

Original Article

Inhibitory effect and mechanism of Aliskiren on LPS-induced angiogenesis of HUVECs

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Abstract: This study is to investigate the inhibitory effect and the possible mechanism of Aliskiren on the lipopolysaccharides (LPS)-induced angiogenesis of human umbilical vein endothelial cells (HUVECs). Routinely cultured HUVECs were randomly divided into control group (Group A), LPS model group (Group B), Aliskiren low-dose group (Group C, 1 μ M), medium-dose group (Group D, 10 μ M) and high-dose group (Group E, 100 μ M). The proliferation of HUVECs was measured by MTT assay. The migration of HUVECs was measured by Transwell assay. The tube forming ability was determined by the formation of luminal structure by HUVECs in Matrigel. The secretion levels of tumor necrosis factor- α (TNF- α), intercellular adhesion molecule 1 (ICAM-1), monocyte chemoattractant protein 1 (MCP-1) were detected by ELISA. RT-PCR and Western blot were respectively used to determine the mRNA and protein levels of Toll-like receptor-4 (TLR4), matrix metalloproteinases-2 (MMP-2) and matrix metalloproteinases-9 (MMP-9) in the HUVECs. Aliskiren inhibited the LPS-induced proliferation, migration and tube formation of HUVECs in a dose-dependent manner. It also inhibited the LPS-induced secretion of TNF- α , ICAM-1 and MCP-1 in a concentration-dependent manner. In addition, Aliskiren could also inhibit the mRNA and protein expressions of TLR-4, MMP-2 and MMP-9. Aliskiren could effectively inhibit LPS-induced angiogenesis of HUVECs, which could be related to its inhibition on the TLR4-mediated inflammation and the expressions of MMP-2 and MMP-9.

Keywords: Aliskiren, angiogenesis, inflammation, TLR4

Introduction

Atherosclerosis (AS) is the pathophysiological basis of cardiovascular and cerebrovascular diseases [1]. The instability of atherosclerotic plaques is closely related to acute cardiovascular events [2]. Recent studies have demonstrated that angiogenesis in plaques is a new measure of plaque stability [3-5]. Angiogenesis can promote the accumulation of inflammatory cells and induce bleeding and plaque rupture, therefore, reduction of angiogenesis in the plaque and stabilization of the plaque become anti-atherosclerosis therapeutic targets [6].

Aliskiren is a recently discovered renin inhibitor, which has anti-oxidative stress and anti-inflammation effects [7]. Proliferation and migration of endothelial cells activated by inflammation can promote the formation of blood vessels

and vascular network in plaques, which plays a key role in the occurrence and development of AS plaques [8]. Thus, Aliskiren may affect the inflammation-induced angiogenesis.

The activation of TLR4 can increase the expressions of monocyte chemoattractant protein 1 (MCP-1) and other chemokines, and thus participates in the aggregation of monocytes and initiates the inflammatory reaction [9]. In addition, such TLR4-activation-mediated production and increase of cytokines and chemokines can stimulate cell migration and proliferation, and can also increase the expressions of matrix metalloproteinases-2 (MMP-2), matrix metalloproteinases-9 (MMP-9) and cathepsin, which are involved in the degradation of extracellular matrix [10]. MMP-2 can promote endothelial cells migrating from the original blood vessels to the outer periphery and forming new blood

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vessels by degrading the basement membrane and remodeling extracellular matrix, and finally caused the occurrence and development of AS [11]. Celletti found that the level of MMP-9 was positively correlated with the expression of VEGF in the AS plaques, suggesting the MMP-9 level might affect the angiogenesis in the plaques [12]. Pepper *et al.* have demonstrated that MMP-9 played important roles in the angiogenic process of a variety of tumors [13].

In this study, the inhibitory effects of Aliskiren on the angiogenesis in the atherosclerotic plaques were investigated, and the possible mechanism through influencing the TLR4-mediated inflammation and production of MMPs were discussed. It suggested that Aliskiren could increase the stability of the plaques and thus delay the occurrence and development of AS.

