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Original Article

Chemerin stimulates aortic smooth muscle cell proliferation and migration via activation of autophagy in VSMCs of metabolic hypertension rats

Juan Wen[1*], Jiajie Wang[2*], Lan Guo[1], Weijun Cai[2], Yang Wu[1], Wei Chen[1], Xiaohong Tang[1]

[1]Department of Cardiology of The Third Xiangya Hospital, Central South University, Changsha, China; [2]Department of Histology and Embryology, School of Basic Medicine, Central South University, Changsha, Hunan, China. [*]Equal contributors.

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Abstract: Vascular remodeling is a characteristic pathogenesis of hypertension and a main cause of abnormal construction and function of organs because of hypertension. Chemerin is a new adipokine that is elevated in states of obesity and metabolic syndrome (MS). However, the molecular mechanisms behind these pathological processes are not fully clarified. An animal model of metabolic hypertension was created to evaluate the role of metabolic chemerin in hypertension. In this study, the expression of chemerin/CMKLR-1 and autophagy in the arteries of metabolic hypertension rats undergoing vascular remodeling was investigated and the effect and mechanisms on the regulation of human aortic smooth muscle cells (HA-SMCs) were explored. The vascular remodeling *in vivo* was more serious in the metabolic hypertensive rat model, and the expression of chemerin and its receptor CMKLR1 were remarkably higher in the media layer of the thoracic aorta and the mesenteric artery in metabolic hypertension rats. In addition, there was an increased number of autophagosomes in SMCs and an up-regulation of the autophagy-related protein LC3 and beclin-1 levels in metabolic hypertension rats. *In vitro*, chemerin significantly stimulated HA-SMC proliferation and migration, as determined by MTT assay and scratch assay, respectively. Chemerin significantly increased LC3 and beclin-1 levels, as measured by western blot analysis, while this effect was inhibited by the autophagy inhibitor 3-MA. It is demonstrated that chemerin stimulates SMC proliferation and migration via autophagy, which may lead to vascular structural remodeling in metabolic hypertension.

Keywords: Blood pressure, metabolic hypertension, chemerin, vascular remodeling, autophagy

Introduction

Hypertension remains a major public health problem despite continuous research and development in cardiovascular medicine. Greater than 60% of risk factors for hypertension are metabolic, although genetic and secondary etiologies play an important role in the development of hypertension [1]. Therefore, Zhu et al introduced the concept of metabolic hypertension, which is defined as high blood pressure that is caused by metabolic disturbances but not other classic secondary factors and can be ameliorated by correction of these metabolic abnormalities [2]. Arterial remodeling plays a key role in the pathogenesis of metabolic hypertension. The exploration of possible therapeutic targets and related molecular mechanisms is meaningful for the prognosis of metabolic hypertension.

Adipose tissue is closely associated with vascular remodeling. Chemerin was identified as a chemoattractant that stimulates the recruitment of dendritic cells and macrophages to lymphoid organs and the sites of injury [3]. Yet, novel roles for chemerin in cardiovascular diseases have recently been revealed. Research data show that chemerin is a new adipokine that is elevated in states of obesity and metabolic syndrome [4, 5]. Chemerin is implicated in angiogenesis [6] and vascular inflammation [3], which are hallmarks of vascular injury in hypertension [7]. However, the effect and mechanism of chemerin on vascular remodeling remain obscure. A recent report has demonstrated that chemerin is expressed in perivascular adipose tissue and served as an endogenous vasoconstrictor [7]. Chemerin promotes the proliferation and migration of vascular smooth muscle and increases mouse blood pressure [8].

Therefore, it is likely that chemerin may affect the development of hypertensive vascular remodeling by acting on SMCs.

Autophagy is a well-conserved mechanism in cells from yeast to mammals that plays a crucial role in maintaining cellular homeostasis. Autophagy has numerous functions, such as regulating differentiation, proliferation, and cell death, and functions in numerous cell types, including those comprising the cardiovascular system. Accumulating evidence shows that an autophagy deficiency in VSMCs contributes to atherosclerosis and restenosis [9]. Recent evidence indicates that autophagic activity is regulated by stimuli or stressors evoked during the course of vascular diseases, resulting in the modulation of the phenotype and viability of VSMCs. In particular, certain growth factors and cytokines, reactive oxygen species (ROS), and drugs trigger autophagy in smooth muscle cells. It seems that the fact that autophagy controls VSMC function supports the idea that modulating its activity may be a viable therapeutic option for preventing or mitigating vascular structure. Therefore, we hypothesized that autophagy effectively intervenes in the action of chemerin on the vascular system.

In the present study, the possible role of chemerin in SMC proliferation and migration *in vitro* and in a rat model of metabolic hypertension *in vivo* was examined to gain a better understanding of its role in vascular remodeling. Furthermore, we determined the potential regulatory function of autophagy on its action to explore the mechanism of chemerin's action on the vascular system.

Material and methods

Ethics statement

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Animal Ethics Committee of the 3rd Xiangya Hospital of Central South University. All efforts were made to minimize suffering.

Materials

The reagent sources were as follows: recombinant human chemerin (PeproTech, USA) and 3-methyladenine (Selleck, USA). The antibody sources were as follows: chemerin (Antibodies-online, Germany); CMKLR-1 (NOVUS, USA); LC-3 (CST, USA); Beclin-1 (abcam, USA); and β-actin (Sigma Co, USA), p-Akt (Santa Cruz, USA) p-mTOR (Santa Cruz, USA).

Metabolic hypertension model

Male Wistar rats weighing 250-290 g were supplied by the Beijing Vital River Laboratory Animal Technology Co Ltd. After 7 days of acclimatization, the rats were randomly assigned to 2 groups. The control group (Ctrl, n = 10) received standard rat chow diet (animal experiment center of the Third Xiangya Hospital, Central South University, Changsha) with tap water, and the metabolic hypertension group (MH, n = 12) was fed with a high-salt, high-fat diet (HSHF, standard diet 58%, lard stearin 12%, yolk power 10%, sugar 5%, peanut 5%, milk power 5%, salt 4%, and sesame oil 1%) together with 20% fructose in the drinking water for 6 to 30 weeks. Based on the diagnostic criteria of the National Cholesterol Education Program Adult Treatment Panel III (NCEP ATP III), a diagnosis of metabolic syndrome can be made when 3 of 5 characteristics (obesity, high triglycerides, low HDL cholesterol, blood pressure, or fasting glucose) are present [10]. In our experiment, SBP is a necessary component, and metabolic hypertension can be diagnosed when at least 2 of the other 4 (obesity, high triglycerides, low HDL cholesterol, or fasting glucose) characteristics are present.

Body weight, blood glucose, serum lipid and SBP measurements

After fasting for 12 hours at night, weight by an electronic scale, blood glucose by an Abbott fast blood glucose meter, and serum lipids such as TC, TG, LDL and HLDL by an automatic biochemical analyzer were measured. The SBP was detected using a non-invasive computerized tail-cuff system (NIBP, Shanghai Alcott Biotech). Before the measurement, rats were warmed for 10-20 min at a temperature of 28°C to detect the steady arterial pulsations of the tail artery. A set of 3 measurements of SBP was averaged for each animal.

Tissue preparation

After feeding for 30 weeks, the thoracic aorta and mesenteric artery were removed. The tis-

sues were fixed in 4% paraformaldehyde solution and were then transferred to a 0.02% sodium azide solution for 24 hours. All of the tissues were saved at 4°C until paraffin embedding and were used for hematoxylin and eosin (H&E) staining and immuno-histochemistry and were soaked in a glutaraldehyde solution for VSMC detection under a transmission electron microscope.

Histological analysis

Hematoxylin and eosin staining was performed on 5-um-thick vessel sections for microscopy, and measurements of the wall-to-lumen ratio in the thoracic aorta and the mesenteric artery were made. At the same time, the fixed tissues were sectioned for immuno-histochemistry, using antibodies targeting chemerin (Antibodies-online, Germany), CMKLR-1 (NOVUS, America), LC3 (CST, America) and Beclin-1 (Abcam, England). The quantification was done using Image Pro Plus 6.

Transmission electron microscope

Thoracic aortas in a 2.5% glutaraldehyde solution were detected by electron microscopy at Xiangya Medical School.

