Original Article
The role of leptin in the ventricular remodeling process and its mechanism

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Abstract: Objective: This study aims to explore the role of leptin in the ventricular remodeling process and its mechanism in the diabetic rats' model. Methods: The diabetic SD rats model induced by streptozotocin was established. The SD rats were randomly divided into 4 groups: control group (20 rats treated with citric acid/sodium citrate buffer); M0 group (10 rats treated with physiological saline); M1 group (10 rats treated with 50 μg/kg LP); M2 group (10 rats treated with 100 μg/kg LP). Ang-II was detected by ELISA. The expression levels of LP and Ob-Rb were detected by RT-PCR. MAPK phosphorylation changes were detected by western blotting. Myocardial morphology was observed. Results: Compared with control group, the blood glucose concentration and Ang-II significantly increased in diabetic model groups (P < 0.01) and body weight decreased (P < 0.05). The expression levels of LP and Ob-Rb increased and heart function decreased in diabetic model groups. Conclusions: LP may be involved in the myocardial cell hypertrophy through the neuroendocrine system and associated with the JAK-STAT, Ras-Raf-MEK-MAPK and PI-3K signaling pathway, which provides a new concept for the pathogenesis of cardiac hypertrophy.

Keywords: Leptin, streptozotocin, ventricular remodeling, Angiotensin II, Ob-Rb, MAPK

Introduction

Ingalls found a new "obese gene" in 1950's, its mutation can lead to the occurrence of diabetes and obesity. The cytokine encoded by this gene can regulate hypothalamus through the chemical pathway to control feeding, the cytokine was named leptin (LP) [1]. LP is a kind of a hydrophilic peptide produced and secreted by adipose tissue, its biological effects occurs mainly by binding to a receptor to activate related signal pathway. Leptin Recepter (LR) has been found to have 6 subtypes of LRa LRb LRc LRd LRe and LRf isomers [2]. Leptin is a multifunctional cytokine with a variety of physiological functions including secretion, energy adjustment, comprehensive immune and animal estrus. Leptin and its receptor are widely distributed and participates in many physiological process, so abnormal LP or its receptor will lead to corresponding pathological reaction such as obesity of excess nutrients, cardiovascular diseases and tumor etc [2, 3].

Cardiac hypertrophy is a complex process including many factors. Generally it is thought to be to an effective compensatory process but produce slowly. The specific mechanism for the occurrence of cardiac hypertrophy remains unclear. Leptin was related with the thickness of left ventricular wall [4, 5], there was few reports about how to cause ventricular remodeling. In this study, we observed the effects of different dose of exogenous leptin on ventricular remodeling in diabetic rats model and explored its mechanism.

Materials and methods

Experimental animals

A total of 60 SPF grade adult healthy SD male rats weighing 170 to 190 g were obtained from the animal experimental center of Tianjin Medical University. The rats were kept under clean and quiet environment with room temperature 18-25°C, provided with 12D:12N photoperiod cycle (6:00 AM-6:00 PM). The rats had free access to food and drinking water and were pre feeding for one week to adapt to the environment. They were randomly divided into 2 groups: control group (20 rats) and diabetic group (40) rats. After fasting for 12 h, citric
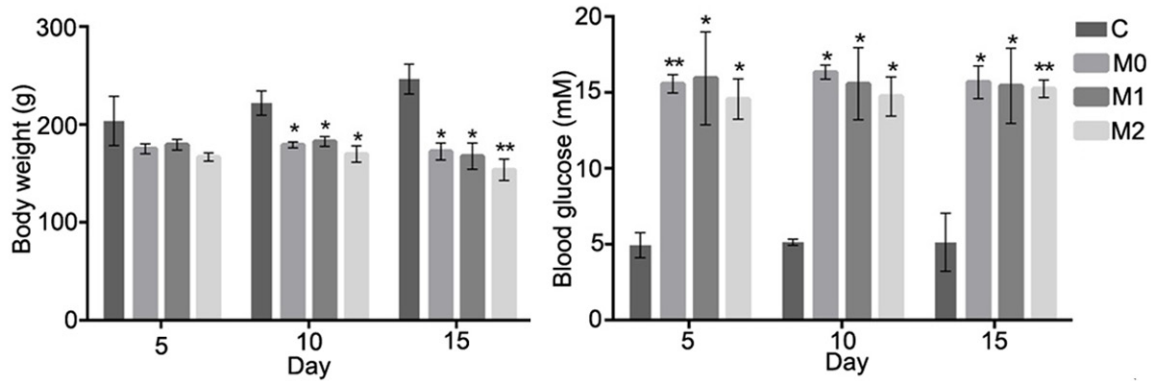
Figure 1. Blood glucose and Body weight. Compared with control group, *P < 0.05, P < 0.01.