Materials and methods

Reagents

Lipopolysaccharide (LPS) and trypsin was purchased from Sigma Co. (CA, USA). DMEM low glucose medium was purchased from Gibco Co. (NY, USA). Fetal bovine serum was obtained from Hyclone Co. (UT, USA). ELISA kits for the detection of TNF- α , MCP-1 and ICAM-1 and the primary antibodies for TLR4, MMP-2 and MMP-9 were bought from BosterBio Co. (Wuhan, China). Aliskiren was purchased from Novartis Pharmaceuticals Co. (Basel, Switzerland).

Cell culture

Human umbilical vein endothelial cell line (HUVEC) was purchased from Thermo Fisher Scientific Inc. (MA, USA). The cells were cultured in DMEM medium with supplementary of 5% FBS under a humid atmosphere with 5% CO₂ at 37°C.

MTT assay

HUVECs at logarithmic growth phase were seeded in 96-well plates at a concentration of 4×10^3 cells/well. The cells were divided into control group (Group A), LPS model group (Group B, 10 mg/L LPS), low-dose group (Group C, 1 μ M Aliskiren + 10 mg/L LPS), medium-dose group (Group D, 10 μ M Aliskiren + 10 mg/L LPS) and high-dose group (Group E, 100 μ M Aliskiren + 10 mg/L LPS). Group C, D and E were treated with different concentrations of

Aliskiren for 24 h, and then all groups except Group A were added with 10 mg/L (final concentration) of LPS and incubated for another 24 h. After that, new medium containing 0.5% MTT was changed for all groups and incubated for 4 h. Then, 150 μ L of DMSO was added into each well and the absorbance at 490 nm was measured.

Transwell assay

HUVECs at a concentration of 2×10^6 cells/mL (100 μ L) were added into the upper wells of a Transwell chamber. Meanwhile, various concentrations of Aliskiren were added into Groups C-E. In the lower wells, all groups except Group A were added with LPS as the stimulator. After 6 hours of incubation, the cells were fixed with 4% paraformaldehyde for 10 min, and then stained with crystal violet for 30 min. The wells were washed with PBS three times and the upper cells were removed. The cell migration was observed and photographed under a microscope. The number of cells through the membrane was counted under $100 \times$ magnification in triplicate. The migrated cells were expressed with relative cell number to reflect the migration ability of HUVECs.

Lumen formation

The lumen formation was detected in Matrigel [14]. Briefly, Matrigel was pre-cooled and melted, and then added into 24-well plates and solidified at 37°C for 1 h. Pre-treated HUVECs (as above mentioned) were seeded onto the Matrigel at a concentration of 1×10^4 /well. After cultured for another 12 h, the lumen formation was observed and photographed under an inverted optical microscope. The images were processed using IPP software and the number of lumen was counted in three different views.

ELISA

The HUVECs were seeded in 6-well plates at a concentration of 6×10^5 cells/well. For Groups C-E, cells were incubated with various concentrations of Aliskiren for 24 h. Then, all groups except Group A were added with LPS at a final concentration of 10 mg/L and incubated for another 24 h. The supernatants were collected and the levels of TNF- α , ICAM-1 and MCP-1 were determined with ELISA kits.

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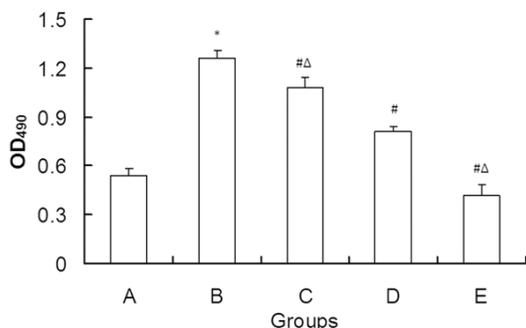


Figure 1. The inhibitory effect of Aliskiren on the LPS-induced proliferation of HUVECs. Control group (Group A), LPS model group (Group B), Aliskiren low-dose group (Group C, 1 μ M), medium-dose group (Group D, 10 μ M) and high-dose group (Group E, 100 μ M). * P <0.05 vs Group A; # P <0.05 vs Group B; ΔP <0.05 vs Group D.