Cell culture

HA-VSMCs (a gift from the central laboratory in Xiangya Hospital of Central South University) were cultured in high-glucose Dulbecco's modified Eagle's medium (DMEM, Gibco).

Cell proliferation assay

For the determination of viable cells, HA-VSMCs were plated in 96-well plates at a density of about 2000-2500 cells/well in DMEM without fetal bovine serum. Twenty-four hours after passaging, the cells were treated with chemerin with or without 3-MA or IGF as indicated in each experiment. The number of viable cells was determined using an MTT Assay kit (Amresco, USA) according to the manufacturer's instructions. The absorbance was read at 490 nm with a 96-well plate reader (Bio-Tek, USA). VSMC viability was calculated as the percentage of the average MTT absorbance in the matched control cells.

Scratch wound healing assay

HA-VSMC migration was measured using the scratch wound assay. Briefly, HA-VSMCs were plated in 6-well cell culture plates and were allowed to grow to 70% confluency. A p200 pipet tip was used to create a scratch on the bottom of each well, and the cells were stimulated with chemerin with or without 3-MA as indicated in each experiment. The distance between the edges of the scratch was measured immediately after scratching and again after 24 h. Migration was quantified and expressed as follows: % gap distance = [(distance immediately after scratching - distance 24 h after scratching)/distance immediately after scratching] ×100.

Immunofluorescence staining and confocal imaging

HA-VSMCs (1*10^5 cells per well) were plated in 6-well cell culture plates with a glass slide, and after the appropriate treatment, they were fixed in 4% paraformaldehyde solution for 20 min and were then stimulated with 0.3% PBST for 15 min. The cells were then incubated for 1 h at room temperature (RT) in a blocking solution (5% BSA in PBS), followed by incubation for 1 h with a 1:1000 dilution of anti-LC3 primary antibody at RT. A 1:200-diluted solution of goat anti-mouse IgG Alexa Fluor 555 secondary antibody (Amresco, USA) was used for incubation after treatment with the primary antibody. After three washes with PBS, cells were stained with DAPI for 10 min at RT. GFP-LC3 puncta were visualized using a Nikon TE-2000E2 microscope interfaced with a Nikon A1 confocal system. DAPI was used to visualize nuclei. Prior to imaging live cells, the cells were placed in phenol red-free DMEM supplemented with 25 mmol/L HEPES (pH 7.4). The individual fluorophores were illuminated as indicated below.

Western blot

HA-SMCs treated with various agents as indicated in each experiment were harvested, and the cells were homogenized with RIPA buffer (Kaiji, China). The protein concentration was measured by the BCA method. Equal amount (20 μg) of proteins were fractionated on a 10% SDS-PAGE and were transferred to a PVDF membrane. The membrane was blocked with PBST buffer containing 5% bovine serum albumin (BSA) for 2 h at room temperature and was then incubated with a specific antibody against LC-3 (1:1000) or Beclin-1 (1:1000) in PBST buffer containing 5% non-fat dry milk overnight at 4°C. Following secondary antibody incubation

Table 1. Physiological and metabolic characteristics between groups

	Ctrl Group (n = 10)			MH Group (n = 12)		
	0 W	6 W	30 W	0 W	6 W	30 W
BW (g)	271 ± 16	336 ± 27#	526 ± 42#	263 ± 14	387 ± 39*,#	598 ± 16*,#
SBP (mmHg)	127 ± 7	124 ± 7	128 ± 4	132 ± 8	144 ± 5*,#	186 ± 6*,#
FBS (mmol/L)	5.2 ± 0.6	5.7 ± 0.5	6.0 ± 0.4#	5.1 ± 0.4	5.6 ± 0.6	7.0 ± 0.5*,#
CHOL (mmol/L)	1.81 ± 0.25	1.76 ± 0.42	2.20 ± 0.56	1.80 ± 0.39	2.21 ± 0.20*,#	3.36 ± 0.46*,#
TC (mmol/L)	0.54 ± 0.08	0.66 ± 0.24	1.03 ± 0.12#	0.61 ± 0.20	0.99 ± 0.27*,#	2.28 ± 0.42*,#
HDL-C (mmol/L)	0.83 ± 0.15	0.70 ± 0.13	1.19 ± 0.24#	0.79 ± 0.12	0.50 ± 0.09*,#	0.52 ± 0.09*,#
LDL-C (mmol/L)	0.21 ± 0.05	0.28 ± 0.04#	0.36 ± 0.10#	0.25 ± 0.08	0.26 ± 0.09	0.40 ± 0.08#

Notes: *P<0.05 compared to control group; #P<0.05 compared to baseline in same group.

(1:2000), the signal was visualized using a Typhoon scanner (GE Healthcare, USA) after exposure to ECL detection reagent. β-actin was used as an internal control. The specific signals were quantitated by the Image J program, and the data are presented as the fold change compared to the vehicle control after normalizing the protein input based on β-actin levels.

Statistical analysis

The data are presented as the means ± SEM. One-way analysis of variance (ANOVA) following post hoc Student-Newman-Keuls test was used to determine the differences among multiple groups. A *p* value of less than 0.05 was considered statistically significant.

Results

Intimal hyperplasia in thoracic aorta and mesenteric artery in MH rats

To determine whether the thoracic aorta and mesenteric artery underwent remodeling in MH rats, the rats were fed a high-sucrose/high-fat diet to create a metabolic hypertension model in rats. Compared to control rats, MH rats showed a marked increase in the body weight, SBP, total cholesterol (CHOL) and triglyceride (TC) and a decrease in HDL-C after they were fed for 6 wk and 30 wk. The fasting glucose increased only at 30 wk but not at 6 wk. MH rats showed a similar LDL-C level compared to the control group (**Table 1**). Based on the diagnostic standard of MH listed above, we created an MH rat model after feeding for 6 wk, with a success rate of 70%.

Hematoxylin-eosin was used to examine the morphology of the thoracic aorta and mesenteric artery. The result showed that a thicker wall and a higher ratio of medial thickness/internal diameter were found in both of the arteries obtained from MH rats compared to the control group (**Figure 1A-D**). It is interesting that the effect was observed at 30 wk but not at 6 wk.

Upregulated chemerin/CMKLR-1 expression in the thoracic aorta and mesenteric artery in MH rats

To examine the difference in the expression of chemerin and CMKLR-1 in the thoracic aorta and the mesenteric artery between control and MH rats, immunohistochemistry analysis was used. Compared with control rats, the expression of chemerin and CMKLR-1 significantly increased both in the thoracic aorta and mesenteric artery after the rats were fed for 30 wk (P<0.01) but not for 6 wk (**Figure 2A-D**).

Increased autophagosome and up-regulated autophagy-related proteins in MH rats

To examine the autophagy level in MH rats, we first assessed the expression of autophagy in the thoracic aorta and mesenteric artery by examining the specific autophagic marker microtubule-associated protein light chain 3 (LC3) (**Figure 3A**, **3B**) and the autophagy-related protein Beclin-1 formation (**Figure 3C**, **3D**). Compared to the control group, LC3 expression in the thoracic aorta and mesenteric artery, as analyzed by western blot, was significantly increased by 2.5-fold and 4.6-fold, respectively, after feeding for 30 wk in the MH group as shown in **Figure 3E**. As shown in **Figure 3F**, Beclin-1 expression in the thoracic aorta and mesenteric artery was also significantly increased by 1.7-fold and 2.2-fold, respectively,

