential effects of oxidative stress and autophagy remain unclear after our research, additional published evidence has shown that both activation of mTOR and generation of reactive oxygen species were required in leptin-mediated effects.  

CONCLUSIONS

In conclusion, our results have shown that leptin had depressing effects on intracellular free calcium and myocardial systolic function, and that the mechanism involved is oxidative stress and autophagy. Although our study shed some light on the role of leptin on adult rat cardiomyocytes, the precise mechanism of action behind leptin-associated oxidative stress and autophagy response still merits further investigation.

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