Table 1. Comparison of chemical indexes in different group

Group	Leptin (μg/L)	Ang-II (mg/L)
Control (n = 20)	5.3 ± 0.3	63.2 ± 18.3
M0 (n = 10)	12.7 ± 0.2	99.7 ± 9.4*
M1 (n = 10)	17.0 ± 1.2*	114.5 ± 22.4*
M2 (n = 10)	23.7 ± 1.7**	139.3 ± 36.2**,Δ

*: Compared with control group: *P < 0.05; **P < 0.01; Δ: Compared with M1 group, ΔP < 0.05.

acid/sodium citrate buffer was injected in control group. STZ solution diluted with citric acid/sodium citrate buffer was injected in diabetic group (65 mg/kg) 2 times with 1 times a day to establish the diabetic model. The blood glucose concentration was detected after 72 h and those > 11.1 mmol/L was selected as successful model. A total of 30 successful rats were randomly divided into 3 groups (M0 group treated with physiological saline; M1 group treated with 50 μg/kg LP; M2 group treated with 100 μg/kg LP).

Housing and procedures involving experimental animals were in accordance with the NIH Animal Care and Use Committee guidelines. All experimental procedures were approved by the Care of Experimental Animals Committee of our hospital.

Ultrasonic Heartbeat diagram determination

Rats were fixed on the operation table after being anesthetized with 10% Chloral hydrate. Cardiac function indexes such as left ventricular end diastolic pressure and ejection fraction were determined by Color Doppler ultrasonography.

ELISA

The chemical indexes LP and Ang-II were detected using ELISA kits according to the manual. Briefly, Blood was collected by abdominal vein puncture before the rats were sacrificed. A total of 50 μl serum and 50 μl sample dilution were added into 96 well plate coated with specific antibody and incubation at 37°C for 2 h. The plates were washed 5 times, 100 μl substrate of enzyme reaction was added and incubation at 37°C for 2 h. Then plates were washed 5 times and 100 μl chromogenic reaction liquid was added and incubation at 37°C for 30 min, the termination liquid was added to terminate the reaction. The absorbance was detected using microplate reader at 450 nm wavelength.