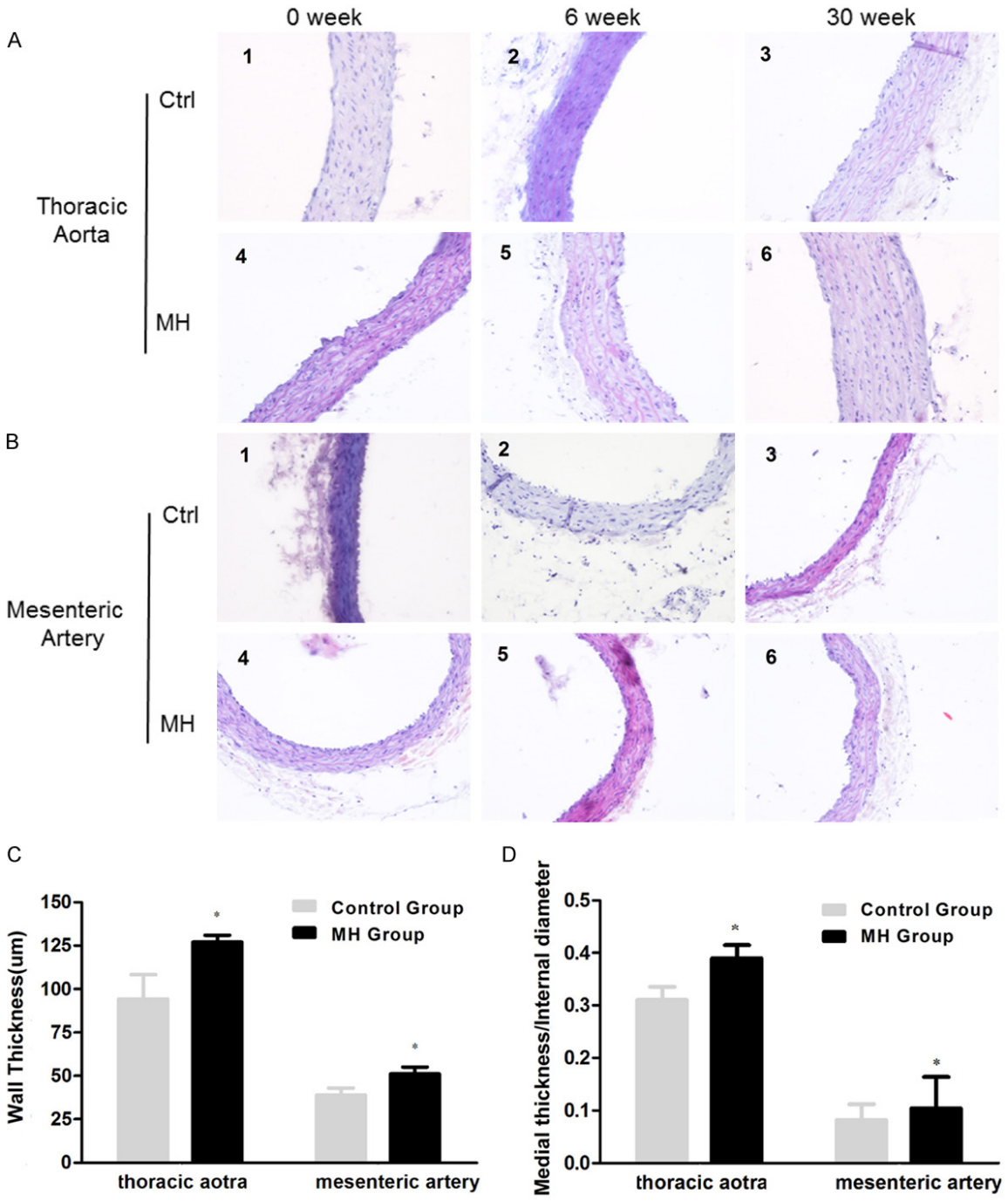
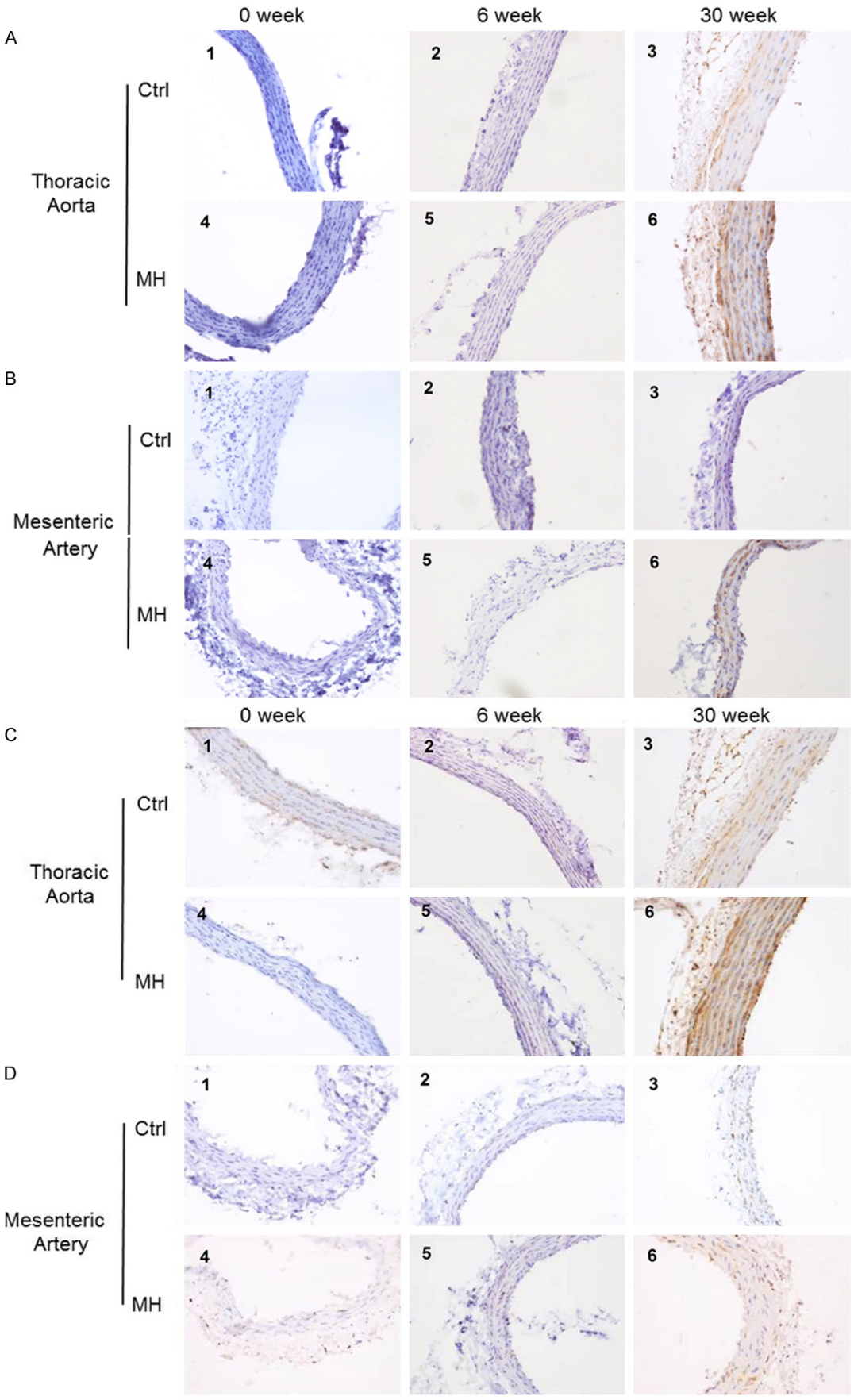


Figure 1. Morphological changes in the thoracic aorta and mesenteric artery in rats. Hematoxylin-eosin staining: (A, B) 22 male Wistar rats were randomly fed with a standard diet or a high-sucrose/high-fat diet for 6 wk or 30 wk. The thoracic aorta and mesenteric arteries were collected, and the morphological analysis of the arteries was examined by H&E staining. (C, D) Quantification of the wall thickness (IMT) and the ratio of the media thickness and the internal diameter (MT/ID) was performed. The wall thickness and the ratio of MT/ID were higher in MH rats compared with that of the control rats at 30 wk (*$P<0.05$; $n = 5$). All of the values are denoted as the mean ± SEM. Magnification = ×200.

after feeding for 30 wk in the MH group compared to the control group. However, no such effect was observed in MH rats fed for 6 wk.

