Original Article
Berberine protects vascular endothelial cells in hypertensive rats

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Received May 25, 2015; Accepted August 2, 2015; Epub September 15, 2015; Published September 30, 2015

Abstract: Objective: This study is to investigate the effect and mechanism of berberine on vascular endothelial cell injury. Methods: The isolated aortic endothelial cells were divided into negative control group, spontaneous hypertension group, and berberine group (1.25, 2.5, and 5 μmol/L berberine). CCK-8 assay was performed to detect cell proliferation. Annexin V-FITC flow cytometry and Hochest33342/PI staining were used to measure cell apoptosis. Expression of TLR4, Myd88, and NF-κB was detected with Western blotting analysis. Level of IL-6 and TNF-α was measured with ELISA. Results: Compared with spontaneous hypertension group, cell proliferation in berberine group was significantly improved (P < 0.05). Flow cytometry showed that cell apoptosis was reduced in berberine group in a dose-dependent manner and there was statistically significant difference between spontaneous hypertension group and berberine group (P < 0.05). This result was further confirmed by Hochest33342/PI staining. Expression levels of TLR4, Myd88 and NF-κB were increased in spontaneous hypertension group but significantly reduced in berberine group than those in spontaneous hypertension group (P < 0.05). Similarly, levels of IL-6 and TNF-α were increased in spontaneous hypertension group and decreased in berberine group. Conclusion: Berberine plays a protective role in vascular endothelial cell injury through inhibiting apoptosis and expression of TLR4, Myd88, NF-κB, IL-6 and TNF-α.

Keywords: Berberine, spontaneous hypertension, vascular endothelial cell injury, TLR4, Myd88, NF-κB, IL-6, TNF-α

Introduction

Vascular endothelial cells could produce many vasoactive substances, such as nitric oxide, prostacyclin, and endothelin-1, through autocrine, endocrine, or paracrine secretion [1, 2]. Vascular endothelial cells play important roles in regulating vascular tension, inhibiting thrombosis, repressing proliferation of smooth muscle cells and inhibiting inflammation of the vessel wall [3]. When vascular endothelial cells are stimulated by factors such as oxidative stress, renin-angiotensin system, oxidized low density lipoprotein, and homocysteine, the production of vasodilator factors is decreased whereas the production of vasoconstrictor factors is increased [4-7]. This could break the homeostasis of vasoconstriction and vasodilation, resulting in a series of cardiovascular events [8]. There is endothelial damage in almost all patients with essential hypertension [9]. It is believed that endothelial damage is secondary to hypertension [10, 11]. And, endothelial damage is the initiating event of atherosclerosis associated with hypertension [12]. Therefore, drug intervention against endothelial cell dysfunction has become a new trend in the field of cardiovascular disease research [13].

The myeloid differentiation factor 88 (MyD88) dependent toll-like receptor 4 (TLR4) signaling pathway expresses in human embryonic kidney cells (HEK293), cardiac cells, and microvascular endothelial cells [14]. It may play a role in endothelial damage induced by hypertension [15, 16]. It could activate interleukin-1 receptor associated kinase (IRAK-1), nuclear factor-κB (NF-κB) and activator protein-1 (AP-1), leading to production of large amounts of inflammatory cytokines (such as COX-2, IL-1, and IL-6) and resulting in endothelial cell injury [17].
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Berberine is the active ingredient extracted from the rhizome of Ranunculaceae Coptis [18]. Studies have found that berberine has clinical application in the treatment of cancer, diabetes, cardiovascular disease, high cholesterol, inflammation, and bacterial and viral infections [19-22].

In this study, the protective effect of berberine on vascular endothelial cell injury was investigated. Whether this protective effect is related with MyD88 dependent TLR4 signaling pathway was also studied.

Materials and methods

Animals

SD rats and rats with spontaneous hypertension were purchased from Model Animal Research Center of Nanjing University (Nanjing, China). They were kept in standard conditions with free access to food and water. All animal experiments were conducted according to the ethical guidelines of Harbin Medical University.

