Original Article
Effects of chemerin/CMKLR1 in obesity-induced hypertension and potential mechanism

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Abstract: Background: Obesity-induced hypertension (OIH) has a high morbidity and mortality, and its prevention and treatment has been a major challenge in clinical practice. Chemerin is a newly discovered adipokine closely related to OIH. Methods: Male Wistar rats (8W) were divided into either a high-fat diet or a regular diet group. Body weight and blood pressure were measured every two weeks. After 20 weeks, serum, adipose tissue and aortic arteries were collected. Arterial tensions were detected; Immunohistochemistry, ELISA and Western blotting were used to detect the expression of chemerin, CMKLR1, Rock2 and P-MYPT1 in the aorta and perivascular adipose tissues. Results: After 4 weeks, the body weight, systolic blood pressure, diastolic blood pressure and mean arterial pressure were all significantly higher in the high-fat diet group (**P <0.05). There was no significant difference in serum chemerin concentration between the OIH group and the control group. However, chemerin and CMKLR1 protein expression was higher in aortic arteries and perivascular adipose tissues of the OIH group (**P <0.05). The arterial tension induced by chemerin 9 (1 μM) and the expression of Rock2 and P-MYPT1 were higher in the OIH group (**P <0.05). Conclusion: OIH positively correlated with chemerin in tissues but not serum. Arterial tension was increased by chemerin 9. Rock2/P-MYPT1 might be involved in the pathogenesis of increased vascular tone in OIH rats.

Keywords: Obesity-induced hypertension, chemerin, CMKLR1.

Introduction
Recent studies have shown that adipokines imbalance due to the adipose lesions play important roles in the occurrence and development of OIH [7]. Chemerin is a recently identified adipokine that is closely related to the pathogenesis of metabolic syndrome [8]. It is secreted in the form of prochemerin which is then hydrolyzed by the serine protease and cysteine protease into chemerin 9 and chemerin 15 [9]. Chemerin was initially identified to act on the chemokine-like receptor 1 (CMKLR1) to induce the chemotaxis of macrophages and dendritic cells. CMKLR1 is a Gi protein-coupled receptor and a major receptor of chemerin. Variety of studies revealed that plasma chemerin, closely related to the blood pressure, increased significantly in hypertensive patients [10, 11]. The pressure lowering drug amlodipine is proved to help reduce plasma chemerin concentration [12]. Intraperitoneal injection of chemerin at 6 mg/kg/day for consecutive 6 weeks might dramatically increase the systolic
blood pressure (SBP) as compared to controls [13]. Taken together, chemerin plays a crucial role in the occurrence and development of hypertension, but the role of chemerin playing in the pathogenesis of OIH is still poorly understood.

Evidence shows that chemerin is able to induce vasoconstriction. CMKLR1 agonist may induce the constriction of thoracic aorta and superior mesenteric artery of Sprague-Dawley rats in a dose dependent manner, and can inhibit the phenylephrine or prostaglandin F2α induced vasoconstriction [14]. It is indicated that chemerin may induce vasoconstriction via a CMKLR1 dependent manner. However, in OIH rat model, the mechanism underlying the chemerin induced vasoconstriction remains unclear. The constriction of vascular smooth muscle is involved in both MLC phosphorylation dependent pathway and non-MLC phosphorylation dependent pathway. RhoA/ROCK/P-MYPT1 signaling pathway is one of the classical MLC phosphorylation dependent pathways [15]. Small GTPase Rho activates effector ROCK, which may directly induce MLC phosphorylation or indirectly act on the myosin phosphatase target subunit 1 (MYPT1) of MLCP, leading to its phosphorylation. In addition, the activation or phosphorylation of myosin phosphatase phosphorylation dependent inhibitor (CPI-17) may inhibit the MLCP activity, which promotes the MLC phosphorylation, increasing the constriction of VSMCs [16]. Further studies are necessary on whether chemerin has an effect on the ROCK2/P-MYPT1 pathway of VSMCs to affect the VSMCs constriction and then participate in the pathogenesis of OIH.

Materials and methods

Approval of animal experiments

Animal care and treatment were done in accordance with the Institutional Guidelines of The Central South University and the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals. This study was approved by the Ethical Committee of the Central South University.

