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Original Article

Ulinastatin attenuates monocyte-endothelial adhesion via inhibiting ROS transfer between the neighboring vascular endothelial cells mediated by Cx43

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Abstract: Ulinastatin is widely used in the treatment of pancreatitis and sepsis, because of its excellent anti-inflammatory and antioxidant effects. However, its effects on atherosclerosis, an inflammatory vascular disease, are rarely reported. Therefore, in present study, we explored effects of ulinastatin on monocyte-endothelial adhesion, the initiator of atherosclerosis. We used U937 monocytes and angiotensin II-stimulated human umbilical vein endothelial cells (HUVECs) to build the model of monocyte-endothelial adhesion. Different methods were used to change the function of connexin43 (Cx43), the level of ROS, the activation of JAK2/STAT3 signaling pathway and its downstream MMP2 and MMP9 expression, and then the influences of ulinastatin on U937-HUVECs adhesion and the adhesion molecules were observed. The results showed that ulinastatin could attenuate ROS transmission between the neighboring HUVECs via inhibiting Cx43 function. With the decrease of ROS, JAK2/STAT3 signaling pathway and its downstream MMP2 and MMP9 expression were downregulated. Ultimately, important adhesion molecules expression, such as VCAM-1, ICAM-1, sVCAM-1 and sICAM-1, and U937-HUVECs adhesion, were both reduced. Thus, we can conclude that ulinastatin attenuates adhesion molecules expression and monocyte-endothelial adhesion, mechanism of which is related that ulinastatin inhibits ROS transfer between the neighboring vascular endothelial cells mediated by Cx43, resulting in the inactivation of JAK2/STAT3 signaling pathway, and its downstream MMP2 and MMP9 expression decrease.

Keywords: Ulinastatin, Cx43, ROS, JAK2/STAT3 signaling pathway, MMP2, MMP9

Introduction

The pathological process of atherosclerosis is very complicated. In short, damaged endothelial cells express adhesion molecules, resulting in monocytes adhering to endothelial cells. Adhered monocytes migrate into the subendothelial intima through the endothelium. Once here, monocytes differentiate into macrophages and engulf modified lipoproteins, transforming them into lipid-rich foam cells, which will show the feature of early-stage atherosclerosis, the fatty streak [1, 2]. Therefore, the increase of monocyte-endothelial adhesion is always thought to be the initiating factor of atherosclerosis [3], understanding the underlying mechanism of which will be beneficial to employ more effective and reasonable strategies for the prevention and treatment of atherosclerosis.

As a serine protease inhibitor, ulinastatin protects organ functions from various harmful factors via scavenging oxygen free radicals and eliminating inflammatory cytokines [4]. Because of its excellent anti-inflammatory and antioxidant effects, ulinastatin is widely used in the treatments of pancreatitis, sepsis and sepsis-related multiple organ dysfunction, reducing the mortality effectively [5]. Atherosclerosis is essentially an inflammatory vascular disease [6]. As an effective anti-inflammatory agent and antioxidant, whether ulinastatin can prevent the development of atherosclerosis has not been reported so far. Thus, in present study, we investigated the influence of ulinastatin

on monocyte-endothelial adhesion, the initiator of atherosclerosis.

We demonstrated that angiotensin II could upregulate Cx43 expression on HUVECs and result in U937 monocytes adhering to HUVECs. Our previous studies showed that Cx43, especially expressing on HUVECs, played an important part in monocyte-endothelial adhesion [1, 7]. In present study, we find that ulinastatin attenuates the function of gap junctions (GJs) composed of Cx43, which is the important biological basis for ulinastatin inhibiting angiotensin II-induced monocyte-endothelial adhesion. GJs composed of Cx43 mediate the direct cell-to-cell signaling transfer, allowing small molecules transmission between the neighboring cells [8]. ROS is one of the few substances that can be transmitted through GJs [8]. Ulinastatin attenuates ROS transfer between the neighboring HUVECs via inhibiting GJs function composed of Cx43, resulting in the downregulating of JAK2/STAT3 signaling pathway and its downstream MMP2 and MMP9 expression. Both MMP2 and MMP9 are often highly expressed in patients with atherosclerosis, and their effects on the occurrence and development of atherosclerosis have been confirmed by multiple studies [9]. More importantly, we find that inhibiting MMP2 and MMP9 can affect the expression of VCAM-1, ICAM-1, sVCAM-1 and sICAM-1. This viewpoint has never been reported. In summary, we believe that the present study clarifies the function of ulinastatin on monocyte-endothelial adhesion and its possible mechanism for the first time, providing valid therapies and a series of potential targets for preventing atherosclerosis.