Although LC3 formation is a validated marker of autophagy and Beclin-1 is an autophagy-related protein, changes in the ultrastructure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Thoracic Aorta
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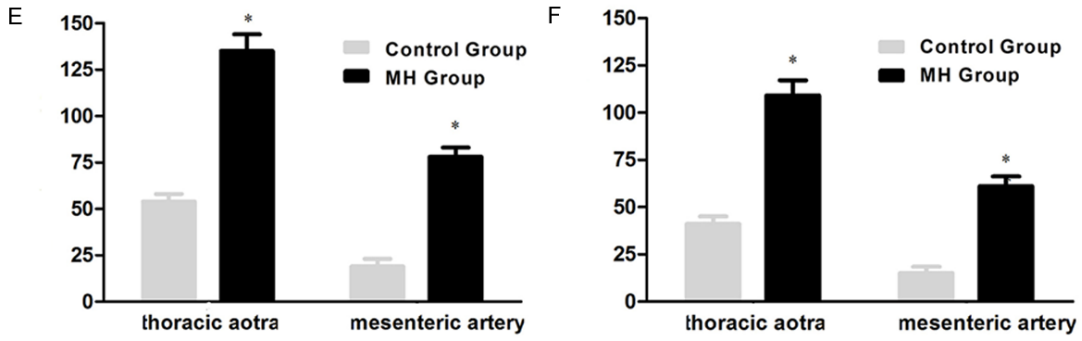


Figure 2. Chemerin/CMKLR-1 expression in the arteries of rats. Immunohistochemical analysis: (A, B) 22 male Wistar rats were randomly fed with a standard diet or a high-sucrose/high-fat diet for 6 wk or 30 wk. Chemerin expression was detected by IHC at 6 wk and 30 wk. (C, D) CMKLR-1 expression was detected in the thoracic aorta and mesenteric artery by IHC at 6 wk or 30 wk. (E) Quantification of chemerin expression by Image pro plus software from Panel (A and B). (F) Quantification of CMKLR-1 expression by Image pro plus software from panels (C and D). Compared with control rats, chemerin or CMKLR-1 was significantly up-regulated in the thoracic aorta and mesenteric artery in MH rats. *P<0.05 vs. the control group. All of the values are denoted as the mean ± SEM. Magnification = ×200.

showing the formation of autophagosomes is considered the "gold-standard" for documenting autophagy. Hence, to examine ultrastructural changes, the cytoplasm of the media of the SMCs in the thoracic aorta was visualized by transmission electron microscopy. Transmission electron micrographs showed that SMCs in the thoracic aorta of MH rats had increased single-membrane autophagic vacuoles (some containing electron-dense material) as well as early double-membrane vacuoles (**Figure 4A-F**).

Collectively, these structural changes are in concordance with our immunological and fluorescence-imaging data and suggest that the autophagic program was increased in MH rats.

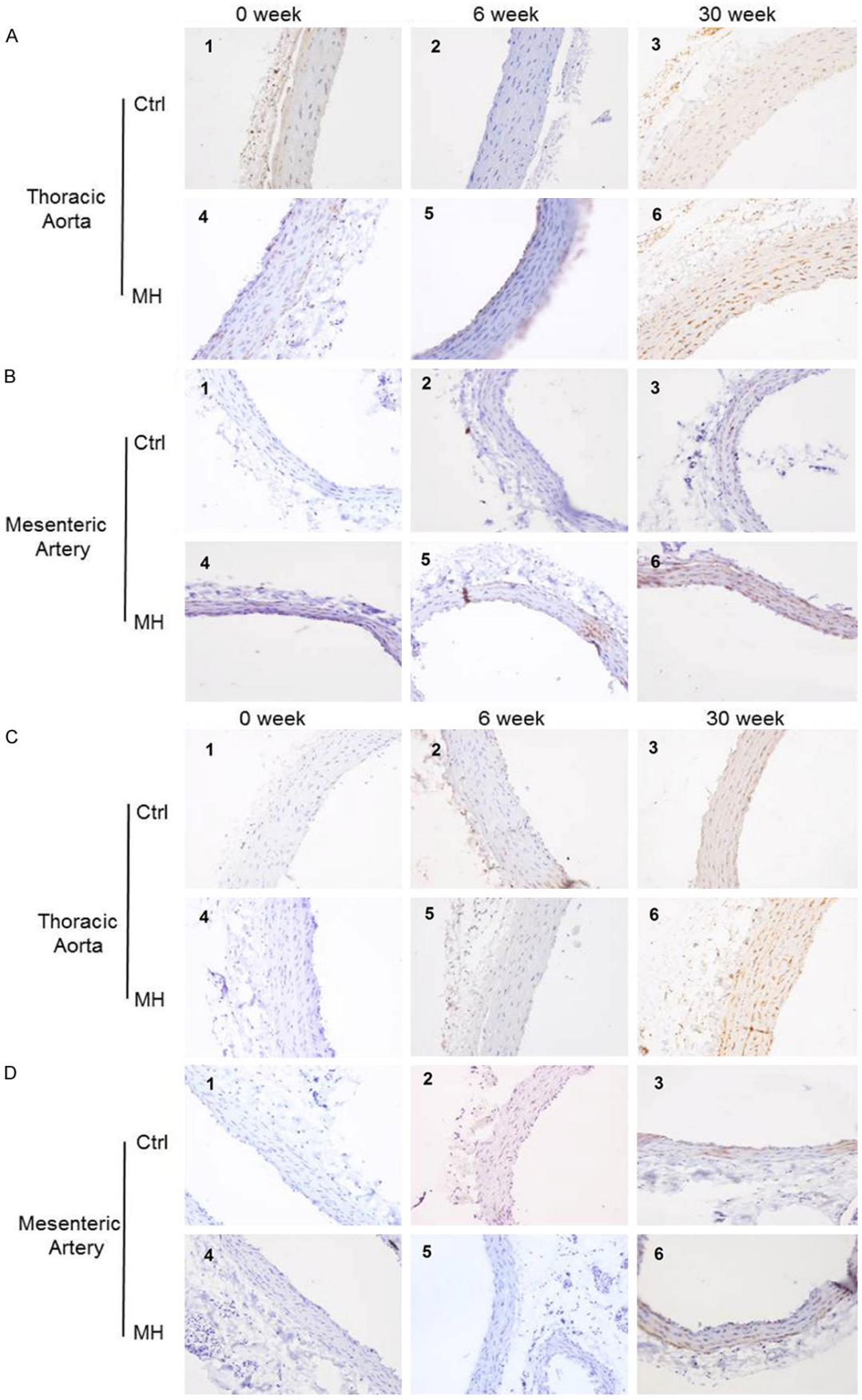
Chemerin promotes cell proliferation and cell migration in HA-SMCs

The impact of chemerin on HA-SMCs in vitro was evaluated using a scratch wound healing assay to assess cell migration. SMCs were treated in the presence or absence of chemerin for 24 h to view and quantify the impact of the protein on the migration rate of cells into the denuded area of the culture. Compared to the control group, it appeared that the closure of the scratch was more complete in SMCs exposed to chemerin at doses of 10, and 100 ug/L (**Figure 5A**, **5B**), indicating that the presence of chemerin impacted the cell migration rate in culture. An observed dose-dependent improvement in culture migration was observed in the presence of chemerin, which exhibited a 48.7% and 78.9% improvement in wound closure at doses of 10 and 100 ug/L, respectively, relative to untreated control cells, by 24 hours (**Figure 5B**). However, no statistical difference was observed between SMCs treated with vehicle control and chemerin at a dose of 1 ug/ul.

To determine whether chemerin affects cell proliferation in HA-SMCs, the cells were treated with various doses of chemerin. As shown in **Figure 5C**, treatment with chemerin at doses ranging from 0.1 to 100 ug/ul for 12 to 48 h produced a dose- and time-dependent induction of cell proliferation as evidenced by the increase in viable cell number. The viable cell number in the 100 ug/L-treated chemerin group was significantly increased compared to the control group (P<0.01). After 24 h of chemerin treatment, the viable cell number increased by 5.8%, 41.8%, 58% (P<0.05), and 71% (P<0.05) compared with the control at doses of 0.1, 1, 10, and 100 ug/L, respectively. After 48 h of chemerin treatment, the viable cell number increased by 40%, 56%, 55% (P<0.01), and 56.2% (P<0.01) compared with the control at doses of 0.1, 1, 10, and 100 ug/L, respectively.