Isolation and culture of aortic endothelial cells

Aortic endothelial cells were isolated from SD rats and rats with spontaneous hypertension as previously described [23, 24]. Briefly, rats were anesthetized with ether and the thoracic aortas were isolated under sterile condition. After washing with PBS and removing vascular adventitia, vascular intima was exposed. The vascular intima was digested with 2.0 g/L of type I collagenase (Beyotime Institute of Biotechnology, Shanghai, China) for 1 h and then the vascular intima were cultured with DMEM/F12 medium (General Electric Company, South Logan, Utah, USA) in flasks coated with collagen. After culturing for 3 to 4 days, endothelial cells were released from the intima tissue. Endothelial cells were cultured in DMEM/F12 medium supplemented with 10% fetal bovine serum (General Electric Company, South Logan, Utah, USA) and 0.1% penicillin-streptomycin (Beyotime Institute of Biotechnology, Shanghai, China) in an incubator at 37°C with 5% CO₂.

Immunohistochemistry

Immunohistochemistry was performed to identify aortic endothelial cells. As previously described [25], the endothelial cell specific antigen von Willebrand factor (vWF) was detected. The isolated endothelial cells were cultured on glass slides and then incubated with 0.3% H₂O₂-methanol solution at room temperature for 10 min. After washing with PBS, the slides were blocked with 10% normal goat serum (General Electric Company, South Logan, Utah, USA) for 1 h. Then the primary antibody of rabbit anti-rat vWF (Cell Signaling Technology, Danvers, MA, USA) was added and incubated at 4°C overnight. After washing with PBS for 3 times, the secondary antibody of HRP-labeled goat anti-rabbit antibody (Cell Signaling Technology, Danvers, MA, USA) was added and incubated in the dark at room temperature for 1 h. Finally, the slides were developed with DAB reagent. After dehydration and transparency, the slides were mounted with neutral gum and observed under a fluorescent microscope (Biological Microscope XSP-8C, SHANGHAI ZOUSUN OPTICAL INSTRUMENT CO., LTD., Shanghai, China).

Cell grouping

The cells were divided into negative control group (aorta endothelial cells isolated from SD rats), spontaneous hypertension group (aorta endothelial cells isolated from rats with spontaneous hypertension), berberine group (aorta endothelial cells isolated from rats with spontaneous hypertension + berberine (1.25, 2.5, and 5 μmol/L)). Cells in berberine group were treated with berberine (1.25, 2.5, and 5 μmol/L) for 24 h.

CCK-8 assay

The isolated endothelial cells at logarithmic growth phase were seeded in 96-well plates at a concentration of 1 × 10⁵/L. After culturing for 24 h, CCK-8 was added to each well and cultured for 1 h. The OD value was measured at 450 nm using a microplate reader (SpectraMax M3, Molecular Devices, Sunnyvale, CA, USA). The cell viability was represented by OD450 values.

Flow cytometry analysis after Annexin V-FITC staining

Cells were collected, washed twice with cold PBS, and suspended in Annexin V buffer (cell concentration 1 × 10⁶/ml). Then 5 μl Annexin V-FITC and 5 μl PI (Beyotime Institute of
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Hochest33342 and PI double staining

Cells were collected and suspended in 1 ml culture medium. Then 10 μL Hoche 33342 stock solution (100 mg/L, dissolved in distilled water) was added. Cells were observed under a fluorescent microscope (XSP-17CE, Shanghai Changfang Optical Instrument Co., LTD, Shanghai, China).

Western blotting

Total proteins were extracted and separated by SDS-PAGE. Then proteins were transferred onto nitrocellulose membrane. After blocking with non-fat milk, the membrane was incubated with primary antibodies of rabbit anti-rat polyclonal TLR-4 antibody (Cell Signaling Technology, Danvers, MA, USA), rabbit anti-rat polyclonal NF-κB antibody (Cell Signaling Technology, Danvers, MA, USA), rabbit anti-rat polyclonal NF-κB antibody (Cell Signaling Technology, Danvers, MA, USA) and rabbit anti-rat polyclonal β-actin antibody (Cell Signaling Technology, Danvers, MA, USA) at 4°C overnight. After washing, the membrane was then incubated with goat anti-rabbit monoclonal antibody (Cell Signaling Technology, Danvers, MA, USA) at room temperature for 1 h. Finally, the membrane was developed by enhanced chemiluminescence plus reagent. The developed film was scanned using the Alphalmager gel imaging systems (Alphalmager, Santa Clara, California, USA). And the Western blot images were analyzed using Image-Pro...
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Plus analysis software (Media Cybernetics, Inc., Rockville, MD, USA). β-actin was used as an internal control.

