Original article

The role of p38MAPK in asymmetric dimethylarginine-induced cytoskeleton and cellular permeability changes in cultured endothelial cells

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Background: Asymmetric dimethylarginine (ADMA) induces endothelial cell barrier dysfunction via cytoskeleton activation and contraction. It is supposed that activated p38 mitogen-activated protein kinase (MAPK) would trigger the formation of stress fibers and increase cellular permeability.

Objective: Explore p38 MAPK as a potentially important enzyme in ADMA-mediated endothelial cell contractile response and permeability change.

Methods: Human umbilical endothelial cells (HUVECs) were cultured, where ADMA and/or SB203580 (the specific inhibitor of p38MAPK) were used to stimulate HUVECs. Immunofluorescent staining was carried out to examine the expression and distribution of F-actin, flow cytometry was used to quantify F-actin, and Transwell was applied to test cellular permeability with FITC-labelled human serum albumin (HSA). Scanning electronic microscopy (SEM) was utilized to observe the changes of intercellular contact.

Results: ADMA induced significant p38MAPK activation in a dose-dependent manner, which correlated with increased stress fibers. SB-203580 attenuated the formation of actin stress fiber and the increase of cellular permeability induced ADMA in the HUVECs ($p < 0.01$, LSCM; $p < 0.01$, cytometry; $p < 0.05$, Transwell). Widened intercellular space induced by ADMA was detected and could be inhibited by SB-203580 (SEM). SB-203580 alone had no effect on cytoskeleton and cellular permeability.

Conclusion: p38MAPK activation participated in cytoskeleton and cellular permeability changes induced by ADMA in HUVECs.

Keywords: Asymmetric dimethylarginine, capillary permeability, cytoskeleton, F-actin, p38MAPK

ADMA is a normal metabolite in vivo, widely existing in tissues, plasma, and urine. It is produced by various cells, including endothelial cells. Asymmetric dimethylarginine (ADMA) was identified as a small molecular toxin [1]. It is also well known as a predictive and risk factor of cardiovascular diseases. Elevated ADMA can increase the morbidity of cardiovascular diseases in patients with chronic kidney diseases (CKD). It is believed that ADMA further leads to cardiovascular diseases. According to Cooke [2], when dysfunction of vascular endothelial cells takes part in cardiovascular diseases, ADMA

inhibits endothelium-dependent vasodilatation by inhibiting the activity of nitric oxide (NO) synthase.

Cytoskeleton usually refers to a cellular skeleton contained within the cytoplasm, and made up of three kinds of protein filaments, actin filaments (or microfilaments), intermediate filaments, and microtubules. Microfilaments are most related to vascular permeability. Under the stimulation of some inflammatory mediators, F-actin rearranges, redistributes, and forms non-polarized bundles of filaments (or stress fibers). They can make cells contract just as myofibrils contract muscle. When contractility of stress fibers is stronger than intercellular adhesion, intercellular gap is enlarged and cellular permeability increases [3].

Previous studies showed that ADMA could activate p38MAPK/Caspase-3 pathway [4], and

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activated p38MAPK would trigger the formation of stress fibers [5, 6]. Stress fibers are composed of F-actin, a kind of cytoskeleton protein. When contractility of F-actin is stronger than intercellular adhesion, cellular permeability increases [3]. Therefore, it is speculated that p38MAPK might take part in cytoskeleton and cellular permeability changes induced by ADMA.

In this study, we used ADMA and SB203580 (the specific inhibitor of p38MAPK) to stimulate HUVECs (human umbilical vein endothelial cells), and detected the expression and distribution of F-actin as well as the changes of cellular permeability and intercellular contact.

Materials and methods

Cell culture and grouping

HUVECs were received from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China), and verified by factor VIII. The 7-10th passages were used in this experiment. Cultured cells were kept in 50 ml culture flask in 3 ml endothelial cell medium (ECM, Sciencell, USA) consisting of 5% fetal bovine serum (FBS) with penicillin, streptomycin and endothelial cell growth supplements. Cells were maintained at 37°C in 5% CO₂, passaged prior to confluence, and fed twice a week. Equal number of cultured HUVECs in vitro was put into 24-well plates. They were divided into control group and treated group at random. Treated group was subdivided into three groups, including ADMA group, SB203580 group, SB203580+ADMA group, three wells per group. Control group was treated with neither ADMA nor SB203580.

