Berberine suppresses in vitro migration of human aortic smooth muscle cells through the inhibitions of MMP-2/9, u-PA, AP-1, and NF-κB

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INTRODUCTION

Vascular remodeling is the major cause of restenosis after coronary artery bypass graft (CABG), coronary artery stenting and angioplasty (1-3). The abnormal migration of vascular smooth muscle cells (VSMCs) is one of the main pathological features of vascular remodeling (4). After vessel injury, VSMCs migrate into the intima, causing intimal thickening and narrowing of the arterial luminal space. The migration of VSMCs requires degradation or remodeling of the extracellular matrix (ECM) (5). Matrix metalloproteins (MMPs) are a family of structural and functional related endopeptidases and are capable of degrading both the collagenous and noncollagenous components of the ECM (6). MMPs facilitate migration of VSMCs in the arterial wall and play an important role during the process of vascular remodeling after injury (7).

Berberine, a type of isoquinoline alkaloid isolated from Chinese medicinal herbs, has been reported to have various pharmacological activities. Studies have demonstrated that berberine has beneficial effects on vascular remodeling and alleviates restenosis after vascular injury. However, its mechanism of action on vascular smooth muscle cell migration is not fully understood. We therefore investigated the effect of berberine on human aortic smooth muscle cell (HASMC) migration. Boyden chamber assay was performed to show that berberine inhibited HASMC migration dose-dependently. Real-time PCR and Western blotting analyses showed that levels of matrix metalloproteins (MMP)-2, MMP-9, and urokinase-type plasminogen activator (u-PA) were reduced by berberine at both the mRNA and protein levels. Western blotting assay further confirmed that activities of c-Fos, c-Jun, and NF-κB were significantly attenuated. These results suggest that berberine effectively inhibited HASMC migration, possibly by down-regulating MMP-2, MMP-9, and u-PA; and interrupting AP-1 and NF-κB mediated signaling pathways. [BMB Reports 2014; 47(7): 388-392]

RESULTS

Berberine inhibited the migration of HASMCs

Effects of berberine on cell migration of HASMCs were investigated using a modified Boyden chamber assay and results are shown in Fig. 1A. The migration of HASMCs was induced significantly by 10% FBS. Treatments with 25, 50 and 100 μM berberine for 6 h inhibited FBS induced cell migration effectively and these effects were dose-dependent. Western blotting results also showed that the protein expression of MMP-2, MMP-9, u-PA was elevated in FBS treated HASMCs (Fig. 1B).

Keywords: Berberine, Human aortic smooth muscle cells, Matrix metalloproteins, Migration, Restenosis

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Berberine inhibited levels of MMP-2, MMP-9, and u-PA in HASMCs
The mRNA and protein levels of migration-associated gene, such as MMP-2, MMP-9, and urokinase-type plasminogen activator (u-PA) were examined by real-time PCR and Western blotting respectively. As shown in Fig. 2, treatment with 100 μM berberine significantly reduced the expression of MMP-2, MMP-9, and u-PA, at both the mRNA and protein levels.

Berberine down-regulated the activity of AP-1 in HASMCs
Phosphorylation levels of c-Fos and c-Jun in cell lysates were found to be significantly reduced after treatment with 100 μM berberine for different time (6, 12, 24 h), as demonstrated by Western blotting (Fig. 3A), whereas β-actin levels (loading control) remained unchanged. These data indicate that berberine effectively down-regulated the activity of AP-1 in HASMCs.

Berberine inhibited levels of NF-κB in HASMCs
As shown in Fig. 3B, Western blotting results indicated that, under the treatment of berberine, the level of NF-κB was lower than the corresponding control. However, the level of IκB was higher than that of the control. Moreover, Western blotting results also revealed that the nuclear translocation of NF-κB was inhibited by the treatment of berberine (Fig. 4A).

Fig. 2. Berberine inhibited levels of MMP-2, MMP-9, and u-PA in HASMCs. (A) mRNA levels of MMP-2, MMP-9, and u-PA in HASMCs after exposure to berberine as examined by real-time PCR. (B) Protein expression of MMP-2, MMP-9, and u-PA in HASMCs treated with 100 μM berberine for different times (6, 12, 24 h) was assessed by Western blotting. Densitometry of different groups was normalized to β-actin. *P < 0.05 compared with the control group.

Fig. 3. Berberine down-regulated AP-1 and NF-κB in HASMCs. (A) Shows representative results of the phosphorylation levels of c-Jun and c-Fos as measured by Western blotting in HASMCs after treated with 100 μM berberine for different times (6, 12, 24 h). Densitometry of different groups was normalized to β-actin. *P < 0.05 compared with the control group. (B) Shows representative results of the protein expression of NF-κB and IκB as measured by Western blotting in HASMCs after treated with 100 μM berberine for different times (6, 12, 24 h). Densitometry of different groups was normalized to β-actin. *P < 0.05 compared with the control group.
Inhibition of c-fos, c-jun, and NF-κB resulted in down-regulation of MMP-2, MMP-9, and u-PA

As ELISA results showed, the expression of MMP-2, MMP-9, and u-PA was down-regulated under the treatment of c-fos siRNA, c-jun siRNA, or NF-κB inhibitor PDTC (Fig. 4B).