Real-time RT-PCR and Western-blotting

Total RNA was extracted from heart tissue using RNA extraction Kit according to the manufacturer's protocol. 1 μg total RNA was subjected to reverse transcription using reverse transcription kit. Real-time PCR were performed using SYNBR Green PCR Master Mix. At the end of each reaction, a melting curve analysis was performed to confirm the absence of primer dimmers. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene was used as an internal control for normalization of RNA quantity and quality differences in all samples. Quantifications of target genes mRNA was performed using the $2^{-\Delta\Delta Ct}$ method. Primer sequences of Ob-Rb gene were as follows: forward 5'-GTGTGAGCATCTCTCCTGGAG-3'; reverse 5'-ACCACACCAGACCCTGAAAG-3'. ΔCT = Ct (Ob-Rb)-Ct (GAPDH); Δ(ΔCT) = ΔCT-ΔCT (control).
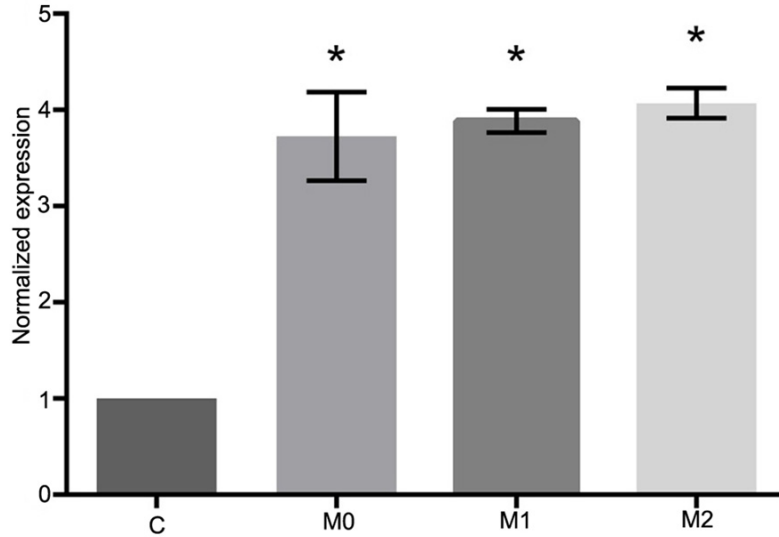
Figure 2. mRNA expressions of leptin receptor Ob-Rb. Compared with control group, *P < 0.05.
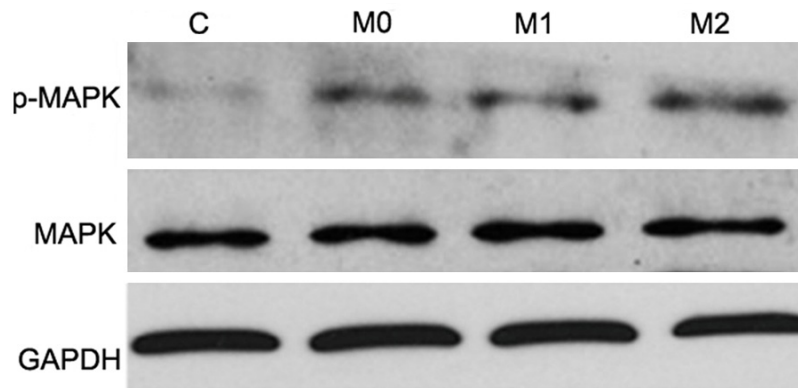

Figure 3. The changes of MAPK and activated-MAPK detected by Western blotting.

Total proteins were extracted from heart tissue and analyzed with SDS-PAGE electrophoresis. Then it was electrotransferred to the PVDF membrane. The membrane containing the proteins was used for Immunoblotting with required antibodies. They were blocked with 5% non-fat milk in TBST (10 mM Tris-HCl (pH 8.0), 150 mM NaCl, and 0.1% Tween-20) for 2 h, then incubated with the primary antibodies at 4°C overnight. Then they were incubated with secondary antibodies conjugated with horseradish peroxidase at room temperature for 1 h. Antibody binding was detected.

Myocardial morphology observation

The rats were sacrificed and the heart was taken out. The heart was rinsed with water and dried with paper to weigh its weight (HW). Heart atrium was cut to weigh left ventricular mass (LVM). Left ventricular mass index was calculated. Apical tissue was stained with hematoxylin and eosin to observe cellular morphology.

Statistical analyses

The results are expressed as mean ± SD. The SPSS software package 19.0 was used for the statistical tests. Variance analysis and t-test were used to compare among groups. P < 0.05 was considered statistically significant.

Results

Successful establishment of diabetic rat model

We detected the content of blood glucose and weight after induction for 5-15 days to observe whether the diabetic rat model was established successfully. Compared with control group, the average blood glucose level increased and weight decreased in model rats (P < 0.01, **Figure 1**), so the diabetic rat model was established successfully.

