Regular Article

Berberine Attenuates Vascular Remodeling and Inflammation in a Rat Model of Metabolic Syndrome

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Berberine is a natural product that shows benefits for metabolic syndrome (MS). However, the effects of berberine on the improvement of vascular inflammation and remodeling in MS remain unclear. This study aimed to investigate whether berberine could prevent vascular remodeling and inflammation in the MS condition. A rat model of MS was established, and MS rats were divided into two groups: MS group without berberine treatment, and MS+B group with berberine treatment (each group $n=10$). Ten normal Wistar rats were used as controls (NC group). Vascular damage was examined by transmission electron microscopy and pathological staining. Compared to the NC group, the secretion of inflammatory factors was increased and the aortic wall thicker in the MS group. The MS+B group exhibited decreased secretion of inflammatory factors and improved vascular remodeling, compared to the MS group. In addition, the levels of p38 mitogen-activated protein kinase (p38 MAPK), activating transcription factor 2 (ATF-2) and matrix metalloproteinase 2 (MMP-2) were significantly decreased in the MS+B group compared to the MS group. In conclusion, our data show that berberine improves vascular inflammation and remodeling in the MS condition, and this is correlated with the ability of berberine to inhibit p38 MAPK activation, ATF-2 phosphorylation, and MMP-2 expression.

Key words metabolic syndrome; inflammation; vascular remodeling; berberine; rat

In addition to their individual effects, the combined effects of abdominal obesity, impaired glucose metabolism, dyslipidaemia and hypertension, which are together known as metabolic syndrome (MS), significantly increase the risk of cardiovascular disease (CVD).^{1,2} These risk factors may lead to changes in the macrovascular structure and function in MS patients, which may contribute to the development of CVD. Previous studies^{3–5} have shown that MS patients experience increased arterial intima-media thickness (IMT) and vessel endothelium stiffness and dysfunction, and these characteristics predict early pathological vascular changes. Thus, because of their high CVD-related morbidity and mortality, it is important to examine lifestyle factors, study the mechanisms of vascular remodeling, delay the progression of CVD with multifactorial intervention and effectively decrease cardiovascular end-point events in patients with MS.

Berberine is a quaternary ammonium salt from the protoberberine group of isoquinoline alkaloids. Several studies have reported the effects of berberine on inflammation and cardiac remodeling.^{6,7} However, the mechanisms by which berberine improves inflammation-mediated vascular remodeling in MS remain elusive.

Recent studies have focused on identifying important inflammatory markers of MS, and tumour necrosis factor- α (TNF- α) and interleukin-6 (IL-6)^{8,9} have been highlighted as key mediators of MS. Compared with controls, patients with MS show increased levels of C-reactive protein (CRP), IL-6, TNF- α and intercellular adhesion molecule (ICAM).^{10–12} In addition, p38 mitogen-activated protein kinase (p38 MAPK)

pathway, a branch of MAPKs signalling pathway, is characterised as a signal transmission of inflammation.¹³ In different cells, p38 MAPK can be activated by different stimuli, including high glucose, free fatty acids, cholesterol and proinflammatory molecules.^{14,15} The activation of p38 MAPK can regulate multiple gene transcription and expression, such as nuclear factor-kappa B (NF- κ B) and activating transcription factor 2 (ATF-2).^{16,17} To date, numerous studies have found that p38 MAPK activation is correlated with vascular remodeling.^{18–20}

The aims of the present study were to evaluate the effects of berberine on vascular inflammation and remodeling, and elucidate the underlying molecular mechanisms in rat model of MS with obesity, hypertension, insulin resistance and hypercholesterolaemia.

MATERIALS AND METHODS

Animal Study All animal care and experimental protocols complied with the National Institutes of Health guidelines and with Shandong University's guidelines for the care and use of laboratory animals. Throughout the experiment, the rats were housed in a room under controlled temperature (23–25°C) and humidity conditions and with a 14h light/10h dark cycle.