Reagents and materials

100-200 µmol/L ADMA (Sigma, MO, USA) were added to the cultured cells for 16-24 hours, 25 µmol/L SB203580 (Sigma, MO, USA) was used to pretreat the cultured cells. 5 µg/mL FITC labeled phalloidin (Sigma, MO, USA) was used to stain the intracellular F-actin. Twenty µg fluorescein isothiocyanate (FITC)-labeled human serum albumin (HSA, Biosynthesis Biotechnology Co, Beijing, China) were used to test the cellular permeability.

Cell viability assay

Cell viability was determined using the 3-(4, 5-dimethylthiazol-2-yl) -2, 5-diphenyltetrazolium bromide (MTT) reduction assay. MTT is reduced in

metabolically active cells to yield an insoluble purple formazan product. Cells were cultured in 96-well culture dishes with ADMA for 6-24 hours. Then, 20 µl per well of MTT solution (5g/L) was added. Four hours later, the supernatants were discarded and 150 µl of dimethyl sulphoxide (DMSO) per well were added to dissolve the formazan. The absorbance was measured using an enzyme-linked immunosorbent assay (ELISA) reader (Bio-Tek Instruments, VT, USA) at 490 nm.

Cell viability was calculated as follows:

$$\text{Cell viability} = (\text{mean OD in treatment group}) / (\text{mean OD in control group}) \times 100\%. \quad (1)$$

Immunofluorescent localization of F-actin

In ADMA group, HUVECs were treated with ADMA for 24 hours, the concentration of which ranged from 10 µmol/L, 25 µmol/L, 50 µmol/L to 100 µmol/L. In SB203580 group, HUVECs were treated with 25 µmol/L SB203580 for 24 hours. In SB203580+ADMA group, HUVECs were pretreated with 25 µmol/L SB203580 for 30 minutes. Then, SB203580 was totally removed and 100 µmol/L ADMA was added into each well. After 24-hour incubation, immunofluorescence was used to examine the localization of F-actin. Endothelial cells were rinsed with phosphate buffered saline (PBS) for 2x5 minutes, fixed in 4% paraformaldehyde for 30 minutes, and permeabilized with 0.2% Triton X-100 for 10 minutes. Cells were then washed with PBS, and incubated with 5 µg/mL FITC-conjugated phalloidin (Sigma, MO, USA) for one hour at 37°C. After being washed with PBS for 2x5 minutes, coverslips were mounted on slides with anti-fade glycerine (pH 8.4) and analyzed under a laser scanning confocal microscope (LEICA TCS SP5, Wetzlar, Germany). Leica Microsystems LAS AF was used to analyze the immunofluorescent intensity with six random fields in each group.

Quantification of F-actin with flow cytometry

Cells were grouped and treated as above, and cell suspension was prepared by 0.25% trypsin. Four percents paraformaldehyde was used to fix the cells for 30 minutes. Cell suspension was centrifuged at 1000 rpm for five minutes. Supernatant was discarded, and the cell cluster was washed with PBS for 2x5 minutes and recentrifuged. The cells were incubated in 5 µg/mL FITC-conjugated phalloidin away from light for one hour, and washed with PBS for 2x5 minutes

again. The immunofluorescent intensity of 10,000 cells in each group was quantified with flow cytometry. The immunofluorescent intensity stood for the relative amount of F-actin.

Measurement of cellular permeability with Transwell

Measurement of cellular permeability was performed as previously described [7]. HUVECs were placed in Millicell (1 μ m pore size, 9.3 mm in diameter) (Millipore, MA, USA) at cell density of 10,000 cells/well, and grown until 90% confluence. Control group was treated with neither ADMA nor SB203580. In ADMA group, HUVECs were treated with 200 μ M ADMA for 16 hours. In SB203580 group, HUVECs were treated with 25 μ M/L SB203580 for 30 minutes. In SB203580+ADMA group, HUVECs were pretreated with 25 μ M/L SB203580 for 30 minutes, and incubated with 200 μ M/L ADMA for 16 hours. Liquid inside and outside the Millicell was drained out, and 1 ml culture medium without phenol red was added into the lower chamber. Two hundred μ l FITC-HSA (20 μ g) was rapidly filled into the upper chamber, and the Millicell was placed into a 5% CO₂ cell incubator at 37°C in dark. After six hour incubation, the liquid both in the upper and lower chamber was collected, respectively, and placed into 96-well black plate for measurement. Fluorescent absorbance was tested by fluorescent microplate reader (BioTek Synergy4, VT, USA). Excitation wavelength and emission wavelength were 485 nm and 520 nm, respectively. The concentration of FITC-HSA was measured by using a pre-charted fluorescence-protein standard curve. The transmission rate (%) of HSA equals the concentration of FITC-HSA in lower chamber/that in upper chamber.