RT-PCR

The cells were treated as described above and then collected. The total RNA was extracted and reverse transcribed into cDNA according to the manufacturer's instruction of MMLV reverse transcription kit. The specific primers for TLR4, MMP-2, MMP-9 and GAPDH were as follows:

TLR4 primers: 5'-GGCATCATCTTCATTGTCCTTG-3', 5'-AGCATTGCTCCTCCACTCG-3'; MMP-2 primers: 5'-AAGAAGTAGCTGTGACCGCC-3', 5'-TTGCTGGAGACAAATTCTGG-3'; MMP-9 primers: 5'-CCCTACTGCTGGTCTTCTGAG-3', 5'-AATTGGCTTCCTCCGTGATTCG-3'; GAPDH primers: 5'-CCCTACTGCTGGTCTTCTGAG-3', 5'-AATTGGCTTCCTCCGTGATTCG-3'.

RT-PCR for TLR4, MMP-2 and MMP-9 was performed at the following conditions: 95°C 5 min, followed by 35 cycles at 95°C for 30 s, 55°C for 30 s and 72°C for 30 s, and finally at 72°C for 10 min.

RT-PCR for GAPDH was performed at the following conditions: 95°C 5 min, followed by 35 cycles at 95°C for 30 s, 60°C for 30 s and 72°C for 30 s, and finally at 72°C for 10 min.

The PCR products were separated by 1.5% agarose gel electrophoresis, and then photographed with UVP gel imaging analysis system. The gray densities of the target genes were analyzed and the ratios of their gray densities to that of the internal standard GAPDH were used as the quantitative indices.

Western blot

The cells were treated as above mentioned. The proteins were extracted from the collected cells with cell lysis solution. The proteins were separated with SDS-PAGE and transferred onto a PVDF membrane, and blocked with 5% skim milk for 1 h. The primary antibody (1:500) was added and incubated at 4°C overnight. After washed with TBST, the secondary antibody was added and incubated for 1 h. The chemiluminescent reaction was carried out and photographs were immediately taken. The signal intensity of primary antibody binding was quantitatively analyzed with Sigma Scan Pro 5 and normalized to a loading control GAPDH.

Statistical analysis

The experimental data was expressed as mean \pm standard deviation. The statistical analysis was performed with the statistical software SPSS 18.0. ANOVA was used for comparisons between groups, while SNK-q test was used for pairwise comparisons. A P value P <0.05 was considered as statistically significant.