Forty male Wistar rats (6 weeks old) were randomly allocated into either the control group ($n=10$) or the model group ($n=30$). The control rats were fed standard chow and tap water. The model group rats were fed with obese-diet (2% fat, 10% sucrose, 6% salt and 8% defatted milk powder) and high

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sugar drinking (20% sucrose solution). After 16 weeks, 24-h fasting blood samples were collected, and final body weight (BW) and systolic blood pressure (SBP) were measured. Compared with the controls, the obese-fed rats with MS were defined using criteria analogous to Adult Treatment Panel III (ATP III) as ≥ 3 of the following: hypertriglyceridemia, low high-density lipoprotein-cholesterol (HDL-C), high fasting glucose, excessive waist circumference, and hypertension. Twenty rats had developed MS and they were further divided into the following two groups: the MS group ($n=10$), which continued to consume the obese-diet, and the MS+B group ($n=10$), which continued the obese-diet and received treatment with berberine 50 mg/kg/d^{-1} *via* gavage. The treatments lasted for 8 weeks.

Body weight and heart rate were measured every 2 weeks in the morning throughout the experiment. Similarly, systolic blood pressure (SBP) and heart rate (HR) was measured every 2 weeks *via* the tail-cuff method using a rat tail manometer provided by Japanese and Chinese Friendly Hospital (RBT-1; Beijing, China), as described previously.²¹⁾ Rats were anesthetized by intraperitoneal injection of pentobarbital sodium (40 mg/kg). Onemilliliter blood were collected from the jugular sinus every 2 weeks after a 12-h fast, and serum concentrations of triglycerides (TGs), cholesterol, HDL-C, low-density lipoprotein-cholesterol (LDL-C), fasting blood glucose (FBG), fasting insulin (FINS) and inflammatory factors were quantified by the Department Clinical Laboratory (Qilu Hospital affiliated with Shandong University, Jinan, China). The homeostasis model assessment-estimated insulin resistance (HOMA-IR) index was calculated as follows²²⁾: $(\text{FBG} \times \text{FINS})/22.5$.

At the end of the experiment, the rats were sacrificed using an overdose of pentobarbital (80 mg/kg), and the thoracic aorta were aseptically excised for subsequent measurement.

Ultrastructural Observation The aortic tissues (approximately $0.5 \times 1 \times 0.5\text{ mm}$) from each group were fixed with 2% glutaraldehyde overnight, washed three times with 0.2 mol/L phosphate buffer, fixed with 1% osmium tetroxide, washed again with 0.2 mol/L phosphate buffer and dehydrated with different concentrations of ethanol. The tissues were cut as 50–70 nm thick sections, and the ultrastructure of the aorta was observed using transmission electron microscopy (TEM; H-7000FA, Hitachi, Tokyo, Japan).

Haematoxylin–Eosin (HE) Staining Briefly, the upper part of the descending aorta was fixed in 4% formaldehyde, embedded in paraffin, and cut as 5- μm thick sections. The paraffin sections were dewaxed and rehydrated, and microwave antigen retrieval was performed. These sections were stained with HE staining. Image analysis (Image-Pro Plus, Version 5.0, Media Cybernetics, Silver Spring, MD, U.S.A.) was used to measure the circumference of the vessel wall and of the outer and inner boundaries of the media, and medial wall thickness (Mt) was calculated.²³⁾ Measurements of all parameters from the three aortic sections per rat were averaged.

Immunohistochemical Staining Paraffin-embedded 5- μm sections of aortic tissue were hydrated, and antigen retrieval was performed by incubating sections with 0.01 mol/L citrate buffer (pH 6.0) at 98°C for 10 to 15 min. Endogenous peroxidase activity was quenched using incubation in phosphate buffered saline (PBS) containing 3% hydrogen peroxide. The sections were blocked with goat serum for 30 min, followed by

incubation with primary antibodies for vascular cell adhesion molecule-1 (VCAM-1) and ICAM-1 (Abcam, Cambridge, MA, U.S.A.) overnight at 4°C. The sections were then incubated with secondary-conjugated immunoglobulin G (IgG) antibody for 1 h at 37°C, and diaminobenzidine (DAB) substrate was applied. Negative controls were incubated with PBS instead of primary antibody. The sections were viewed on a confocal FV 1000 SPD Laser Scanning microscope (Olympus, Japan). Three sections per rat and four areas from each arterial section were analysed. The relative levels of VCAM-1 and ICAM-1 were analyzed by comparing the intensity of VCAM-1 and ICAM-1 immunostaining visualized with DAB to an unstained reference to calculate the relative optical density (ROD) using the ImageJ software.