Chemerin induces cell proliferation and migration in HA-VSMCs via autophagy

Up-regulation of chemerin and autophagy were both observed in the MH animal model, which shows a more serious vascular remodeling. Therefore, we hypothesized that autophagy


A
0 week
6 week
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Thoracic Aorta
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MH
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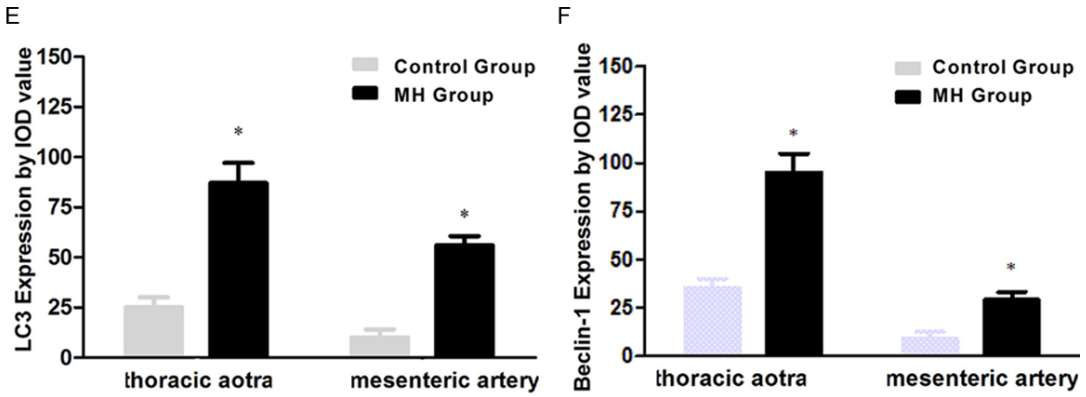

Figure 3. Expression of autophagy-related proteins in VSMCs of the thoracic aorta and mesenteric artery of rats. Immunohistochemical analysis: (A, B) 22 male Wistar rats were randomly fed with a standard diet or a high-sucrose/high-fat diet for 6 wk or 30 wk, and then the thoracic aorta and mesenteric arteries were collected. LC3 expression was detected in the thoracic aorta and mesenteric artery by IHC at 6 wk or 30 wk. (C, D) Beclin-1 expression was detected in the thoracic aorta and mesenteric artery by IHC at 6 wk or 30 wk. (E) Quantification of LC3 expression from panels (A and B). (F) Quantification of LC3 and Beclin-1 expression from panels (C and D). Compared with control rats (n = 8), LC3 or Beclin-1 was significantly up-regulated in the thoracic aorta and mesenteric artery in MH rats (n = 9). * P<0.05 vs. the control group. All of the values are denoted as the mean ± SEM.

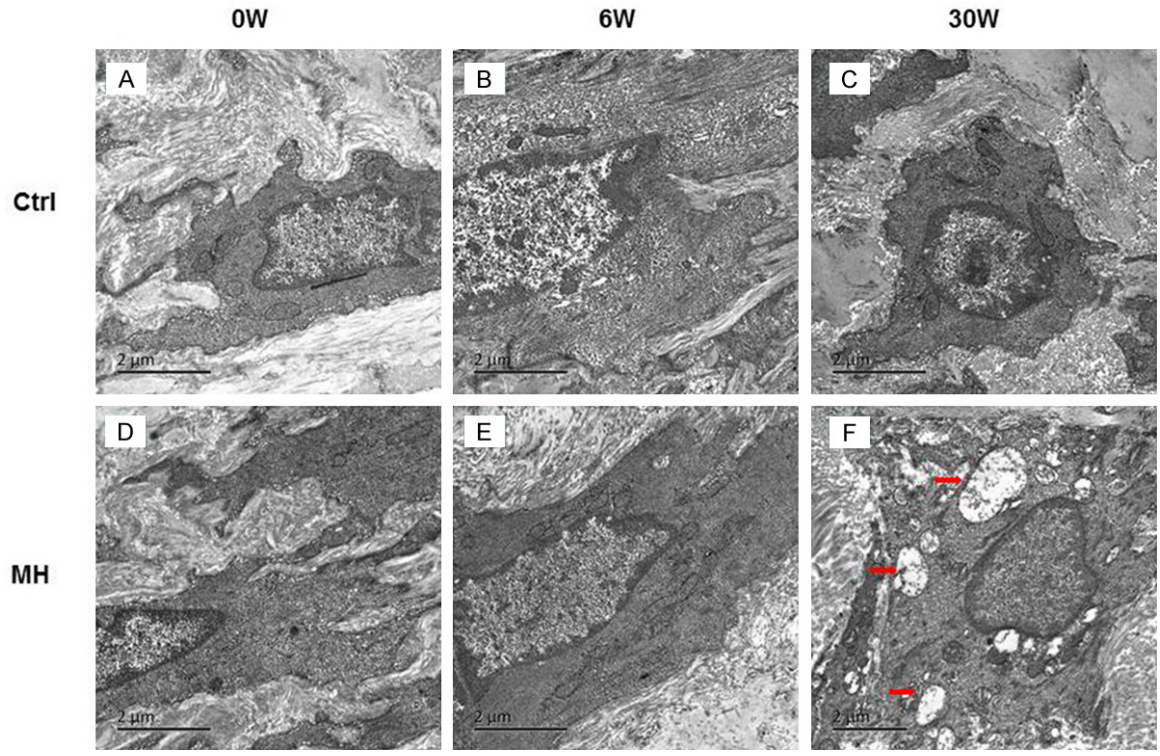

Figure 4. Autophagosome formation and extensive vacuolization in VSMCs of the thoracic aorta in rats. Microscopic evaluation of autophagy and ultrastructure in VSMCs of the thoracic aorta: (A-F) Representative transmission electron micrographs of cells in the thoracic aorta in the control group (A-C) or MH group (D-F). In (F), the light red arrows indicate the autophagic vacuoles or early double membrane structures. Magnification = ×200.

may be important for the function of chemerin on vascular remodeling. To test this hypothesis, we first assessed the effects of chemerin on autophagy in VSMCs by examining the specific