Results

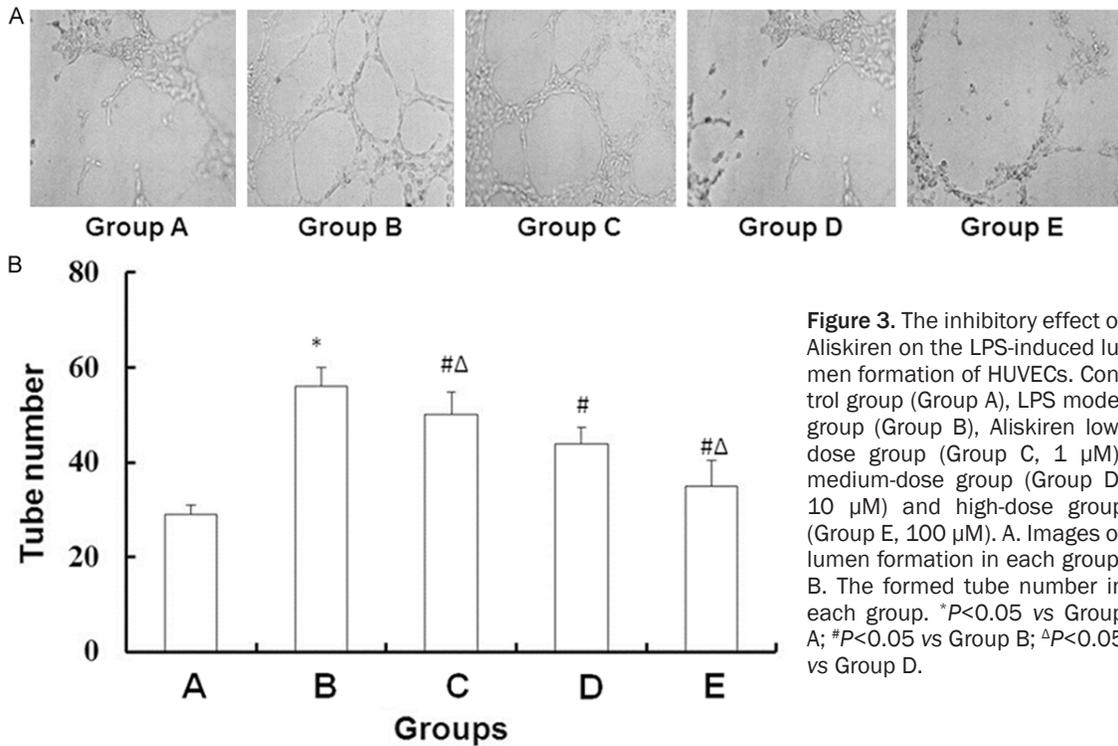
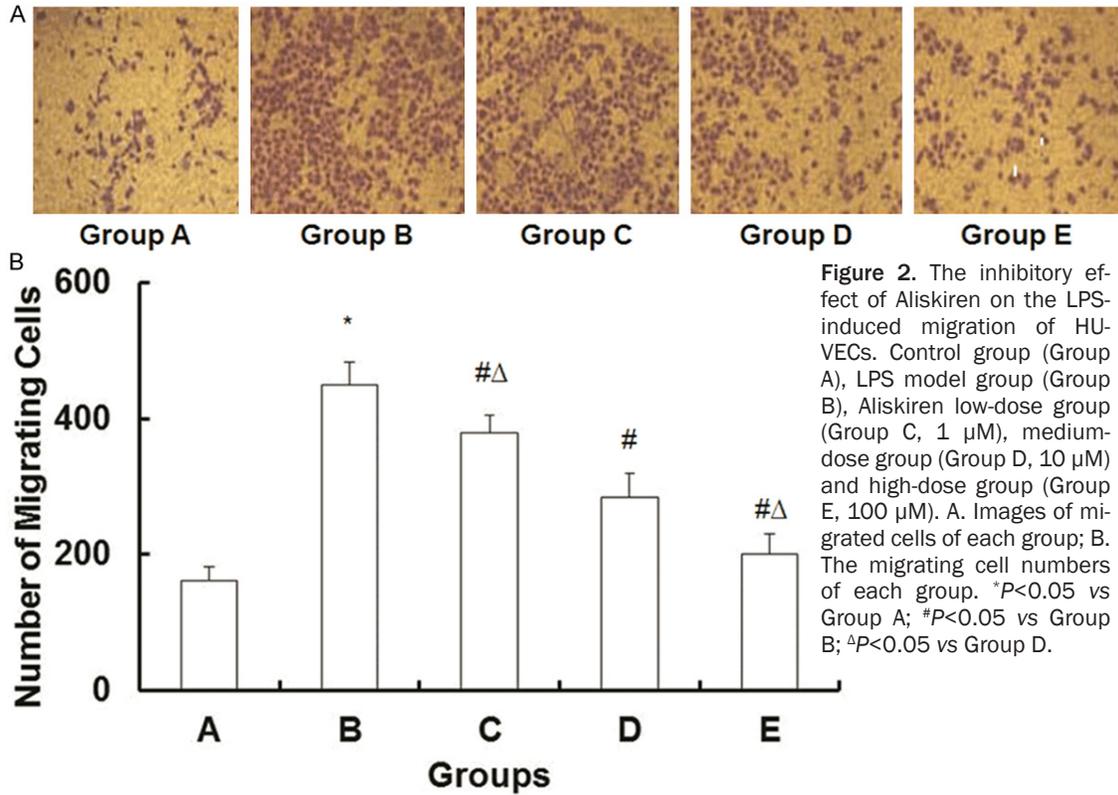
Inhibition of the LPS-induced HUVECs proliferation by Aliskiren

To determine the effect of Aliskiren on the LPS-induced HUVECs proliferation, MTT assay was performed. Compared with Group A, the cell proliferation of Group B was obviously increased (P <0.05) (Figure 1). In comparison with Group B, the cell proliferations in Groups C-E were obviously inhibited (P <0.05). Compared with Group D, the inhibitory effect in Group C was obviously lower (P <0.05), while that in Group E was obviously enhanced (P <0.05). These results indicated that Aliskiren could inhibit the LPS-induced HUVECs proliferation in a dose-dependent manner.