Detection of LP and Ang-II

As shown in **Table 1**, the LP and Ang-II levels in model rats were significantly higher than that of control group, especially in M2 group (P < 0.05); the LP and Ang-II levels in M2 group were significantly higher than that of M1 group (P < 0.05).

Detection of Ob-Rb

The expression levels of leptin receptor Ob-Rb were detected by RT-PCR. It was higher in model rats than that of control group (P < 0.05);

Table 2. Comparison of ultrasonic heartbeat diagram determination in different group

Group	LVEDP (mmHg)	LVIDd (mm)	LVEF (%)
M1 (n = 10)	13.4 ± 2.2*	6.4 ± 0.4*	0.7 ± 0.1*
M2 (n = 10)	18.6 ± 2.3*,Δ	6.6 ± 0.9*,Δ	0.6 ± 0.1*,Δ
M0 (n = 20)	7.5 ± 1.3	4.1 ± 0.5	0.8 ± 0.1

*: Compared with M0 group, *P < 0.05; **P < 0.01; Δ: Compared with M1 group, ΔP < 0.05.

Table 3. Comparison of HW, LVM and LVMI in different group

Group	HW (mg)	LVM (mg)	LVMI (mg/g)
M1 (n = 10)	888.9 ± 28.5	830.8 ± 4.7**	2.8 ± 0.5*
M2 (n = 10)	916.5 ± 27.3*	825.0 ± 5.0*,Δ	2.8 ± 0.4*,Δ
M0 (n = 20)	840.4 ± 25.0	867.2 ± 6.2	3.0 ± 0.5

*: Compared with M0 group, *P < 0.05; **P < 0.01; Δ: Compared with M1 group, ΔP < 0.05.

but there was no difference among M0, M1 and M2 groups (**Figure 2**).

Changes of MAPK phosphorylation

The changes of MAPK and activated-MAPK were detected by Western blotting. There was no significant difference among control, M0, M1 and M2 groups in MAPK but there were significant differences among control, M0, M1 and M2 groups in activated-MAPK (**Figure 3**).

Determination of ultrasonic Heartbeat diagram

The levels of LVEDP and LVIDd in M1 and M2 group were significant higher that of M0 group (P < 0.05) while the level of LVEF in M1 and M2 group was significant lower that of M0 group (P < 0.05). The levels of LVEDP and LVIDd in M2 group were significant higher that of M1 group (P < 0.05) while the level of LVEF in M2 group was significant lower that of M1 group (P < 0.05) (**Table 2**).

The levels of weight and HW in M1 and M2 group were significant higher that of M0 group (P < 0.05). The levels of LVM and LVMI in M1 and M2 were significant lower that of M0 group (P < 0.05) (**Table 3**).

Pathological results

Myocardial cells in M0 group have similar size and arranged neatly with no obvious abnormity in cells or stromal cells. There were different degree of hypertrophy and degeneration in myocardial cells in M1 and M2 groups, arranged in disorder with unequal size, cell necrosis was visible (**Figure 4**).

Discussion

Leptin (LP) is a secreted protein encoded by the obese gene and has widely nervous endocrine and metabolic effects. Previous studies showed that the receptor of leptin mainly distributed in the region of the hypothalamus, heart and large vessels. Long term infusion of leptin could cause persistent elevated arterial blood pressure [1-5].

In recent years, the relationship between leptin and cardiovascular disease has received more and more attention. LP was thought to play an important role in myocardial hypertrophy as an endogenous substance [6]. LP is the product of obesity gene in the mature fat cells [7-10]. LP has extensive biological effects after entering the blood. It can activate the sympathetic nervous system (SNS) and Ang-II receptor by a variety of signaling pathways even through the blood brain barrier. The activation of SNS plays an important role in the cardiac remodeling process. SNS can stimulate the generation of mitogen activated protein kinase (MAP), cause myocardial cell division, which leading to myocardial remodeling and cardiac hypertrophy. Ang-II activates phospholipase C (PLC) and three inositol phosphate (IP3) after combining with its receptor and lead to cardiac hypertrophy directly [11-16].