Reagent

Chemerin 9 (Genscript, Scotch Plains, NJ) and RhoA Activation Assay kit (NewEast Biosciences, Malvern, PA, USA) were used in the present study. Following antibodies were used in the experiments: chemerin, CMKLR1 (Abcam, Cambridge, MA), ROCK2, β-actin (Proteintech Group, Chicago, IL), and P-MYPT1 (Santa Cruz Biotechnology, Santa Cruz, CA).

Modeling

A total of 20 male Wistar rats were randomly assigned into the control group (n=10) and the OIH group (n=10). All rats were housed for 1 week before study. In the OIH group, animals were fed with high fat diet (general chow: 60%; lard: 20%; egg yolk powder: 10%; peanut: 9%; sesame oil: 1%). In the control group, rats were fed with normal chow until the end of study. Body weight was measured once every two weeks; blood pressure was non-invasively measured via the tail vein once every 4 weeks. At 20 weeks, rats were sacrificed after anesthesia. The thoracic aorta was collected, and surrounding vascular and adipose tissues were removed. The vessels were separated for the following detections.

Culture of human aortic smooth muscle cells

HA-VSMCs were maintained in high glucose DMEM containing 10% fetal bovine serum at 37°C in an environment with 5% CO₂. The medium was refreshed once every 48 h. When the cell confluence reached 80%, the cells were digested with 0.25% trypsin for 1 min. When the cells became round and detached from the wall, medium containing serum was added to stop the digestion. After centrifugation, the supernatant was removed. Cells were re-suspended in the 10% of medium containing serum. Cell suspension was divided into two parts. After passaging once, cells were processed for the extraction of protein and RNA.

Detection of vascular tension

The intima of vessels was scratched with blunt forceps 2-3 times to remove the endothelium. Then, the vessels were transferred into a chamber (ph=7.3) equipped with camera. The thoracic aorta bears the cross-wall pressure at 60 mm Hg under continuously monitoring. The solution in outer chamber was heated to 37.5°C, and the inner chamber was flushed with MOPS buffer for 30 min. When the vascular tension became stable, the inner diameter
was recorded. The vascular tension was calculated as the proportion of the reduction in maximal diameter to the maximal diameter. Vessels were treated independently with chemerin, CCX832 and C3 transferase, and then the vascular tension was measured.

Detection of serum chemerin

After water deprivation for 12 h, rats were intraperitoneally anesthetized with 10% chloral hydrate at 0.3 mg/100 g. Then, 1 ml of blood was collected from the inner canthus vein, and serum was separated for the detection of chemerin with corresponding kit. Detection was done at 0, 4 and 20 weeks.

Analysis of RhoA activation

RhoA activity was measured with RhoA Activation Assay kit (NewEast Biosciences, Catalog Number: 80601).

Immunohistochemistry for CMKLR1

Vascular tissues were embedded in paraffin and sectioned (5 μm in thickness). Tissues sections were placed on polylsine treated slides. After deparaffinization and antigen retrieval, sections were incubated with primary antibody and secondary antibody, followed by visualization with DAB. After counterstaining with hematoxylin, dehydration was performed, followed by mounting. Sections were observed and photographed under a light microscope.

Detection of ROCK phosphorylation

After extraction of proteins, total ROCK and phosphorylated MYPT1 were detected by the Western blotting. The phosphorylated MYPT1 was normalized to total ROCK as the ROCK activity. Protein band was analyzed with Image J software, and optical density was detected as the protein expression.
Western blot analysis

HA-VSMCs treated with various agents were harvested, and total cellular proteins were extracted using a lysis buffer (62.5 mmol/l Tris-HCl, pH 6.8, 100 mmol/l dithiothreitol, 2% SDS, 10% glycerol). The protein concentrations were then determined using the Bio-Rad Protein Assay following the manufacturer's instruction (Bio-Rad, Hercules, CA). Equal amounts of proteins were fractionated on a 10% SDS-PAGE and transferred to a PVDF membrane (Amersham Pharmacia Biotech, Piscataway, NJ). The blots were blocked with TBST buffer (500 mmol/l NaCl, 20 mmol/l Tris-HCl, pH 7.4, and 0.1% Tween 20) containing 5% nonfat dry milk (Bio-Rad Laboratories) and then incubated with a specific primary antibody in TBST buffer containing 5% nonfat dry milk overnight at 4°C. Following secondary antibody incubation, the signal was visualized using the Super Signal West Pico Chemiluminescent kit (Pierce Biotechnology, Rockford, IL) and exposed to Kodak X-Max film. β-actin or GAPDH was used as an internal control by incubating a specific antibody against β-actin or GAPDH with the same membrane by stripping the membrane after the first blot. The specific signals were quantitated using the Image J software. The protein levels were expressed as fold of control after normalizing the internal control level.