Quantitative Real-Time Reverse Transcription Polymerase Chain Reaction (RT-PCR) Total RNA was isolated from each rat aorta using TRIzol reagent (Invitrogen, Carlsbad, CA, U.S.A.). The quality of RNA was determined using spectrophotometry (DU®800, Beckman, Palo Alto, CA, U.S.A.). The total RNA was processed using a two-step procedure as described in the SYBR RT-PCR Kit (Perfect Real Time, TaKaRa, Shiga, Japan). The sequences of the oligonucleotide primers are described in Table 1. The primers were synthesised by BioAsia Corp. (Shanghai, China). The efficiency of the PCR was assessed with serial dilutions of a sample of cDNA from the normal control group. Each experiment was performed in duplicate, and the data were analysed using Light Cycler Software 4.0 (Roche Diagnostic, Indianapolis, IN, U.S.A.).

The relative changes in gene expression were analysed with the $2^{-\Delta\Delta C_t}$ method.²⁴⁾ The specificity of the RT-PCR was assessed by analysing melting curves and by gel electrophoresis of the amplicons.

Western Blot Analysis Equal amounts of protein (50 μg) were fractionated on 10% sodium dodecyl sulfate (SDS)-polyacrylamide gels in running buffer (25 mM Tris, 192 mM glycine, 0.1% (w/v) SDS, pH 8.3) at 90 V and then electroblotted to nitrocellulose membranes. The membranes were blocked at 4°C with 5% non-fat milk in Tris-buffered saline (25 mM Tris, 137 mM NaCl and 2.7 mM KCl) containing 0.05% Tween-20 and then incubated overnight at 4°C with the following primary antibodies: P-p38 MAPK (rabbit, 1:400), P-ATF-2 (rabbit, 1:1000) and matrix metalloproteinase 2 (MMP-2) (rabbit, 1:500) (Santa Cruz Biotechnology Inc., Santa Cruz, CA, U.S.A.). Then the membranes were washed three times in TBS-T and incubated with horseradish peroxidase-conjugated goat anti-rabbit secondary antibody at room temperature for

Table 1. The Sequences of Oligonucleotide Primers

Gene	Sense and antisense
GAPDH	5'-TGCCAAGTATGATGACATCAAGAAG-3' 5'-AGCCCAGGATGCCCTTTAGT-3'
VCAM-1	5'-GCGAAGGAACTGGAGAAGACA-3' 5'-ACACATTAGGGACCGTGCAGTT-3'
ICAM-1	5'-TTTCGATCTCCGACTAGGG-3' 5'-AGCTTCAGAGGCAGGAAACA-3'
IL-6	5'-GTCAACTCCATCTGCCCTTC-3' 5'-ACTGGTCTGTTGTGGGTGGT-3'
TNF- α	5'-AGTCCGGGCAGGTCTACTTT-3' 5'-TGAGCCACAATTCCTTTCT-3'

Table 2. Metabolic Parameters at the End of 16 Weeks of Obese-Diet Feeding

	NC group (n=10)	MS group (n=10)	MS+B group (n=10)
Body weight (g)	510.09±21.38	552.79±20.08*	545.12±21.19*
SBP (mmHg)	101.72±3.59	137.81±7.32*	136.89±8.76*
Cholesterol (mmol/L)	1.41±0.19	1.69±0.13*	1.72±0.27*
Triglycerides (mmol/L)	0.83±0.23	0.85±0.28	0.84±0.26
Glucose (mmol/L)	4.83±1.23	7.81±0.69*	7.52±1.18*
Insulin (μIU/L)	13.68±4.62	24.15±5.21**	23.16±5.89**
HOMA-IR	3.08±1.38	8.19±2.21**	7.89±2.98**

SBP: systolic blood pressure; HOMA-IR: homeostasis model assessment-estimated insulin resistance. Values are shown as mean±S.D. * $p<0.01$, ** $p<0.001$ compared with the NC group.