Material and methods

Cell culture

The study protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki with the approval of the Institutional Medical Ethics Committee of the Third Affliated Hospital of Sun Yat-sen University. U937 monocytes and HUVECs were both purchased from American Type Culture Collection (Manassas, VA, USA). U937 monocytes were cultured in RPMI1640 medium (Invitrogen), containing 20% fetal bovine serum (Invitrogen) and 100 U/ml penicillin streptomycin (Invitrogen). HUVECs were cultured with human endothelial SFM (Invitrogen, Carlsbad, CA, USA), containing 20% fetal bovine serum (Invitrogen), 100 μg/ml endothelial cell growth supplement (Becton, Dickinson and Company, Frankin Lakes, NJ, USA), 100 μg/ml heparin (Sigma-Aldrich, St. Louis, MO, USA) and 100 U/ml penicillin-streptomycin (Invitrogen). U937 monocytes and HUVECs were cultured in a 5% CO_2 incubator at 90% humidity (Thermo Fisher Scientific, Waltham, MA, USA).

Cell treatments

HUVECs were stimulated with angiotensin II (1 μM) for 4 hours. Ulinastatin (Tianpu Biochemical Pharmaceutical, Guangzhou, China) was used at 200 U/ml for 4 hours before angiotensin II exposure. Gap27 was used to inhibit Cx43 function (Sigma-Aldrich, 300 μM, 1 hour before angiotensin II exposure). N-acetyl cysteine (NAC, Sigma-Aldrich, 10 mM, 1 hour before angiotensin II exposure) was used to scavenge ROS. SB-3CT (Sigma-Aldrich, 1 μM, 4 hours before angiotensin II exposure) was used to inhibit MMP2 and MMP9.

Adhesion assay

Firstly, U937 monocytes were labeled with calcein-acetoxymethyl ester (Invitrogen, 5 μM, 30 minutes). Secondly, the labeled U937 monocytes were washed twice with PBS (Invitrogen) and resuspended in the medium without serum. Finally, they were added onto confluent monolayers of HUVECs pretreated with or without angiotensin II. The cells were cultured in the 5% CO_2 incubator at 37°C for 1 hour. Then, the plates were rinsed twice with medium without serum. The adherent U937 monocytes were remained on the confluent monolayers of HUVECs. The adherent U937 monocytes were counted with a fluorescence microscope (Olympus IX71, Tokyo, Japan). For each condition, 8 different 200× visual fields in the middle of the dish were chosen for analysis.

Protein detection

Cx43, JAK2, p-JAK2, STAT3, p-STAT3, VCAM-1 and ICAM-1 were tested with western blotting. MMP2, MMP9, sVCAM-1 and sICAM-1 were tested with ELISA. In western blotting, protein samples were quantified with Pierce™ BCA Protein Assay Kits (Thermo Fisher Scientific, Inc.) and the dilutions of antibodies were as fol-

lowing: anti-Cx43 (1:4000, Sigma-Aldrich); JAK2 (1:2000, Sigma-Aldrich); p-JAK2 (1:1000, Sigma-Aldrich); STAT3 (1:2000, Sigma-Aldrich); p-STAT3 (1:1000, Sigma-Aldrich); VCAM-1 and ICAM-1 (1:200, Santa Cruz biotechnology, Santa Cruz, CA, USA), and GAPDH (1:5000, Sigma-Aldrich). The original bands are showed in the supplemental materials. Protein band sizes are valued by Alpha View software (version number: 2.2.14407, Protein Simple, Santa Clara, CA, USA). All of human MMP2, MMP9, sVCAM-1 and sICAM-1 ELISA kits were purchased from Sigma-Aldrich. They were used according to the instructions.