DISCUSSIONS

The results of the present study demonstrated that berberine inhibited the migration of HASMCs, and this is in agreement with earlier reports (19, 20). It was indicated that VSMC migration is accompanied by local degradation of the ECM. The MMPs play the major role in the ECM degradation (6). Among the MMPs, MMP-2 and MMP-9 derive from VSMCs and in amatory cells after vascular injury and play important role in VSMC migration from the arterial wall to the neointima lining after vessel injury by degrading collagenous components of the ECM (21), and contribute to the development of intimal hyperplasia (22). Real-time PCR and Western blotting results showed that both the mRNA expression and protein secretion of MMP-2 and MMP-9 were progressively decreased under the treatment of berberine.

It has been reported that u-PA, which lies upstream of MMP-2 and MMP-9, is responsible for the degradation of the main component of the basement membrane, collagen IV (23), and the combined involvement of u-PA/MMP signaling has been demonstrated during the vascular remodeling process (24). Our data showed that the expression of u-PA was also decreased. Taken together, inhibition of u-PA, MMP-2 and MMP-9 may help to explain the inhibitory effects of berberine on the migration of VSMC.

We further investigated the related molecules and signaling pathways that are involved in the VSMC migration and MMP secretion. It is reported that, the protein expression of MMP-2, MMP-9 and u-PA requires AP-1 and NF-κB (25, 26). In addition, AP-1 and NF-κB are shown to be key molecules involved in regulation of VSMC migration during vascular remodeling process (27, 28). Here we demonstrated that berberine effectively reduced phosphorylation of AP-1 (Fos and Jun). NF-κB lies mainly in the cytoplasm and binds to NF-κB inhibitory subunit IκB, which can block the translocation of NF-κB into the nuclear compartment and thus prohibit NF-κB to enter the nucleus and bind cis-acting IκB sites in the promoters and enhancers of target genes (29). Our results showed that berberine significantly inhibited phosphorylation of NF-κB and its nuclear translocation, whereas enhanced phosphorylation of IκB. Moreover, the regulatory role of c-fos, c-jun, and NF-κB in MMP-2, MMP-9, and u-PA expression was further determined by using c-fos, c-jun specific siRNAs and NF-κB inhibitor. Taken together, these results suggested that the inhibitory effects of berberine on the cell migration, MMP and u-PA expression might be through the inhibitions of both AP-1 and NF-κB signaling pathways.

In conclusion, our data indicated that berberine could inhibit the migration of HASMCs, and offered a molecular explanation for the anti-migratory properties of berberine, possibly through the inhibitions of AP-1 and NF-κB signaling pathways resulting in the attenuation of MMP-2/9, and u-PA expression. Based on these observations, berberine should be considered as a promising therapeutic agent for the treatment of restenosis after CABG, coronary artery stenting and angioplasty.

MATERIALS AND METHODS

Materials
Berberines, dimethyl sulfoxide (DMSO), and NF-κB inhibitor (pyrrolidine dithiocarbamate, PDTC) were obtained from Sigma (St. Louis, MO, USA). Anti-MMP-2, anti-MMP-9, anti-u-PA, anti-NF-κB and anti-IκB, and anti-β-actin antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-phosphorylation-c-Fos, anti-phosphorylation-c-Jun antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA).

Cell culture
HASMCs were purchased from ScienCell (San Diego, CA, USA) and maintained in smooth muscle cell medium (ScientCell) containing smooth muscle cell growth factor supplement, supplemented with 2% fetal bovine serum (FBS) and penicillin/streptomycin in CO2 incubator with a humidified at-
mosphere of 95% air and 5% CO₂ at 37°C in DMEM containing 10% FBS. Once the cells reached 70-80% confluence, they were trypsinized and seeded on 6-well plastic dishes for the following experiments. Cells from passage 4 to 6 were used for the experiments. For the small interfering RNA (siRNA) transfection procedure, cells were grown to 60% confluence and c-Fos, c-Jun or control siRNA (Santa Cruz) were transfected using Lipofectamine™ 2000 (Invitrogen, CA, USA) according to the manufacturer's instructions.

Migration assay
A migration assay was performed using a modified Boyden chamber with 8 μm pore size polycarbonate membranes occluded by a Matrigel basement membrane matrix (BD Biosciences, Oxford, UK). HASMCs were pretreated with or without berberine (25, 50, 100 μM) for 24 h, cells were then trypsinized, re-suspended in serum-free medium. The cells were allowed to settle in DMEM containing 1% FBS for 1 h before the addition of agents in the lower chamber. 10% FBS DMEM was loaded into the lower chamber. Cells were allowed to migrate through the membrane to the underside of the apparatus for 6 h in a humidified atmosphere with 95% air and 5% CO₂ at 37°C. After non-migrating cells on the upper surface of the filters had been scraped with a cotton swab, migrated cells on the lower membrane surface were fixed with methanol/acetic acid solution and stained with crystal violet solution (0.1% crystal violet, 0.1 M borate pH 9.0, 2% ethanol). The crystal violet stain was eluted in 10% acetic acid; optical density and c-Fos, c-Jun or control siRNA (Santa Cruz) were transfected using Lipofectamine™ 2000 (Invitrogen, CA, USA) according to the manufacturer's instructions.

ELISA
Cell culture supernatant was collected. The concentration of MMP-2, MMP-9, and u-PA in the supernatant was measured using commercial ELISA kits (Abcam, Cambridge, MA, USA) according to the manufacturer's instructions.

Statistical analysis
Data are presented as mean ± standard error of the mean (SEM) and each experiment was performed at least three times. The statistical significance of differences was determined by ANOVA followed by Bonferroni's pairwise comparison using Prism 5.0 (GraphPad, San Diego, CA). A P value of <0.05 was considered significant.

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