Table 3. Metabolic Parameters in the Three Groups at the End of the Treatment Period

	NC group (n=10)	MS group (n=10)	MS+B group (n=10)
Body weight (g)	539.81±11.87	606.38±13.21**	558.84±16.42** ^{##}
SBP (mmHg)	101.60±3.95	138.30±6.75**	135.83±7.42**
Cholesterol (mmol/L)	1.50±0.09	1.92±0.13**	1.76±0.24** ^{##}
Triglycerides (mmol/L)	0.76±0.17	0.82±0.16	0.78±0.17
Glucose (mmol/L)	5.15±0.72	8.11±0.69**	7.11±0.57** ^{##}
Insulin (μIU/L)	15.74±3.14	29.70±4.68**	23.33±5.50** ^{##}
HOMA-IR	3.66±1.17	10.65±1.57**	7.40±1.91** ^{##}

SBP: systolic blood pressure; HOMA-IR: homeostasis model assessment-estimated insulin resistance. Values are shown as mean±S.D. ** $p<0.001$ compared with the NC group; ^{##} $p<0.01$, [#] $p<0.05$ compared with the MS group.

Fig. 1. The Expression of Serum Inflammatory Factors

Data are shown as means±S.D. ** $p<0.01$ vs. NC group, ^{##} $p<0.01$ vs. MS group.

2h. Immunoreactive bands were visualised with enhanced chemoluminescence and quantified by an image analyser (AlphaImager 2200, U.S.A.). Protein levels were normalised to β -actin levels.

Statistical Analysis Values are presented as mean±S.D. Results were compared using one-way ANOVA followed by a Tukey–Kramer *post-hoc* test. All statistical analyses were performed using the SPSS 17.0 software, and $p<0.05$ was considered significant.

RESULTS

Induction of MS and the Effects of Berberine on MS Related Disorders The MS model was achieved by feeding male Wistar rats obese-diet for 16 weeks. After 16 weeks, the MS rats developed hypercholesterolaemia, hyperglycaemia, hypertension and obesity compared with the NC group ($p<0.01$) (Table 2). The MS rats also developed insulin resistance, as characterised by a significantly increased HOMA-IR

compared with the NC group ($p<0.001$) (Table 2). No difference was observed in triglyceride levels between the two groups (Table 2). After 8 weeks of treatment with berberine, BW, FBG, cholesterol and FINS were significantly decreased in MS+B group compared to MS rats (Table 3).

Effects of Berberine on Inflammation We found that serum inflammatory marker CRP and cytokines TNF- α and IL-6 were increased in MS rats and that berberine effectively decreased their expression (Fig. 1).

ICAM-1 and VCAM-1 were more abundant in the aortic intima of the MS group compared to the NC group and were more weakly expressed in the MS+B group than in the MS group (Fig. 2).

According to real-time PCR, the aortic expression of IL-6, TNF- α , ICAM-1 and VCAM-1 mRNA in the MS rats was markedly increased ($p<0.01$) compared with the NC group (Fig. 3). Compared with the MS group, the rats in the MS+B group showed a significant decrease in IL-6, TNF- α , ICAM-1 and VCAM-1 mRNA expression after 8 weeks of treatment

Fig. 2. Immunohistochemistry Staining of ICAM-1 and VCAM-1 in Aortic Cross-Sections

A, D: NC group ($n=10$); B, E: MS group ($n=10$); C, F: MS+B group ($n=10$). The panels show representative micrographs of immunohistochemistry staining in the 3 groups. Original magnification $\times 400$. G: Quantitative analysis of ICAM-1 and VCAM-1 protein levels. Data are shown as means $\pm$ S.D. ** $p < 0.01$ vs. NC group; ## $p < 0.01$ vs. MS group.