Parachute dye-coupling assay

This method was used to examine the function of gap junction intercellular communication. In present study, the parachute dye-coupling assay was used to examine gap junction intercellular communication composed of Cx43 on HUVECs. Donor cells were labeled with 5 μM calcein-AM (30 minutes at 37°C). Then, they were trypsinized and seeded onto the receiver cells at a ratio of 1:150 (donor:receiver). The donor cells would attach to the receiver cells to form GJs. After 4 hours, the results were observed with a fluorescence microscope (Olympus DP73, Tokyo, Japan). For each well, 8 different 200× visual fields in the middle of the dish were chosen for analysis. The average number of receiver cells around every donor cell was counted. We considered the data of the control group as 1, and other groups were normalized to the control group. This ratio just represented as adhesion fraction.

ROS detection

After pretreatment with different chemicals, HUVECs were stained with ROS assay kit (6-carboxy-2'-7'-dichlorodihydrofluorescein diacetate [DCFHDA]), and then detected by flow cytometry. The fluorescence intensity of cells for each group was calculated following the manufacturer's instruction.

Statistical analysis

Statistical analysis was performed by using SPSS 15.0 software (SPSS, Inc., Chicago, IL, USA). Multiple comparisons among groups were analyzed using repeated measures one-way ANOVA, followed by Tukey post hoc comparisons. A threshold of $P < 0.05$ was defined as statistically significant.

Results

Ulinastatin attenuates angiotensin II-induced U937-HUVECs adhesion

Recent studies showed that angiotensin II could induce VCAM-1 and ICAM-1 expression on HUVECs [10, 11]. Therefore, we speculated that angiotensin II might result in monocyte-endothelial adhesion increase. In present study, **Figure 1A** demonstrated that U937-HUVECs adhesion was enhanced when HUVECs were pretreated with angiotensin II. Our previous investigation indicated that Cx43 expressing on HUVECs played an important part in monocyte-endothelial adhesion [1, 12]. Thus, we explored effects of Cx43 on angiotensin II-induced U937-HUVECs adhesion. **Figure 1B** and **1C** showed that angiotensin II pretreatment on HUVECs could indeed enhance Cx43 expression and its function that mediated dye spread between neighboring cells, which demonstrated that angiotensin II-induced U937-HUVECs adhesion might be relative with Cx43 expression increase on HUVECs. Ulinastatin, widely used in clinic, is considered a reasonable therapy for vascular barrier dysfunction in inflammatory disorders [13]. We found that it could reduce angiotensin II-induced U937-HUVECs adhesion significantly. Interestingly, ulinastatin could inhibit Cx43 function, but had no effects on its expression (**Figure 1A-C**). This phenomenon has never been reported. It means that ulinastatin protects against monocyte-endothelial adhesion induced by angiotensin II via inhibiting Cx43 function, but not its expression.

Ulinastatin attenuates angiotensin II-induced U937-HUVECs adhesion via inhibiting ROS spread between neighboring cells mediated by Cx43

ROS is one of Cx43-mediated important substances transmitted between neighboring cells, and plays an important role in vascular inflammation injury and monocyte-endothelial adhesion [8, 14]. **Figure 2A** showed that angiotensin II increased the level of ROS, which could be alleviated by Gap27, the specific inhibitor of Cx43. It was mainly because Gap27 blocked ROS transmission between the neighboring