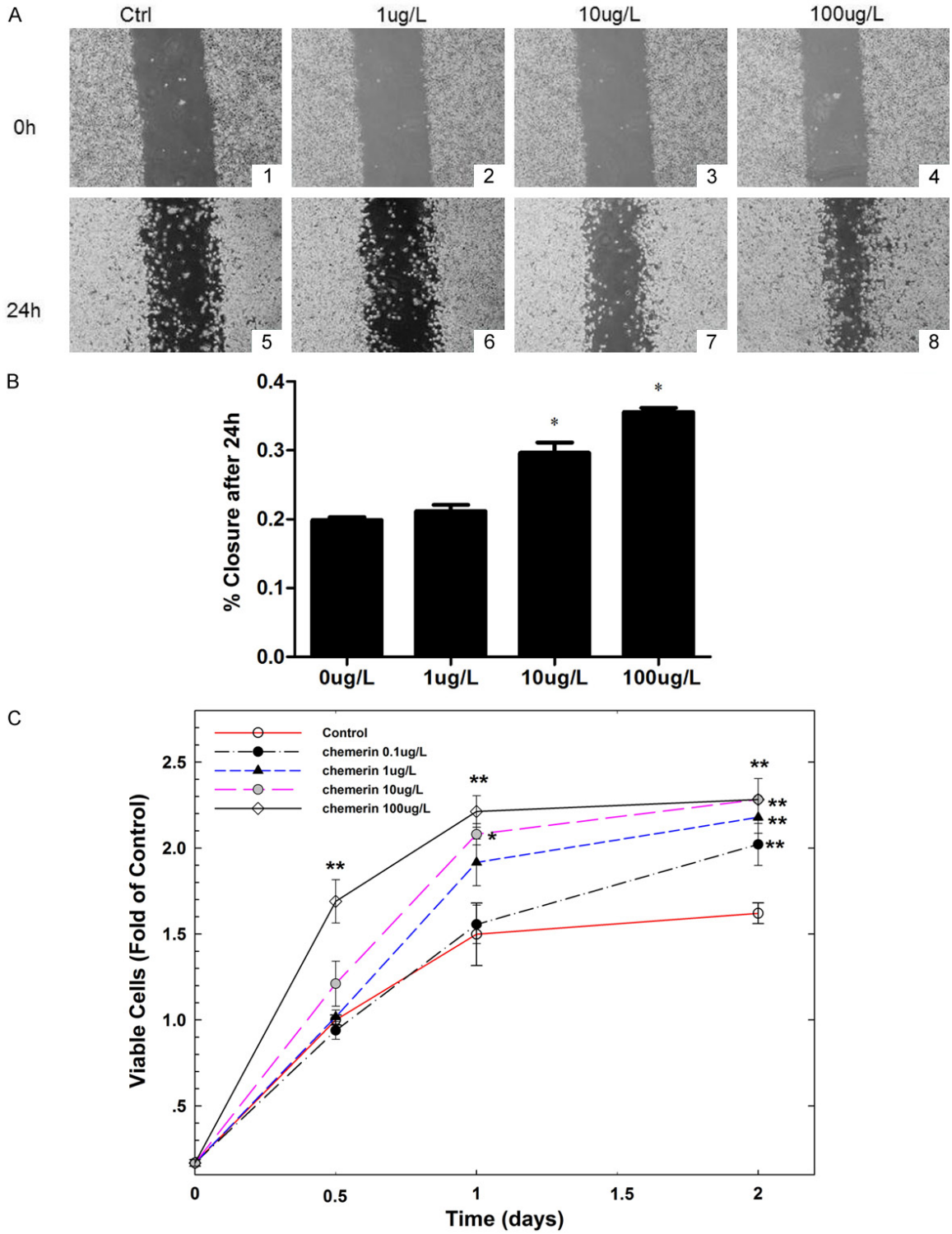

Figure 5. Effect of chemerin on cell proliferation and cell migration in VSMCs. Scratch wound healing assay: (A) VSMCs were plated in 6-well plates, and a straight line was scratched to create a cross in each well. The cells were treated with a vehicle control, chemerin 1 ug/L, 10 ug/L or 100 ug/L as indicated for 24 hours. The gap distance was evaluated using Image J software. (B) Quantification of gap distance closure from panel (A). *P<0.05 vs. the control group. MTT assay: (C) VSMCs plated in 96-well plates were treated with vehicle control (0), chemerin 0.1 ug/L, 1 ug/L, 10 ug/L or 100 ug/L as indicated for 12 hours, 24 hours or 48 hours. The viable cells number was determined at the end of experiments by the MTT assay. The data are expressed as the fold of the corresponding media control of each experiment. **P<0.01, *P<0.05 vs. the control group at the corresponding time. All of the values are denoted as the mean ± SEM.

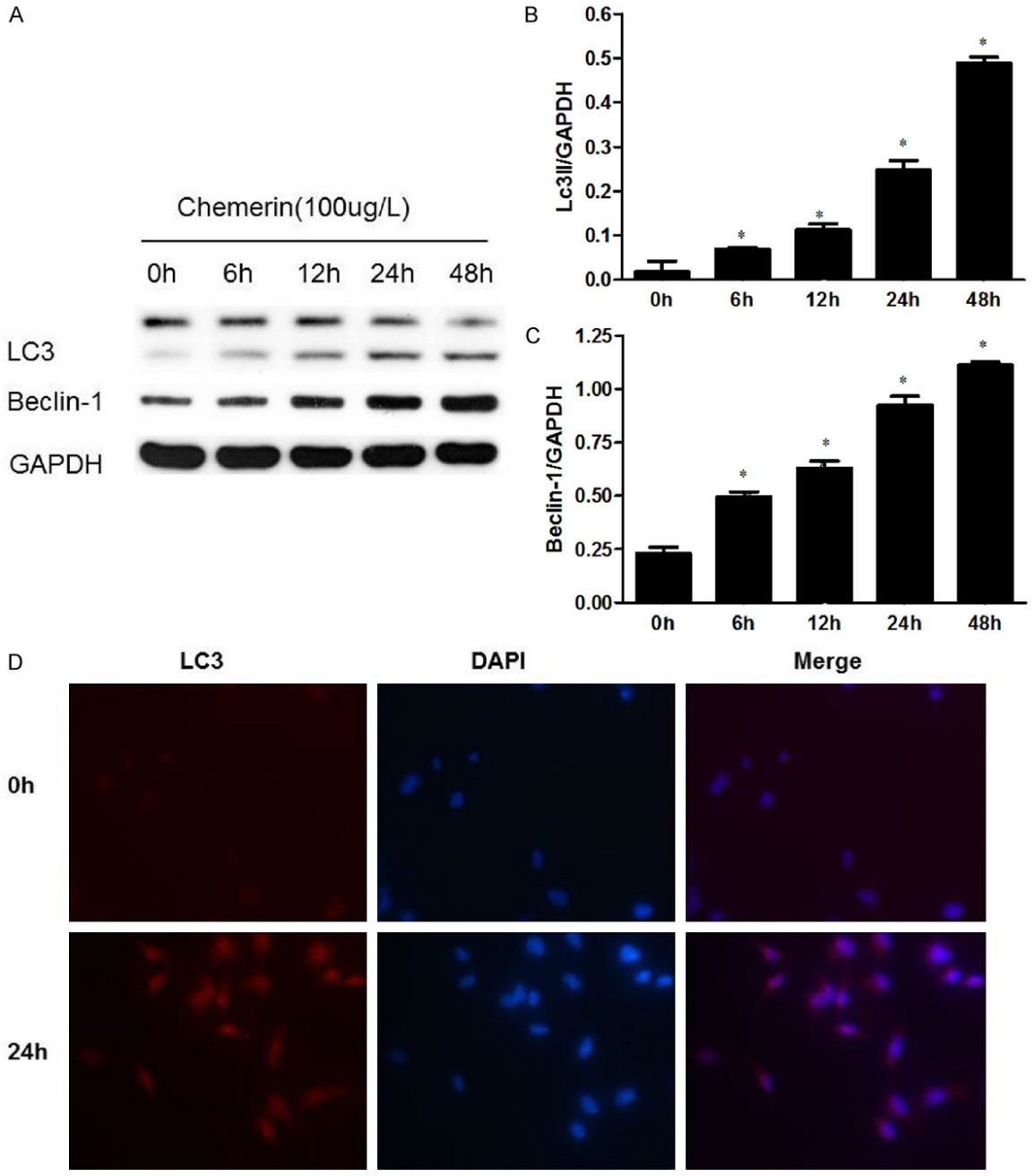

Figure 6. Effect of chemerin on autophagy in VSMCs. Immunoblot analysis: (A) VSMCs were stimulated with chemerin (100 ug/ul) for 6 h, 12 h and 24 h. The abundance of LC3 and Beclin-1 was detected by western blotting; (B) Quantification of LC3 expression from panel (A), the data was expressed as the fold of the GAPDH. (C) Quantification of Beclin-1 expression from panel (A), the data was expressed as the fold of GAPDH. Immunofluorescence analysis: (D) VSMCs were treated with control or chemerin for 24 h, and LC-3 expression was detected by immunofluorescence. *P<0.05 vs. 0 h; All of the values are denoted as the mean ± SEM. Magnification = ×200.

autophagic marker LC3 and the autophagy-related protein Beclin-1 formation. As shown in **Figure 6A-C**, chemerin stimulation caused a time-dependent increase in LC3 and Beclin-1 formation. A significant increase in LC3 and Beclin-1 was observed 6 h after chemerin stimulation, and maximal increases in LC3 and Beclin-1 were obtained 48 h after chemerin treatment, suggesting that chemerin promotes autophagy. In addition, immunofluorescence