($p < 0.01$; Fig. 3).

Effects of Berberine on Vascular Remodeling In the NC and MS+B groups, HE staining demonstrated that the aortic intima were smooth; in the MS group, the intima was coarse and partly thickened, and an increase in vascular intima thickness was clearly evident (Fig. 4).

In the NC group, TEM demonstrated that the endothelium was clear, and the mitochondria appeared to be of normal size and have normal numbers of vascular endothelial cells. In the MS group, phenotypic change in the vascular endothelial cells was evident by endothelial dysfunction and cell loss, and significant structural changes in the mitochondria such as severe swelling and extensive vacuolisation. However, the ultrastructural changes in the MS+B group were clearly improved. The endothelium was arranged in a regular fashion, and the mitochondria exhibited slight swelling and mild vacuolisation in the MS+B group compared to the MS group. TEM

photographs of the aortas of rats from each group are shown in Fig. 5. These results demonstrate macro-vascular remodeling in MS rats and that berberine attenuated macro-vascular remodeling *in vivo*.

Berberine Inhibits the Activation of p38 MAPK and Expression of ATF-2 and MMP-2 in the Rat Aortas To provide mechanistic insight, the levels of p38 MAPK, ATF-2 and MMP-2 were examined by Western blot analysis. The MS rats exhibited increased p38 MAPK, ATF-2 and MMP-2 levels compared with the NC group rats ($p < 0.01$); but their levels were decreased in the MS+B group ($p < 0.01$, Fig. 6). Therefore, berberine effectively inhibited p38 MAPK activation, ATF-2 phosphorylation, and MMP-2 expression.

DISCUSSION

In our rat model, which is valuable for the study of MS,

Fig. 3. IL-6, TNF- α , ICAM-1 and VCAM-1 mRNA Expression Levels Were Detected Using Real-Time PCR, and Each Bar Represents the Mean $\pm$ S.D.

** p <0.01 vs. NC group; ## p <0.01, # p <0.05 vs. MS group. NC group (n =10); MS group (n =10); MS+B group (n =10).

Fig. 4. HE Staining Demonstrates the Histological Morphology of the Rat Aortas

A: NC group (n =10): the intima of the aorta is smooth, and the endothelial cells are flat and attached to the internal elastic lamina. B: MS group (n =10): the intima is coarse and thickened, and there are breakages of endothelial cells; C: MS+B group (n =10) the histological morphology of the aorta is clearly improved compared to the MS group. Original magnification $\times$ 400. D: The intima thickness of blood vessels. Data are shown as means $\pm$ S.D. ** p <0.01 vs. NC group; ## p <0.01 vs. MS group.

Fig. 5. Representative Transmission Electron Microscopy Analysis Photomicrographs of the Rat Aortas

A: NC group: the endothelium is clear, and the mitochondria appear to be of normal size with normal numbers in vascular endothelial cells. B: MS group: endothelial dysfunction is prominent, and significant structural changes in mitochondria, such as severe swelling and extensive vacuolisation, are clearly evident. C: MS+B group: the endothelium is arranged regularly, and the mitochondria exhibit slight swelling and mild vacuolisation compared with the MS group. The arrows indicate swollen mitochondria and sarcomeres (TEM, $\times$ 10000).

we showed that vascular inflammation and remodeling were enhanced and that berberine could decrease vascular inflammation and remodeling.

The available data suggest that many patients with MS have vascular abnormalities at the time of diagnosis.^{10,25} In our study, the rat model of MS showed hypertrophic remodeling of the aorta, and vascular inflammation was enhanced. These abnormalities may contribute to the high prevalence of cardiovascular complications in patients with MS. Epide-

miological, clinical and experimental evidence together show that inflammation is a pivotal factor in the progression and exacerbation of MS and is a hallmark throughout the distinct stages of disease.^{8,26} The risk factors associated with cardiovascular disease, including hypertension, hyperglycaemia and dyslipidaemia, may initiate and advance vascular remodeling. Components of the innate and adaptive immune system, including the characteristic cytokines interleukin-1 and tumour necrosis factor- α , respectively, have prominent functions in