**ELISA**

The assay was carried out according to the manual provided by the ELISA kit (Rat IL-6 ELISA kit and Rat TNF-α ELISA kit, Uscn Life Science Inc., Wuhan China). Briefly, cell culture supernatant was added to the pre-coated microplate and incubated at 37°C for 30 min. After washing for 5 times, detection antibody was added to the wells. After incubation at room temperature for 1 h, the microplate was washed again. Then HRP conjugated antibody was added and incubated at room temperature for 30 min. After washing for 5 times, substrate solution was added and incubated at room temperature for 15 min. Finally, stop solution was added to stop color development and the plate was read at 450 nm using a microplate reader (SpectraMax M3, Molecular Devices, Sunnyvale, CA, USA). The standard curve was generated by 2-fold serial dilutions of the standard samples. The content of IL-6 and TNF-α was calculated according to the standard curve.

**Statistical analysis**

Data was processed using SPSS 19.0 statistical software. All data were presented as mean ± standard deviation. One-Way ANOVA was performed to compare differences among different groups. Mean values between groups were analyzed by SNK method. P < 0.05 was considered statistically significant.

**Results**

*Identification of aortic endothelial cells*

To identify the isolated cells, the isolated cells were first observed under microscope. As
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shown in Figure 1A, cells were flat showing short spindle or polygonal morphology and were arranged in monolayer, like “paving stone”. This morphology is characteristic of endothelial cells. Then, immunohistochemistry was performed to detect the expression of the endothelial cell specific antigen vWF. As shown in Figure 1B, there was positive vWF expression in the isolated cells. Therefore, the isolated cells were identified as aortic endothelial cells.

Effect of berberine treatment on proliferation of aortic endothelial cells

To determine the effect of berberine on proliferation of aortic endothelial cells, CCK-8 assay was performed. Cells in negative control group were aorta endothelial cells isolated from SD rats. Cells in spontaneous hypertension group and berberine group were aorta endothelial cells isolated from rats with spontaneous hypertension. Cells in berberine group were treated with berberine (1.25, 2.5, and 5 μmol/L) for 24 h. As shown in Figure 2, cell proliferation in spontaneous hypertension group was significantly decreased than that in negative control group (P < 0.05). However, after treatment with berberine, cell proliferation was increased. Compared with spontaneous hypertension group, cell proliferation in berberine group was significantly increased (P < 0.05). Thus, proliferation of aortic endothelial cells isolated from rats with spontaneous hypertension is inhibited and this inhibition is alleviated by berberine treatment.

Effect of berberine treatment on apoptosis of aortic endothelial cells by flow cytometry

To investigate the effect of berberine on apoptosis of aortic endothelial cells, flow cytometry analysis was conducted. Cell grouping was described in Materials and methods. Representative flow cytometry results were shown in Figure 3A and quantitative flow cytometry results were shown in Figure 3B. The apoptosis rate in negative control group was 21.02 ± 0.05% whereas in spontaneous hypertension group was 60.69 ± 5.92%. Compared with negative control group, the apoptosis rate in spontaneous hypertension group was significantly higher (P < 0.05). The apoptosis rate in berberine group treated with 1.25, 2.5, and 5 μmol/L berberine was 37.51 ± 0.19%, 31.44 ± 0.25%, and 25.63 ± 0.21%, significantly lower than that in spontaneous hypertension group (P < 0.05). And, this decrease in apoptosis rate was dependent on the dose of berberine. These results indicate that apoptosis of aortic endothelial cells isolated from rats with spontaneous hypertension is increased and this increased apoptosis is inhibited by berberine treatment.

Effect of berberine treatment on apoptosis of aortic endothelial cells by Hochest33342/PI staining

To further verify the inhibitory effect of berberine on apoptosis of hypertensive aortic endothelial cells, Hochest33342/PI staining was performed. Similarly, as shown in Figure 4, in the negative control group, cells were stained blue and apoptotic cells were barely observed. In the spontaneous hypertension group, some cells were stained red, indicating that there were apoptotic cells. However, the number of apoptotic cells was reduced in the berberine group in a dose-dependent manner. This data further suggests that berberine treatment inhibits apoptosis of aortic endothelial cells isolated from rats with spontaneous hypertension.
Effect of berberine on expression of TLR4, Myd88 and NF-κB