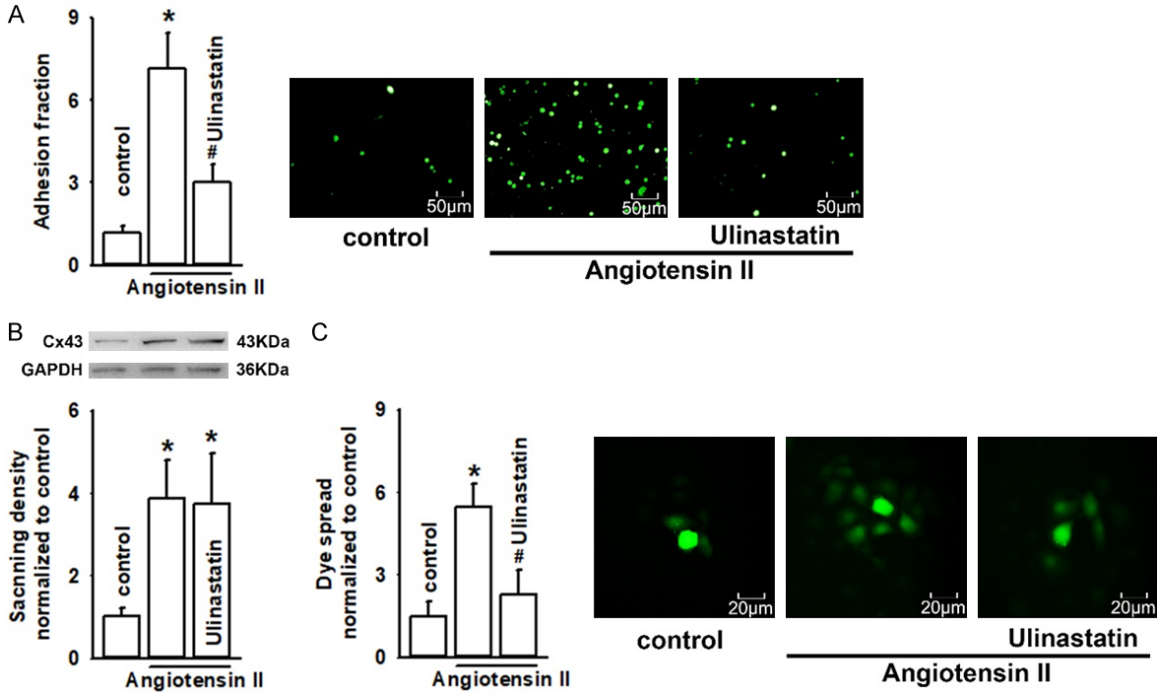

Figure 1. Ulinastatin attenuated angiotensin II-induced U937-HUVECs adhesion and Cx43 channels function, but had no effects on Cx43 expression on HUVECs. A. Ulinastatin attenuated angiotensin II-induced U937-HUVECs adhesion (angiotensin II: 1 μM for 4 hours; ulinastatin: 200 U/ml, 4 hours before angiotensin II exposure; data are shown as mean ± SEM; n=5, *P < 0.05 vs control; #P < 0.05 vs angiotensin II group); B. Ulinastatin had no effect on Cx43 expression on HUVECs (angiotensin II: 1 μM for 4 hours; ulinastatin: 200 U/ml, 4 hours before angiotensin II exposure; data are shown as mean ± SEM; n=5, *P < 0.05 *vs* control); GAPDH is used as a loading control. C. Ulinastatin attenuated Cx43 channels function (Parachute dye-coupling assay) (angiotensin II: 1 μM for 4 hours; ulinastatin: 200 U/ml, 4 hours before angiotensin II exposure; n=5, *P < 0.05 *vs* control; #P < 0.05 *vs* angiotensin II group).

cells and the consequent rise of ROS. The effect of Gap27 was equivalent to NAC, the scavenger of ROS. It demonstrated that inhibition Cx43 function could reduce the level of ROS. Because ulinastatin could inhibit Cx43 function (**Figure 1C**), it also attenuated the level of ROS (**Figure 2A**). As the level of ROS induced by angiotensin II was suppressed by Gap27 and NAC, U937-HUVECs adhesion was also reduced (**Figure 2B**). Based on **Figures 1** and **2**, we can conclude that ulinastatin attenuates U937-HUVECs adhesion via inhibiting ROS transmission between the neighboring cells mediated by Cx43.