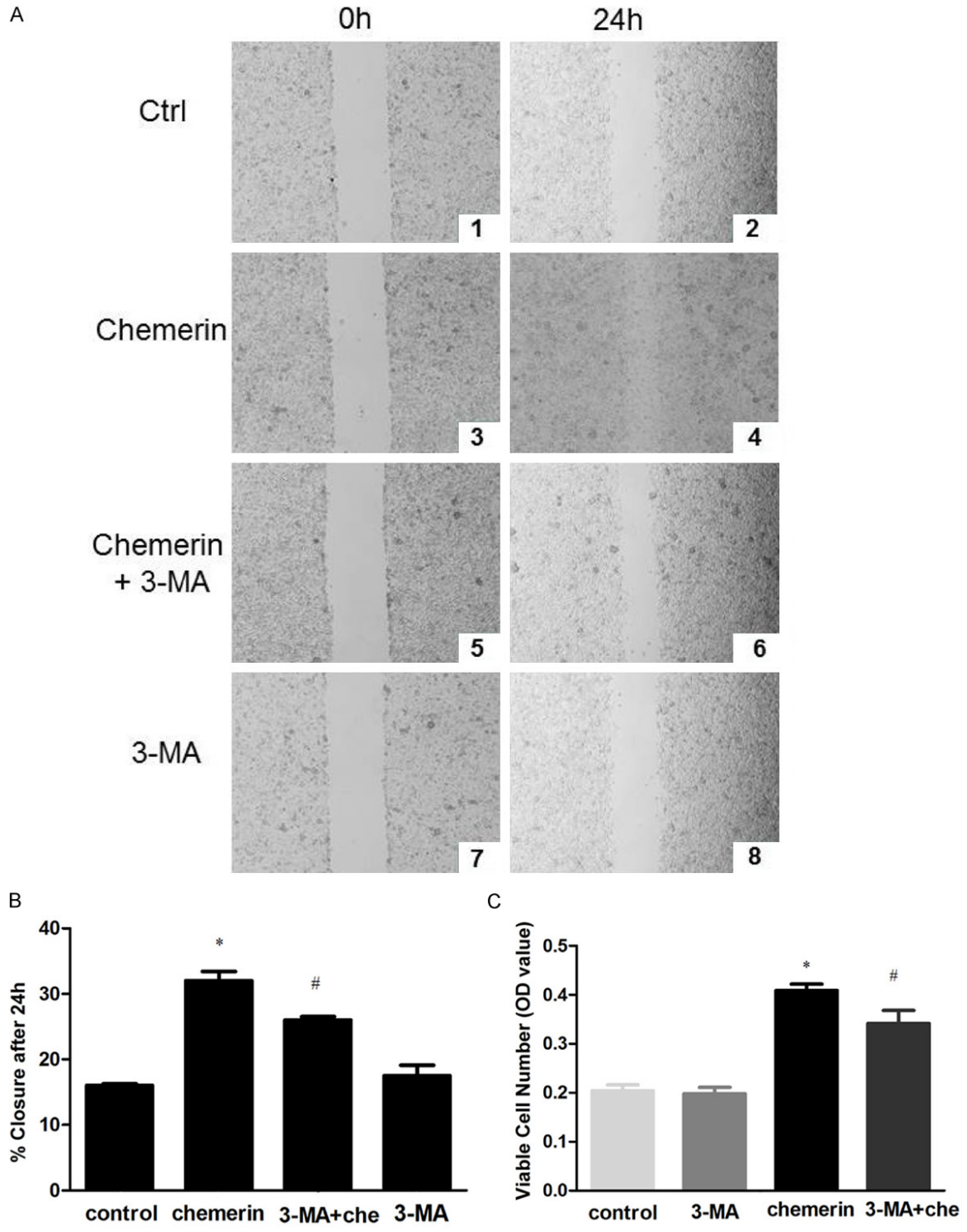

Figure 7. Effect of the autophagy inhibitor 3-MA on chemerin-induced cell proliferation and migration in VSMCs. Scratch wound healing assay: (A) VSMCs were plated in 6-well plates and were pre-treated with or without 3-MA (5 mM, 30 min), and a straight line was scratched to create a cross in each well. The cells were treated with vehicle control or chemerin 100 ug/L as indicated for 24 hours. The gap distance was quantitatively evaluated using Image J software. (B) Quantification of gap distance closure from panel (A). MTT assay: (C) VSMCs were pre-treated with or without 3-MA (5 mM, 10 min) in 96-well plates and were treated with vehicle control or chemerin 100 ug/L as indicated for 24 hours. The viable cell number was determined at the end of the experiments by the MTT assay. *P<0.05 vs. the control group. #P<0.05 vs. the chemerin group. Magnification = ×200. All of the values are denoted as the mean ± SEM.

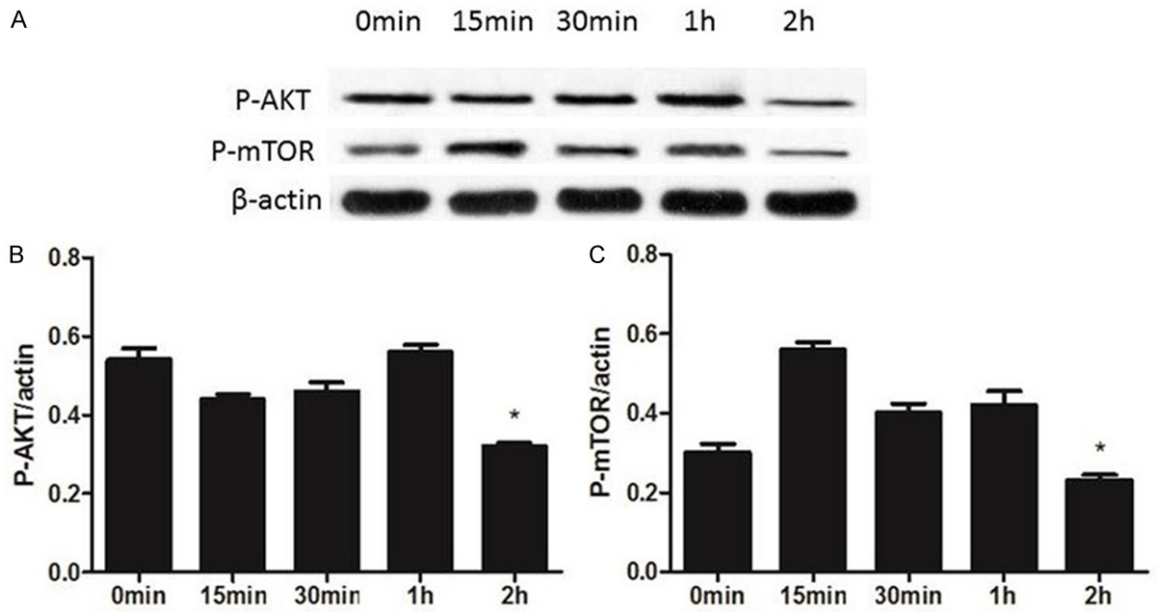

Figure 8. Chemerin down-regulated AKT and mTOR expression in HA-VSMC. Immunoblot analysis of VSMCs after chemerin treatment: (A) Representative Western blots of p-Akt and p-mTOR phosphorylation in VSMCs treated with vehicle control or chemerin 100 ug/L as indicated for 0 min, 15 min, 30 min, 1 hour or 2 hours. Each phospho protein was normalized to its non-phosphorylated total protein band intensity. (B) Quantification of AKT changes in band intensity from groups shown in panel (A) *P<0.05 vs. the 0 min time point. (C) Quantification of mTOR changes in band intensity from groups shown in panel (A). *P<0.05 vs. the 0 min time point.

was further utilized to locate LC3 expression after chemerin treatment for 48 h (**Figure 6D**). Compared to the control group, more LC3 particles were observed, and these particles were only located in the cytoplasm, which was verified by merging the LC3 particles with DAPI.

To determine whether the action of chemerin is mediated via autophagy in SMCs, cells were pretreated with 5 mM 3-methyladenine (3-MA) for 30 min and were then subsequently cultured with or without chemerin (100 ug/L) for 24 h. As shown in **Figure 7A**, **7B**, chemerin-induced cell migration was blocked by addition of 3-MA.

We further examined the mechanism by which chemerin induces cell proliferation in SMCs. Pretreatment with 3-methyladenine (3-MA) (5 mM), a selective PI3K inhibitor and also an autophagosome formation inhibitor, significantly blocked the chemerin (100 ug/L)-induced increase in viable cell number (**Figure 7C**).