Fig. 6. Western Blot Analysis of the Activation of MMP-2, P-p38 MAPK and P-ATF-2 in the Rat Aortas

The upper panels show representative blots of protein in the aorta. The bottom bar graph shows the relative protein levels. Each bar represents the mean $\pm$ S.D. ** $p < 0.01$ vs. NC group; # $p < 0.05$, ## $p < 0.01$ vs. MS group. NC group ($n = 10$); MS group ($n = 10$); MS+B group ($n = 10$).

atherogenesis.^{27,28)} The inflammatory process is essential for the initiation and progression of vascular remodeling, leading to the degradation and reorganisation of the extra-cellular matrix (ECM) scaffold of the vessel wall and the development of atherosclerotic lesions.^{29,30)}

In our study, we found abnormal endothelium and increased expression of ICAM-1 and VCAM-1 in MS rats. Inflammation may not only inhibit the insulin receptor substrate/phosphoinositide-3 kinase (IRS/PI3K) pathway, but also activate the MAPK pathway, leading to insulin resistance and vascular remodeling.³¹⁾ Previous studies indicated that hyperinsulinaemia induced the formation of atherosclerotic lesions by increasing the release of endothelial adhesion molecules, such as ICAM-1 and VCAM-1. Moreover, macrophage recruitment by the abnormal endothelium to developing atherosclerotic plaques is aided by the endothelial expression of adhesion molecules.^{25,32)} Additionally, macrophages play important roles in the progression of atherosclerosis by exhibiting unique characteristics under various stimuli and by contributing to the evolution of plaque instability, thrombus formation and remodeling.

IL-6 and TNF- α are major mediators of inflammation.^{8,9)} They activate several protein kinases in fibroblasts, including the three types of MAPK.³³⁾ The p38 MAPK pathway is a key regulator of pro-inflammatory cytokine biosynthesis; thus, different components of the pathway are potential targets for the treatment of inflammatory diseases, such as MS and atherosclerosis.³⁴⁾

Berberine, isolated from a variety of medicinal plants, has been reported to exhibit anti-inflammatory properties and attenuate left ventricular remodeling through the augmentation of autophagy.^{7,35)} Berberine has also been shown to have cardioprotective effects including improving glucose metabolism,

lipid-lowering, anti-arrhythmic and inotropic actions.^{36–39)} However, little is known about the effects of berberine on vascular remodeling in MS. In the present study, we demonstrated that the berberine markedly reduced vascular medial wall thickness and the expression of inflammatory factors, and attenuated the progression of vascular remodeling in MS. These novel findings reveal the direct beneficial effects of berberine on vascular remodeling in MS.

In our study, we found that inflammation-stimulated p38 MAPK was activated in MS rats. We also found increased phosphorylation of ATF-2, which is the downstream target of p38 MAPK. Moreover, we found that berberine treatment inhibited the inflammation activated by p38 MAPK and suppressed the expression levels of MMP-2 in MS rats. Matrix metalloproteinases (MMPs), a family of proteolytic enzymes that degrade the extracellular matrix (ECM), play important role in the pathogenesis of atherosclerosis.⁴⁰⁾ Released by inflammatory cells and smooth muscle cells, MMPs regulate vascular remodeling.^{41,42)} Mechanistically, our data suggest that beneficial effects of berberine on vascular remodeling in MS are related to the inhibition of p38 MAPK activation, ATF-2 phosphorylation, and MMP-2 expression.

The mechanism by which inflammation enhances vascular remodeling may involve the activation of p38 MAPK and ATF-2 in the aorta, and consequent upregulation of MMP-2 expression. Notably, berberine alleviates vascular inflammation and remodeling in MS condition and this is correlated with its ability to inhibit p38 MAPK activation, ATF-2 phosphorylation, and MMP-2 expression. Therefore, berberine is a potent agent for the treatment of cardiovascular damages in MS.

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Conflict of Interest The authors declare no conflict of interest.

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