Results

Body weight, LEE index, epididymal fat mass and mass index

In the study, the body weight of rats increased in two groups. Since week four, the body weight in the OIH group was significantly higher than in the control group (P<0.05; Figure 1A). The weight index reached the highest at week four, and since then, the LEE index in the OIH group was remarkably higher than in the control group (P<0.05; Figure 1B). In the 23-week period, the epididymal fat mass and mass index in the OIH group were significantly higher than in the control group (P<0.05; Figure 1C, 1D).
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Comparison of SBP, DBP, MAP and HR

Since week four, the SBP, DBP and MAP in the OIH group increased gradually and were significantly higher than in the control group (P<0.05; Figure 2A-C). Compared with the controls, the HR in the OIH group was also significantly higher (P<0.05; Figure 2D).

Chemerin expression

Immunohistochemistry showed that chemerin expression in the adipose tissues of the thoracic aorta, thoracic aorta, mesenteric artery, and renal artery vascular smooth muscle was all significantly higher than in the control group (Figure 3A).

Western blotting was performed to detect the chemerin protein expression in the adipose tissues surrounding the thoracic aorta. Results showed that the chemerin protein expression in the OIH group was significantly higher than in the control group (P<0.05; Figure 3B).

Western blotting was performed to detect the chemerin protein expression in the aorta without endothelium. Results showed the chemerin expression in the OIH group was significantly higher than in the control group (P<0.05; Figure 3C).

The serum chemerin concentration was measured at 0, 4 and 20 weeks. Results showed the serum chemerin concentration was comparable between two groups at different time (P>0.05; Figure 3D).

CMKLR1 expression

Immunohistochemistry showed that CMKLR1 expression in the aorta, mesenteric artery and renal artery vascular smooth muscle in the OIH group increased greatly when compared with the control group (Figure 4).

Western blotting was employed to detect the CMKLR1 protein expression in the aorta without endothelium. Results showed the CMKLR1 protein expression in the aorta without endothelium of the OIH group was significantly higher than in the control group (P<0.05; Figure 4B).

Vascular tension

When the vascular tension was stable for 1 h, chemerin 9 (1 μM) was added. The vascular...
tension increased significantly (Figure 5). In the OIH group, the increase in vascular tension was significantly higher than in the control group (P<0.05; Figure 5).

ROCK2/P-MYPT1 expression in vessels and cells

Western blotting was employed to detect the ROCK expression in the thoracic aorta without endothelium. Results showed the ROCK2 protein expression in the thoracic aorta without endothelium of the OIH group was significantly higher than in the control group (P<0.05; Figure 6A).

Human aortic vascular smooth muscle cells (HA-VSMCs) were treated with 100 μg/L chemerin. At different time points, the ROCK2 protein expression in HA-VSMCs increased significantly (P<0.05; Figure 6B). It reached the highest level at 48 h. Western blotting was employed to detect the P-MYPT1 protein expression in the thoracic aorta without endothelium. Results showed the P-MYPT1 expression in the thoracic aorta of the OIH group was significantly higher than in the control group (P<0.05; Figure 6C).

Discussion

Currently, the specific mechanism underlying the influence of chemerin on hypertension and the potential pathways involved are still poorly understood. A large number of studies showed that chemerin could affect the vascular remodeling, proliferation and migration, and the potential mechanism was also investigated [17, 18]. However, little is known about the mechanism underlying the influence of chemerin on vasoconstriction. The OIH has a high morbidity but a poor control. In this study, the animal model was successfully established to investigate the role of chemerin in the OIH. The
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Figure 5. Chemerin 9 induced arterial contraction. A: The concentration of isolated thoracic aorta without endothelium was induced by chemerin 9 in control group. B: The tension of isolated thoracic aorta without endothelium was elevated by chemerin 9 in OIH group. C: Amplification of artery contraction induced by chemerin 9 in two groups. The data are the mean ± SEM (n=3). Note: arrow: time of chemerin 9 (1 μM) treatment. *P<0.05 vs control group.