In this study we found that LP decreased the heart function of rats in different degree, LP, Ang-II, LVEDP, LVIDd and HW increased and LVEF, LVM and LVMI decreased obviously. Heart function impaired significantly with the increase of leptin. LP significantly affected the expression of Ang-II while Ang-II can play roles by combining with its receptor in myocardial protein synthesis, and then cause myocardial hypertrophy even lead to heart failure. Other studies showed that LP can induce oxidative stress of vascular endothelial cells in myocardium, increase the activity of sympathetic nerve, promote the proliferation and migration of vascular smooth muscle cells, and then promote the increase of blood pressure [17-19]. In
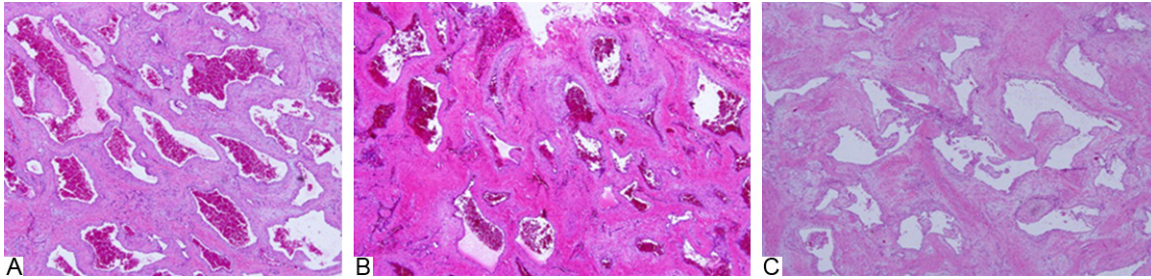
Figure 4. HE staining of myocardial cells. A: M1; B: M2; C: M0.

recent years, the arterial blood pressure is thought to be crucial factors for the formation of myocardial hypertrophy. It does not produce atherosclerosis in leptin-knock out mice. LP induced myocardial cell hypertrophy through the JAK-STAT, Ras-Raf-MEK-MAPK, phosphatidylinositol -3 kinase (PI-3K) signaling pathway, and induced myocardial fiber reconstruction through the inflammation medium and oxidative stress. LP participates in the regulation of sugar, fat and other substances metabolism as a protein hormone, high concentration leptin can combine with its receptors in the heart and blood vessels directly involved in promoting myocardial hypertrophy [20-23].

In summary, LP plays an important role in the myocardial cell hypertrophy through the neuroendocrine system and associated with the JAK-STAT, Ras-Raf-MEK-MAPK and PI-3K signaling pathway, which provides a new concept for the pathogenesis of cardiac hypertrophy.

Acknowledgements

Disclosure of conflict of interest

None.

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References

[1] Zhang Y, Proenca R, Maffei M, Barone M, Leopold L and Friedman JM. Positional cloning of the mouse obese gene and its human homologue. Nature 1994; 372: 425-432.

[2] Park HK and Ahima RS. Leptin signaling. F1000 Prime Rep 2014; 6: 73.

[3] Wazir U, Al Sarakbi W, Jiang WG and Mokbel K. Evidence of an autocrine role for leptin and leptin receptor in human breast cancer. Cancer Genomics Proteomics 2012; 9: 383-387.

[4] Barison A, Aquaro GD, Pugliese NR, Cappelli F, Chiappino S, Vergaro G, Mirizzi G, Todiere G, Passino C, Masci PG, Perfetto F and Emdin M. Measurement of myocardial amyloid deposition in systemic amyloidosis: insights from cardiovascular magnetic resonance imaging. J Intern Med 2015; 277: 605-14.

[5] Harwood SM, Allen DA, Raftery MJ and Yaqoob MM. Calpain is a mediator of myocardial injury in experimental uremia: is it activated by endogenous ouabain? Kidney Int Suppl 2003; S177-180.