Chemerin down-regulates AKT and mTOR expression in HA-VSMC

Activation of autophagy in mammalian cells usually involves the inhibition of the mammalian target of rapamycin (mTOR), an important signaling molecule necessary for the growth and proliferation of cells. During autophagy activation, mTOR is a downstream target of multiple signaling pathways such as the Akt signaling pathway and the AMPK pathway, which inhibit and activate autophagy, respectively. We found that chemerin treatment inhibited both Akt and mTOR phosphorylation level despite the induction of autophagy, which reached its maximum effect after 2 h treatment (**Figure 8**). It is suggested that chemerin-induced autophagy may be mediated by the inhibition of mTOR and Akt.

Discussion

This is the first study to identify a robust animal model of metabolic hypertension required for preclinical development studies. The present study demonstrates a novel role of chemerin in regulating vascular remodeling. We found that chemerin, which promotes the development of VSMC proliferation and migration, was a robust inducer of autophagy and pharmacological inhibition of autophagy by 3-MA blocked this effect.

Recently, numerous studies have shown that metabolic abnormalities alone could contribute

to the pathogenesis of hypertension, and some experts proposed a new classification of the etiology of hypertension and introduce a concept of metabolic hypertension [2]. The common clinical types of metabolic hypertension include obesity-related hypertension, diabetes-induced hypertension, familial dyslipidemia-associated hypertension, metabolic syndrome, hypertension with hyperhomocysteinemia, hypertension with hyperuricemia and salt-sensitive hypertension.

Currently, numerous approaches have induced MS in animals in order to allow researchers to study hypertension in MS. The animal models can be divided into three groups: genetic models, drug-induced models and dietary-induced models of MS. Dietary-induced models are used extensively and are closer to human reality, which presents the complexity of human disease and the association of the different cardiovascular risk factors. They include the use of a single type or a combination of diets, such as high-fructose, high-sucrose, high-fat, high-fructose/high-fat, or high-sucrose/high-fat diets [11]. A high-sucrose/high-fat/high-salt diet was chosen to build our MH model. Our results proved that MH rats showed a marked increase compared to the control group in body weight, SBP, CHOL and TC and a decrease in HDL-C after feeding for 6 wk, which was sustained to 30 wk. Based on the diagnostic standard of MH listed above, we built an MH rat model after feeding for 6 wk, with a success rate of 70%. Furthermore, the fasting glucose increased only at 30 wk but not at 6 wk. Londa et al [12] used a high-fat (21.4%)/high-fructose (25%) diet to feed Wistar rats, and the trend of the body weight was similar to our result, but the fasting glucose increased obviously from 8 wk to 24 wk compared to the control group. This may be explained by the insulin resistance state of the rats before diet inducement, and 20% high-sucrose may be harder to induce the fasting glucose than high-fructose. MH rats had a similar LDL-C level compared to the control group throughout our experiment, and one possibility is that the LDL level is related to cholesterol in the diet. A study showed that 10% cholesterol in the diet could not produce significant hypercholesterolemia [13]. However, no pure cholesterol was added in our experiment.

Arterial remodeling plays a key role in the pathogenesis of metabolic hypertension. The remodeling of resistant arteries raises peripheral resistance and stabilizes BP, and with regard to the large arteries, it increases their stiffness and creates a reflected wave, resulting in an increased pulse BP, systolic BP, and enhanced left ventricular hypertrophy [14].

Marchesi *et al* [15] examined New Zealand Obese (NZO) mice, a metabolic syndrome model, which had insulin resistance, hyperinsulinemia, and BP elevation compared with New Zealand black control mice. The NZO mice exhibited hypertrophic vascular remodeling of both the aorta and the mesenteric resistance arteries. Vascular remodeling was also found in a diet-induced MS animal model [16]. In our experiment, a thicker wall and a higher ratio of medial thickness/internal diameter were found in both the thoracic aorta and mesenteric artery obtained from MH rats compared to the control group. It is interesting that the effect was observed at 30 wk but not at 6 wk. These data suggest that metabolic hypertension deteriorates arterial remodeling in both the aorta and resistance vessel in a time-dependent manner.

Adipose tissue produces many adipokines, including chemerin, a chemoattractant for inflammatory cells that mediates its effects mainly through chemokine-like receptor 1 (CMKLR1). Chemerin is implicated in angiogenesis [6] and vascular inflammation [3], which are hallmarks of vascular injury in hypertension [7]. The expression of chemerin and CMKLR1 is identified in atherosclerotic coronary arteries and aorta [17] and in isolated mesenteric arteries [7]. In our experiment, we found that chemerin and CMKLR1 were widely up-regulated in the both the aorta and mesenteric resistant arteries in MH rats, which indicated that chemerin/CMKLR-1 might regulate the vascular system in MH.

Evidence shows that the migration of VSMCs to the intima and their proliferative capacity are crucial steps in vascular remodeling. In the present study, we aimed to elucidate the effects of chemerin on human aortic SMC proliferation and migration. The chemerin concentration used in the present study is within the pathophysiologically proper range [18]. The results showed that chemerin influenced SMC proliferation and migration in a time- and dose-dependent manner. The effect of chemerin is in accor-

dance with a previous study. Neves *et al* [19] found that chemerin stimulated human SMC proliferation (increased proliferating cell nuclear antigen expression [proliferation marker] and BrdU incorporation [proliferation assay]). Kunimoto *et al* [8], isolated SMCs from the mesenteric artery (MA), and the data demonstrated the stimulatory action of chemerin on MA-SMC proliferation and migration.

Autophagy is a regulated lysosomal pathway involved in the degradation and recycling of long-lived proteins and organelles within cells [20]. Evidence shows that autophagy is affected in numerous vascular disease states, including restenosis, atherosclerosis, and hypertension [21]. However, the autophagy activity in vessels of metabolic hypertension is unknown. In our present study, the structural changes such as arterial remodeling are in accordance with the immunological and fluorescence-imaging data, which suggests that the autophagic program increased in the arteries of MH rats. The immunoblotting results were confirmed by the detection of LC3-II accumulation and Beclin-1 expression. Beclin-1 is necessary for the induction and initiation of autophagosome formation. Once the autophagosomes fuse with lysosomes to form autophagolysosomes, Beclin-1, which is present on the inner autophagosomal membrane, is degraded by the lysosomal proteases. Subsequent studies identified LC3 and Beclin-1 as essential markers for autophagy. These findings suggest that there may be a close link between autophagy and vascular remodeling in MH.

Autophagy is documented in VSMC in response to various stimuli, resulting in the modulation of VSMC functions, including proliferation, migration, matrix secretion, contraction/relaxation, and differentiation. Each of these changes in VSMC functions plays a critical role in the development of vascular diseases. Therefore, we hypothesized that autophagy joins in vascular remodeling in MH rats via acting on SMCs. The role of autophagy in the VSMC phonotype is both complex and controversial. Autophagy is activated in VSMCs by multiple other conditions and signaling agents including starvation (particularly lack of amino acids), ROS accumulation, metabolic stress, hypoxia, reactive species, drugs, growth factors, and cytokines [22]. The form(s) of autophagy elicited by these species are not equal and have different consequences. Previous studies revealed that chemerin induces autophagy in endothelial cells and that the inhibition of autophagy suppresses chemerin-induced angiogenesis in HAECs [23]. It seems that chemerin may function as a stimulator of vascular remodeling via VSMCs. However, the underlying mechanism of its regulatory role in vascular remodeling remains largely unknown. Our findings indicated that chemerin induced autophagy in SMCs. Both the fluorescence of LC3 particles and immunoblotting of the expression of LC3-II and Beclin-1 revealed an increase in the formation of autophagosomes when SMCs were treated with chemerin. Furthermore, the action of chemerin on SMCs was significantly weakened by the autophagy inhibitor 3-MA. These results suggest that autophagy mediated chemerin-induced cell proliferation and migration in SMCs, which may contribute to vascular remodeling in hypertension.

In conclusion, we for the first time found that chemerin induced aortic smooth muscle cell proliferation via activation of autophagy, which may lead to vascular remodeling in metabolic hypertension. Further research is required to better understand the role of chemerin and its receptors in the pathophysiologic processes of vascular remodeling in vivo and their possible use as therapeutic targets.

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Disclosure of conflict of interest

None.

Address correspondence to: Dr. Xiaohong Tang, Department of Cardiology of The Third Xiangya Hospital, Central South University, 138 Tong-Zi-Po Road, Changsha 410013, Hunan, China. E-mail: 77037946@qq.com

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