Figure 6. A: ROCK2 protein expression in the thoracic aortic of both groups. B: ROCK2 expression was induced by chemerin time-dependently in the HA-VSMCs. HA-VSMCs were treated with the vehicle control and chemerin 100 μg/L for various times (0 h, 6 h, 12 h, 24 h, 48 h). The data are mean ± SEM of 4 individual samples from two independent duplicate experiments. C: P-MYPT1 protein expression in the thoracic aorta of two groups. The levels of ROCK2 and P-MYPT1 were expressed as fold of control and the data are the mean ± SEM (n=3). A representative western blot analysis is presented below or next to the bar graph. HA-VSMCs, human aortic smooth muscle cells. *P<0.05 vs control group.

Study has revealed that chemerin may induce vasoconstriction, which is consistent with the findings from the study of Stephanie et al [15]. Further studies indicated that chemerin induced vasoconstriction might be related to MEK-ERK1/2 signaling pathway [14]. In the present study, results presented that chemerin could induce the constriction of chemerin content of perivascular adipose tissues, serum and vascular smooth muscle and the expression of CMKLR1, ROCK2 and P-MYPT1 in the arterial smooth muscle were detected in rats with and without OIH. In addition, the human aortic vascular smooth muscle cells were cultured and then treated with chemerin. The ROCK2 expression was detected at different time points. Results showed that the chemerin expression in the adipose tissues surrounding the thoracic aorta increased significantly and the protein expression of chemerin, CMKLR1, ROCK2 and P-MYPT1 in the arteries elevated dramatically in the OIH rats as compared to the control group. Moreover, chemerin was able to promote the ROCK2 expression in HA-VSMCs. These findings indicated that the elevated expression of chemerin and its receptor CMKLR1 might activate the ROCK2 pathway to induce vasoconstriction.

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aorta, and the chemerin induced vasoconstriction in the OIH rats was more potent than in the healthy rats. In addition, chemerin may also increase the endothelin or phenylephrine induced vasoconstriction. On the basis of important role of RhoA/ROCK2/P-MYPT1 pathway in the maintenance of vasoconstriction [19, 20], and that, the endothelin and phenylephrine may affect the RhoA/ROCK2/P-MYPT1 mediated vasoconstriction [21], we speculated that chemerin might act on its receptor to activate RhoA/ROCK2/P-MYPT1 and then induce vasoconstriction, which affects the occurrence and development of hypertension. To further elucidate the effect of chemerin on the RhoA/ROCK2/P-MYPT1 pathway, serum chemerin content was measured at different time points. Results supported that serum chemerin content was comparable between two groups (Figure 3C), and the chemerin and CMKLR1 expression increased significantly in the peri-vascular and vascular tissues of OIH rats as compared to the control group (Figures 3A, 3B, 4A, 4B). Thus, we proposed that chemerin might function to induce vasoconstriction via paracrine in OIH rats. There was also evidences claimed that negative result in serum chemerin detection could be related to the time point at which detection was done [22]. Study has shown that the chemerin, CMKLR1, ROCK2 and P-MYPT1 expression in the thoracic aorta and peri-vascular adipose tissues increased in the OIH rats. We assumed that the increase in P-MYPT1 was indicative of elevated ROCK2 activity, as we speculated that the relationship between chemerin and OIH is related to the ROCK2 expression and activity. Chemerin may act on RhoA/ROCK2 pathway to induce vasoconstriction, leading to the occurrence of OIH.

However, the specific influences of chemerin on the RhoA/ROCK2/P-MYPT1 pathway warranted to be further investigated, which may provide more solid evidences on the chemerin induced vasoconstriction and subsequent hypertension. In addition, chemerin may also act on inflammatory cytokines, and the role of inflammatory cytokines in the pathogenesis of OIH is also needed to be further elucidated.

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Disclosure of conflict of interest

None.

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