Aliskiren inhibited the LPS-induced HUVECs migration

To determine the effect of Aliskiren on the LPS-induced HUVECs migration, a Transwell experiment was performed. Compared to Group A, the migration ability of HUVECs in Group B was obviously enhanced and the number of migrating cells significantly increased (P <0.05) (Figure 2), while after interfered by various concentra-

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tions of Aliskiren in Groups C-E, the numbers of migrating cells significantly declined ($P < 0.05$). In comparison with Group D, the number of

migrating cells in Group C was obviously higher ($P < 0.05$), and that in Group E was significantly lower ($P < 0.05$). These results indicated

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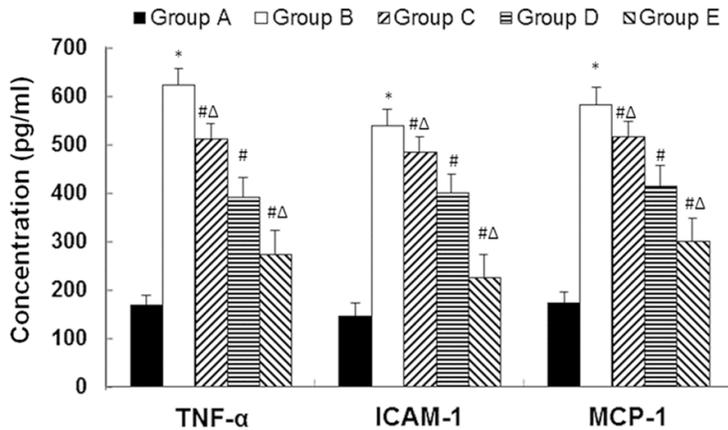


Figure 4. The inhibitory effect of Aliskiren on the LPS-induced secretions of TNF- α , ICAM-1 and MCP-1 by HUVECs. Control group (Group A), LPS model group (Group B), Aliskiren low-dose group (Group C, 1 μ M), medium-dose group (Group D, 10 μ M) and high-dose group (Group E, 100 μ M). * P <0.05 vs Group A; # P <0.05 vs Group B; ΔP <0.05 vs Group D.

that LPS had strong migration-inducing effects on HUVECs, and Aliskiren could inhibit this effect in a dose-dependent manner.

Aliskiren inhibited LPS-induced lumen formation by HUVECs

To observe the influence of Aliskiren on the lumen formation by HUVECs, the formed lumens were counted under a microscope. As shown in **Figure 3**, HUVECs in Group A most formed streak or discontinuous small tube-like structure, but there were only a small number of complete tubes. Compared to Group A, the number of formed tubes in Group B significantly increased. Compared to Group B, after treatment by different concentrations of Aliskiren in Groups C-E, the formation of tubes showed dose-dependent inhibition (P <0.05).

Aliskiren inhibited LPS-induced secretions of TNF- α , ICAM-1 and MCP-1 by HUVECs

To determine the effects of Aliskiren on the LPS-induced secretions of TNF- α , ICAM-1 and MCP-1 by HUVECs, ELISA was performed. As shown in **Figure 4**, compared to Group A, the levels of TNF- α , ICAM-1 and MCP-1 obviously increased (P <0.05). Compared to Group B, the levels of TNF- α , ICAM-1 and MCP-1 in Group C, D and E all significantly decreased (P <0.05). In comparison with Group D, the levels of TNF- α , ICAM-1 and MCP-1 in Group C were obviously higher (P <0.05), while those in Group E were

significantly lower (P <0.05). These results demonstrated that Aliskiren could inhibit the LPS-induced secretions of TNF- α , ICAM-1 and MCP-1 by HUVECs in a dose-dependent manner.