[6] Hamed EA, Zakary MM, Ahmed NS and Gamal RM. Circulating leptin and insulin in obese patients with and without type 2 diabetes mellitus: relation to ghrelin and oxidative stress. Diabetes Res Clin Pract 2011; 94: 434-441.

[7] Maisch B, Alter P and Pankuweit S. Diabetic cardiomyopathy--fact or fiction? Herz 2011; 36: 102-115.

[8] Hall JE, da Silva AA, do Carmo JM, Dubinion J, Hamza S, Munusamy S, Smith G and Stec DE. Obesity-induced hypertension: role of sympathetic nervous system, leptin, and melanocortins. J Biol Chem 2010; 285: 17271-17276.

[9] Magge SN. Cardiovascular Risk in Children and Adolescents with Type 1 and Type 2 Diabetes Mellitus. Curr Cardiovasc Risk Rep 2012; 6: 591-600.

[10] Marroqui L, Gonzalez A, Neco P, Caballero-Garrido E, Vieira E, Ripoll C, Nadal A and Quesada I. Role of leptin in the pancreatic beta-cell: effects and signaling pathways. J Mol Endocrinol 2012; 49: R9-17.

[11] Konner AC and Bruning JC. Selective insulin and leptin resistance in metabolic disorders. Cell Metab 2012; 16: 144-152.

[12] Rahmouni K, Sigmund CD, Haynes WG and Mark AL. Hypothalamic ERK mediates the anorectic and thermogenic sympathetic effects of leptin. Diabetes 2009; 58: 536-542.

[13] De Mello WC. Mechanical stretch reduces the effect of angiotensin II on potassium current in

cardiac ventricular cells of adult Sprague Dawley rats. On the role of AT1 receptors as mechanosensors. J Am Soc Hypertens 2012; 6: 369-374.

[14] Larson JE, Rainer PP, Watts VL, Yang R, Miller KL, Phan A and Barouch LA. Dependence of beta3-adrenergic signaling on the adipokine leptin in cardiac myocytes. Int J Obes (Lond) 2012; 36: 876-879.

[15] Zibadi S, Cordova F, Slack EH, Watson RR and Larson DF. Leptin's regulation of obesity-induced cardiac extracellular matrix remodeling. Cardiovasc Toxicol 2011; 11: 325-333.

[16] Abel ED, Litwin SE and Sweeney G. Cardiac remodeling in obesity. Physiol Rev 2008; 88: 389-419.

[17] Abel ED and Sweeney G. Modulation of the cardiovascular system by leptin. Biochimie 2012; 94: 2097-2103.

[18] De Mello WC and Frohlich ED. On the local cardiac renin angiotensin system. Basic and clinical implications. Peptides 2011; 32: 1774-1779.

[19] Brand S, Amann K and Schupp N. Angiotensin II-induced hypertension dose-dependently leads to oxidative stress and DNA damage in mouse kidneys and hearts. J Hypertens 2013; 31: 333-344.

[20] Chen W, Chen W, Xia Y, Zhao X, Wang H, Yu M, Li Y, Ye H and Zhang Y. Therapy with Astragalus polysaccharides rescues lipotoxic cardiomyopathy in MHC-PPARalpha mice. Mol Biol Rep 2013; 40: 2449-2459.

[21] Jahan S, Zinnat R, Hassan Z, Biswas KB and Habib SH. Gender differences in serum leptin concentrations from umbilical cord blood of newborn infants born to nondiabetic, gestational diabetic and type-2 diabetic mothers. Int J Diabetes Dev Ctries 2009; 29: 155-158.

[22] Lieb W, Sullivan LM, Aragam J, Harris TB, Roubenoff R, Benjamin EJ and Vasan RS. Relation of serum leptin with cardiac mass and left atrial dimension in individuals > 70 years of age. Am J Cardiol 2009; 104: 602-605.

[23] Kruger M, Babicz K, von Frieling-Salewsky M and Linke WA. Insulin signaling regulates cardiac titin properties in heart development and diabetic cardiomyo pathy. J Mol Cell Cardiol 2010; 48: 910-916.