Change of intercellular contact by SEM

HUVECs were seeded onto cover-slips in 24-well plates and cultured with ECM till total confluence. After 16 hours' incubation, cells were rinsed in PBS for thrice and fixed in 3% glutaraldehyde for two hours at room temperature. After being rinsed in PBS for 2x10 minutes, cells were post-fixed in osmium tetroxide for one hour, washed in distilled water, and dehydrated through a graded series of ethanol solutions. The samples were critical-point-dried from CO₂, glued onto aluminium stubs with colloidal silver adhesive, sputter-coated with 20 nm platinum, and examined in TM-1000 scanning electron microscope (Hitachi,

Tokyo, Japan) operating at 15kV.

Statistical analysis

The data were expressed as mean $\pm$ standard deviation (SD). The p-values were calculated using SPSS 11.5 software. A p-value of <0.05 was considered to be statistically significant. One-factor analysis of variance was used to show statistical significance of optical density among groups. Dixon's Q-test was used to examine the statistical significance of optical density between groups.

Results

ADMA decreased cell viability

The effect of ADMA treatment on HUVEC proliferation was examined using the MTT assay. HUVECs treated with ADMA were suppressed in growth and proliferation rates compared to untreated cells. Further, the MTT results suggested that cell viability of HUVECs decreased with increase of ADMA concentration and culture time applied to cells.

Figure 1 shows cell viability in different groups that were calculated based on equation 1. To optimize the drug effect on HUVECs and meantime keep cell viability over 90%, 24 hours incubation of 100 μ M/L ADMA or 16 hours incubation of 200 μ M/L ADMA were used in cytometry, Transwell and SEM assays.

ADMA changed the location of F-actin in endothelial cells

Figure 2 shows laser confocal microscopic images of endothelial change in F-actin with immunofluorescence. Apparently, HUVEC treated with ADMA (100 μ M/L, 24 hours) became less polarized morphologically, showing increased formation of F-actin (**C-F**), as compared with untreated controls (**A**) and SB203580 group (**B**). With the increase of dose, the effect of ADMA on F-actin became more and more obvious. By contrast, in ADMA+SB203580 group, the effect of ADMA on cytoskeleton could be reversed (**G-J**).

The fluorescent integrated density volume (IDV) was calculated using Leica Microsystems LAS AF software. **Figure 3** shows Fluorescent IDV in ADMA and SB203580+ADMA groups. In ADMA group, ADMA-induced increase of F-actin was dose-dependent and ADMA in all the concentration had statistical significance compared to control group (**A**). Statistical significance was neither found within SB203580+ADMA group, nor found between control

group and SB203580 group. ADMA-induced increase of F-actin could be reversed in SB203580+ADMA group and there was statistical significance between any concentration pair (**B**).

ADMA increased the formation of F-actin

Flow cytometry analysis of F-actin among different groups demonstrated that the mean fluorescent intensity of SB203580 and SB203580+ADMA group had no statistical significance compared to control. The mean fluorescent intensity of ADMA group had statistical significance compared to control and SB203580+ADMA group ($p < 0.01$) as shown in **Figure 4**.

ADMA increased permeability of HUVECs

The mean permeability of HSA by Transwell had

no statistical significance among SB203580, SB203580+ADMA and control group. However, the mean permeability of HSA in ADMA group had statistical significance compared to that in control, SB203580 and SB203580+ADMA group ($p < 0.01$) as can be seen in **Figure 5**.

ADMA widened intercellular contact

Figure 6 shows SEM images of ADMA-induced change of intercellular contact. Interestingly, in control group, intercellular contact was tight and intercellular space was narrow (**A, a, C, c, D, d**), while in ADMA group, intercellular contact was sparse and intercellular space is widened (**B, b**). Besides, ADMA could even interrupt the intercellular contact, as shown by arrow in **b**.

Figure 1. Cell viability in different groups. **A:** ADMA-induced growth suppression in a dose-dependent manner, **B:** 200 μmol/LADMA-induced growth suppression in a time-dependent manner

Figure 2. Endothelial change in F-actin with immunofluorescence (LSCM $\times 1000$). F-actin arranged in cellular periphery beneath cell membranes, forming organized bundles, and the cellular morphology is normal in control (A) and SB203580 group (B). In ADMA group, F-actin rearranged and redistributed with stress fibers increased and cells shrinking in a dose-dependent manner (C-F). In ADMA+SB203580 group, ADMA-induced cytoskeleton change could be inhibited by SB203580 (G-J).