Aliskiren inhibited LPS-induced mRNA expressions of TLR4, MMP-2 and MMP-9 by HUVECs

To determine the mRNA expression levels of TLR4, MMP-2 and MMP-9 in HUVECs induced by LPS, RT-PCR was performed. Compared to Group A, the mRNA expressions of TLR4, MMP-2 and MMP-9 in Group B significantly increased (P <0.05) (**Figure 5**). Compared to Group B, the mRNA expressions of TLR4,

MMP-2 and MMP-9 in Groups C-E obviously decreased (P <0.05). Comparing to Group D, the mRNA expressions of TLR4, MMP-2 and MMP-9 in Group C were obviously higher (P <0.05), while those in Group E were obviously lower (P <0.05). These results indicated that Aliskiren could inhibit LPS-induced mRNA expressions of TLR4, MMP-2 and MMP-9 in HUVECs in a dose-dependent way.

Aliskiren inhibited LPS-induced protein expressions of TLR4, MMP-2 and MMP-9 by HUVECs

To determine the Aliskiren influences on the LPS-induced expressions of TLR4, MMP-2 and MMP-9, Western blot was performed. Compared to Group A, the protein expressions of TLR4, MMP-2 and MMP-9 in Group B significantly increased (P <0.05) (**Figure 6**). In comparison with Group B, the protein expressions of TLR4, MMP-2 and MMP-9 in Groups C-E obviously decreased (P <0.05). When comparing with Group D, the protein expressions of TLR4, MMP-2 and MMP-9 in Group C were obviously higher (P <0.05), while those in Group E were obviously lower (P <0.05). These results indicated that Aliskiren could inhibit LPS-induced protein expressions of TLR4, MMP-2 and MMP-9 in HUVECs in a dose-dependent manner.

Discussion

AS is a chronic, slowly progressive disease with lipid deposition on the vascular walls. The for-

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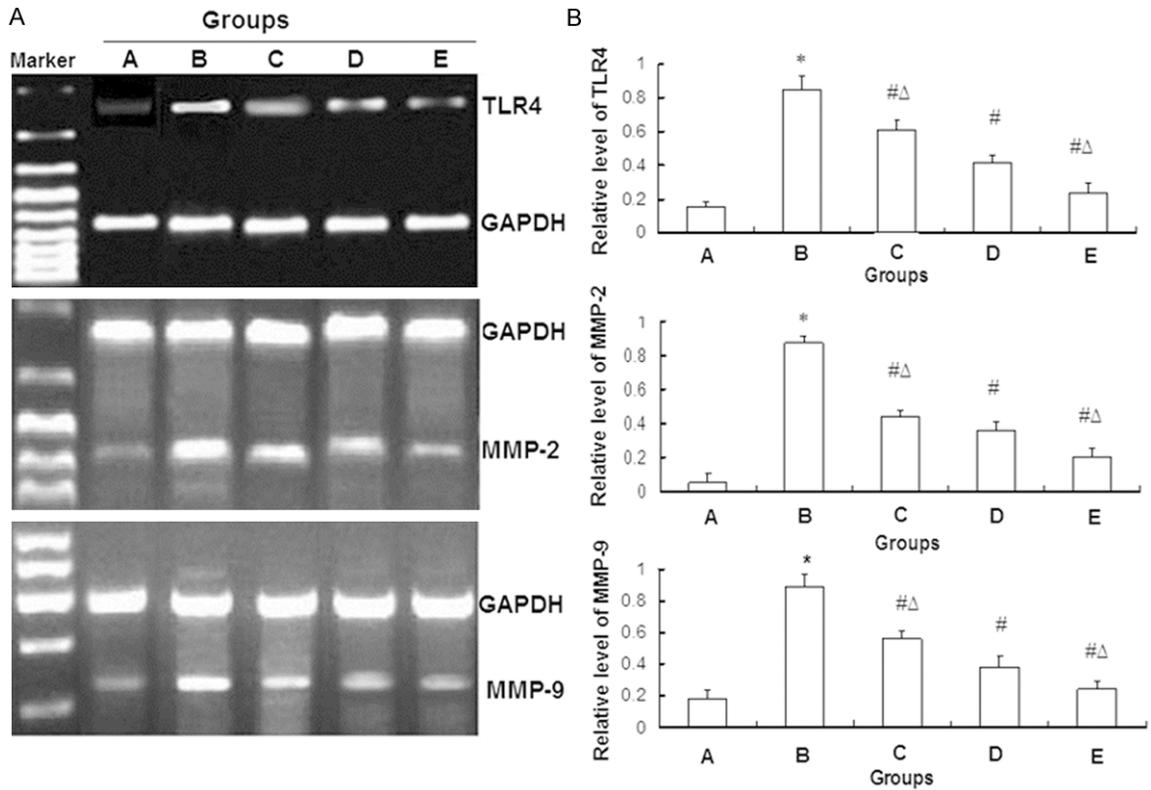