To detect the expression of TLR4, Myd88 and NF-κB in aortic endothelial cells after berberine treatment, Western blotting analysis was performed. As shown in Figure 5, the expression levels of TLR4 (Figure 5A), Myd88 (Figure 5B) and NF-κB (Figure 5C) were relatively low in the negative control group. However, their expression levels were increased in the spontaneous hypertension group, significantly higher than those in the negative control group (P < 0.05). After treatment with berberine, the expression levels of TLR4, Myd88 and NF-κB in the berberine group were further decreased. Statistically, the expression levels of TLR4, Myd88 and NF-κB in the berberine group were significantly lower than those in the spontaneous hypertension group (P < 0.05). Thus, these findings showed that berberine treatment inhibited the expression of TLR4, Myd88 and NF-κB in aortic
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Effect of berberine on expression of IL-6 and TNF-α

ELISA was carried out to measure the levels of IL-6 and TNF-α in culture supernatant of aortic endothelial cells. The levels of IL-6 (Figure 6A) and TNF-α (Figure 6B) in the spontaneous hypertension group were significantly higher than those in the negative control group (P < 0.05). However, after berberine treatment, the levels of IL-6 and TNF-α decreased. Statistically, the berberine group had significantly lower levels of IL-6 and TNF-α than the spontaneous hypertension group (P < 0.05). Therefore, the levels of IL-6 and TNF-α in culture supernatant of aortic endothelial cells isolated from rats with spontaneous hypertension are decreased by berberine.

Discussion

There is vascular endothelial cell injury during the development of hypertension [26]. However, the mechanism underlying hypertensive vascular endothelial cell injury is not clear. It is generally considered that vascular endothelial cell injury is secondary to hypertension [27]. Vascular endothelial cell injury is accompanied with inflammation and apoptosis [28, 29]. Meanwhile, TLR4 pathway is an important pathway involved in inflammation [30]. As a traditional Chinese medicine, berberine is widely used in the treatment of heart failure, arrhythmia, high cholesterol, and inflammatory diseases [31]. The results of this study showed that berberine could improve the proliferation of cells isolated from rats with spontaneous hypertension. Compared with spontaneous hypertension group, the difference was statistically significant. Thus, berberine has certain protective effect on hypertensive vascular endothelial injury.

To further reveal the underlying mechanism of the protective effect of berberine on vascular endothelial cells, we detected cell apoptosis after berberine treatment. Flow cytometry analysis showed the apoptosis rate in hypertension group was significantly higher than that in neg-
Vacate control group. However, after treatment with berberine, the apoptosis rate was significantly reduced in a dose dependent manner. Similarly, Hochest33342/PI staining showed that many cells in hypertension group were stained red. And, the number of cell with red staining in berberine group was obviously lesser. These results suggest that apoptosis of aortic endothelial cells isolated from rats with spontaneous hypertension is increased and this increased apoptosis is inhibited by berberine treatment.

Hypertensive vascular endothelial cell injury is related with TLR4 signaling pathway [32, 33]. TLR4 signaling pathway is involved in inflammation and immune response through promoting the synthesis and release of cytokines [34]. The results of this study showed that expression levels of TLR4, Myd88 and NF-κB were increased in hypertension group. After the intervention with berberine, expression levels of TLR4, Myd88 and NF-κB was significantly reduced.

TNF-α and IL-6 play important roles in endothelial damage, inflammation, smooth muscle proliferation and vascular remodeling in hypertension patients [35, 36]. IL-6 is a glycoprotein with a molecular weight of 26 kD and is produced by a variety of tissues [37]. It can induce activation of B cells to produce immunoglobulins, promote T cell growth, and induce the generation of hepatic acute phase protein [38]. TNF-α, which is mainly produced by mononuclear macrophages, is a kind of peptide with a molecular weight of 17 kD [35]. It is an important inflammatory mediator and immunomodulatory factor that is involved in many pathophysiological processes, such as immune response, tissue repair, and cell apoptosis [39, 40]. In this study, levels of IL-6 and TNF-α in spontaneous hypertension group were significantly higher than those in negative control group. However, after treatment with berberine, IL-6 and TNF-α levels were significantly decreased, compared with spontaneous hypertension group.

In conclusion, our findings indicate that berberine may protect vascular endothelial cells isolated from hypertensive rats from injury. And, this protective effect may be acted through regulating apoptosis and the expression of TLR4, Myd88, NF-κB, IL-6 and TNF-α.

Acknowledgements

We thank Lanfeng Wang from Department of CCU, the First Affiliated Hospital, Harbin Medical University for his valuable help during the preparation of the manuscript.

Disclosure of conflict of interest

None.

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