Figure 3. Fluorescent IDV in ADMA and SB203580+ADMA groups. **A:** ADMA in all the concentration led to the increase of F-actin, which had statistical significance compared to control group. Fifty μmol/L and 100 μmol/L ADMA groups both had statistical significance compared to 10 μmol/L and 25 μmol/L ADMA groups. **B:** There was also a significant difference of ADMA group vs. SB203580+ADMA group at the same ADMA concentration in F-actin change. *significant difference between this group vs. control ($p < 0.01$). #significant different between this group vs. 10 μmol/L ADMA group ($p < 0.01$). %significant difference between this group vs. 25 μmol/L ADMA group ($p < 0.01$).

Figure 4. Fluorescent intensity of F-actin in different treated groups. F-actin quantification with flow cytometry was shown above. **A:** With the same cell number, fluorescent intensity in ADMA group is stronger than that in any other group. **B:** Histogram was drawn to reveal the difference in detail. *significant difference of this group vs. ADMA group ($p < 0.01$). There was no significant difference between any other two groups.

Figure 5. ADMA-induced change of cellular permeability. The mean permeability of HSA stood for cellular permeability. *significant difference between this group vs. ADMA group ($p < 0.05$). There was no significant difference between the other two groups.

Figure 6. ADMA-induced change of intercellular contact under SEM (A, B, C, D: x250; a, b, c, d: x1500). After 16 hours incubation, HUVEC in control (A, a), SB203580 group (C, c) and SB203580+200 $\mu\text{mol/L}$ ADMA group (D, d) grew well with intercellular contact tight, while in 200 $\mu\text{mol/L}$ ADMA group (B, b) cell growth was inhibited with intercellular contact sparse. Arrow in b shows interrupted intercellular contact.

Discussion

Endothelial dysfunction often exists in the patients with cardiovascular diseases. NO plays a key role in protecting endothelial function. Since ADMA is an endogenous competitive inhibitor of NO synthase, it inhibits vascular NO production at concentrations found in pathophysiological conditions. Increased ADMA levels are associated with reduced NO synthesis as assessed by impaired endothelium-dependent vasodilation or reduced NO metabolite levels [8]. Therefore, ADMA may become a promising drug target in reversing endothelial dysfunction [9]. Currently, rare pathway studies of ADMA-induced endothelial dysfunction are documented.

Change of endothelial cytoskeleton leads to cellular contraction, which further causes the change of cellular permeability. Cellular contraction is mostly influenced by actin and myosin. Phosphorylation of myosin light chain will activate the ATPase in the head of myosin heavy chain, which energizes F-actin to make cell contraction. Studies showed p38MAPK could increase stress fibers by two pathways as follows. Firstly, p38MAPK phosphorylates and activates the mitogen-activated protein kinase-activated protein kinase-2/3/5 (MAPKAP-K2/K3/K5), leading to the phosphorylation of one of its substrates, heat shock protein (HSP-27). Phosphorylated HSP-27 reduces actin polymerization,

and stress fibers form [5]. Secondly, p38 MAPK activation leads to caldesmon phosphorylation and dissociation of caldesmon-myosin complex, leading to actin cytoskeleton rearrangement and stress fiber formation [6]. Jiang et al. [4] did some research on ADMA-induced HUVEC apoptosis, and found which was achieved via p38 MAPK/caspase-3-dependent signaling pathway in endothelial cells.

All these results revealed that ADMA could induce cytoskeleton changes in HUVECs and p38MAPK might take part in this process. SB203580 is the specific inhibitor of p38MAPK, which cannot only combine the active p38MAPK, but also combine non-active p38MAPK, and decrease its activation rate [10, 11]. In the normal state, F-actin arranged in the cellular periphery beneath cell membranes, forming organized bundles, and the cellular morphology was normal. In the ADMA-treated group, F-actin rearranged and redistributed with stress fibers increased and cells shrinking. ADMA could enlarge intercellular space and increase intercellular permeability.

In conclusion, in cultured HUVECs in vitro experiment, SB203580 might improve the ADMA-induced endothelial dysfunction and offered a brand-new way to treat endothelial dysfunction in CKD patients. Blocking p38MAPK cascade might relieve inflammatory reaction. Nevertheless, MAPKs cascade is a complicated and reciprocal system, and inhibition of some pathway in partial and whole body

may lead to unexpected outcome or disturbed physiological processes.

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