Figure 5. The inhibitory effect of Aliskiren on the LPS-induced mRNA expressions of TLR4, MMP-2 and MMP-9 in HUVECs. Control group (Group A), LPS model group (Group B), Aliskiren low-dose group (Group C, 1 μ M), medium-dose group (Group D, 10 μ M) and high-dose group (Group E, 100 μ M). A. The electrophoresis of the mRNA of TLR4, MMP-2 and MMP-9 in each group of HUVECs; GAPDH was used as the internal standard; B. The mRNA expression levels of TLR4, MMP-2 and MMP-9 in each group of HUVECs, expressed as the relative gray densities of the bands. * $P < 0.05$ vs Group A; # $P < 0.05$ vs Group B; $\Delta P < 0.05$ vs Group D.

mation of unstable plaques and even plaque rupture in AS are the main pathological basis causing acute coronary syndrome and other serious clinical complications [14]. Angiogenesis is one of the most important features of the unstable plaques [15]. Angiogenesis is a forming process of new blood vessels in the form of budding or non-budding from pre-existing blood vessels through proliferation and migration of vascular endothelial cells. The process can be divided into three phases: endothelial cell proliferation, migration and lumen formation. The new blood vessels in the plaques are thin and without muscle support, and thus liable to cause plaque instability, plaque hemorrhage and even plaque rupture [16]. Moulton KS *et al.* found that angiogenesis inhibitor drugs could inhibit the formation of new blood vessels in atherosclerotic lesions, stabilize the plaques and slow their progression [17]. Aliskiren has great significance in the treatment of AS and coronary heart disease [18, 19]. HUVECs are

the major cells for the study of angiogenesis, so the inhibitory effect of Aliskiren on angiogenesis was evaluated from the aspects of its influences on the proliferation, migration and lumen forming abilities of HUVECs in this study. The results showed that Aliskiren could inhibit the proliferation, migration and lumen formation of HUVECs in a dose-dependent manner.

Ross believed that inflammatory response was present in every stage of the AS occurrence and development [20]. Researches have indicated that inflammatory response is one of the important reasons causing angiogenesis in atherosclerotic plaques [21]. LPS is the most commonly used inflammation-stimulating factor in experiments, which can mediate the expressions of chemokines, inflammatory cytokines and so on [22]. In this study, LPS was used as the inflammatory stimuli to observe the effect of TLR4 signaling pathway activation on the formation of new blood vessels, and discuss the

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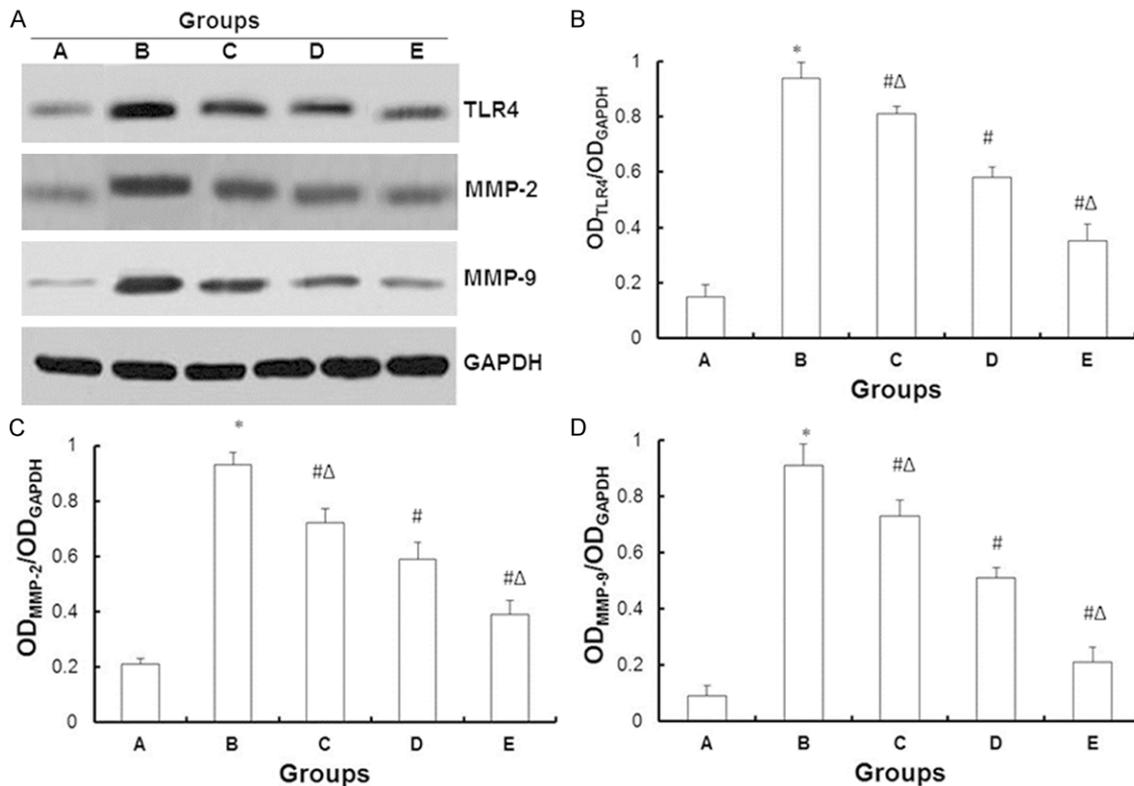


Figure 6. The inhibitory effect of Aliskiren on the LPS-induced protein expressions of TLR4, MMP-2 and MMP-9 in HUVECs. Control group (Group A), LPS model group (Group B), Aliskiren low-dose group (Group C, 1 μ M), medium-dose group (Group D, 10 μ M) and high-dose group (Group E, 100 μ M). A. The Western blot of TLR4, MMP-2 and MMP-9 in each group of HUVECs; B. The protein expression levels of TLR4 in each group of HUVECs; C. The protein expression levels of MMP-2 in each group of HUVECs; D. The protein expression levels of MMP-9 in each group of HUVECs. * $P < 0.05$ vs Group A; # $P < 0.05$ vs Group B; $\Delta P < 0.05$ vs Group D.

mechanism of angiogenesis inhibition by Aliskiren. The results indicated that Aliskiren inhibited the expression of TLR4 and the secretions of TNF- α , ICAM-1 and MCP-1 in a dose-dependent manner, which suggested that Aliskiren might inhibit the angiogenesis and stabilize the plaques through the inhibition of TLR4 signaling pathway activation.

The metabolism of extracellular matrix in plaques is another important factor affecting the stability of atherosclerotic plaques, and the matrix metalloproteinase family is a key factor influencing the decomposition of matrix [23]. When AS occurs, the activated endothelial cells and monocyte-macrophages can secrete MMP-2, MMP-9 and vascular endothelial growth factors, thus stimulating the vascular endothelial cells proliferation, migration and accelerating angiogenesis [24]. In addition, matrix metalloproteinases can promote endothelial cell migration from existing blood vessels to the outer periphery to form new blood vessels by degrad-

ing the basement membrane and remodeling the extracellular matrix. When HUVECs was cultured on basement membrane-like materials, the endothelial cells would be arranged in a straight line soon and formed tubes [25]. Gelatin zymography showed that the supernatant contained a large number of activated MMP-2 and MMP-9, suggesting that MMP-2 and MMP-9 were closely related to the formation of new blood vessels [26]. The results of this study indicated that Aliskiren could inhibit the mRNA and protein expressions of MMP-2 and MMP-9 in a dose-dependent manner, suggesting that Aliskiren might inhibit the angiogenesis and stabilize the plaques through suppressing the expressions of MMP-2 and MMP-9.

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Disclosure of conflict of interest

None.

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