Ulinastatin attenuates angiotensin II-induced JAK2/STAT3 signaling pathway activation via inhibiting ROS spread between neighboring cells mediated by Cx43

Figure 3A showed that angiotensin II activated the JAK2/STAT3 signaling pathway, manifested as the increase of p-JAK2 and p-STAT3 expression (JAK-2 and STAT3 had no changes). When HUVECs were pretreated with NAC, the JAK2/STAT3 signaling pathway activation induced by angiotensin II was inhibited, which demonstrated that ROS played an important part in the process of angiotensin II-induced JAK2/STAT3 signaling pathway activation. Combining with the result that Cx43 inhibition attenuated ROS transmission between the neighboring cells (**Figure 2A**), we speculated that Cx43 inhibition could suppress JAK2/STAT3 signaling pathway activation via regulating the level of ROS. Our results supported this hypothesis. Gap27 application attenuated the activation of JAK2/STAT3 signaling pathway (**Figure 3A**). With the changes of JAK2/STAT3 signaling pathway activity, the downstream MMP2 and MMP9 were affected, both which were considered to be responsible for atherosclerosis [15]. Gap27 and NAC application inhibited angiotensin II-induced MMP2 and MMP9 expression via influencing

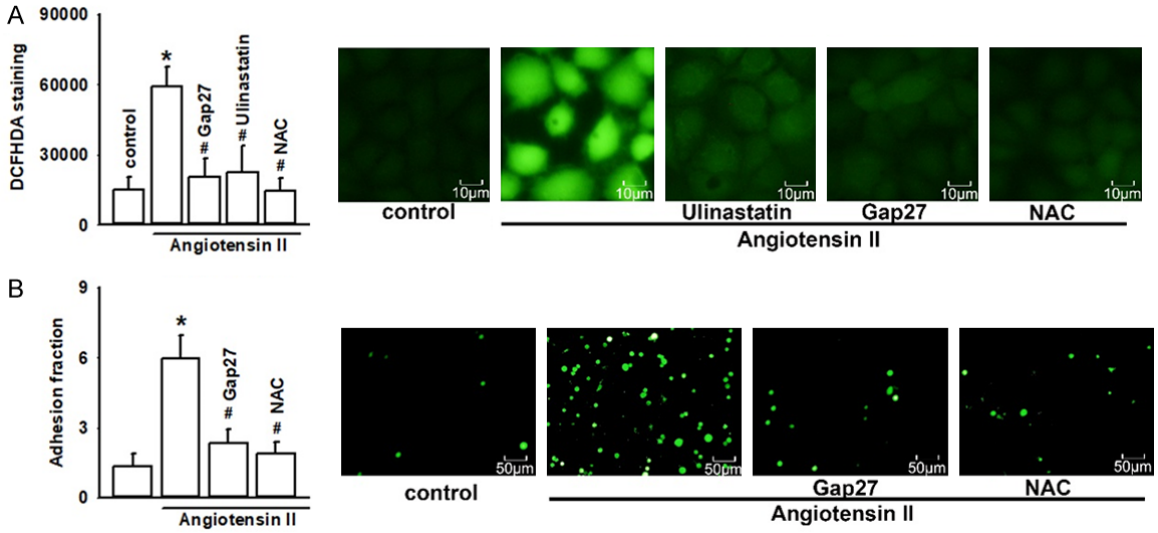

Figure 2. Ulinastatin, Gap27 and NAC attenuated angiotensin II-induced the increase of ROS and U937-HUVECs adhesion. A. Ulinastatin (200 U/ml, 4 hours before angiotensin II exposure), Gap27 (300 μM, 1 hour before angiotensin II exposure) and NAC (10 mM, 1 hour before angiotensin II exposure) attenuated angiotensin II-induced the increase of ROS (DCFHDA staining) (data are shown as mean ± SEM; n=5, **P* < 0.05 *vs* control; #*P* < 0.05 *vs* angiotensin II group); B. Gap27 (300 μM, 1 hour before angiotensin II exposure) and NAC (10 mM, 1 hour before angiotensin II exposure) attenuated angiotensin II-induced U937-HUVECs adhesion (data are shown as mean ± SEM; n=5, **P* < 0.05 *vs* control; #*P* < 0.05 *vs* angiotensin II group).

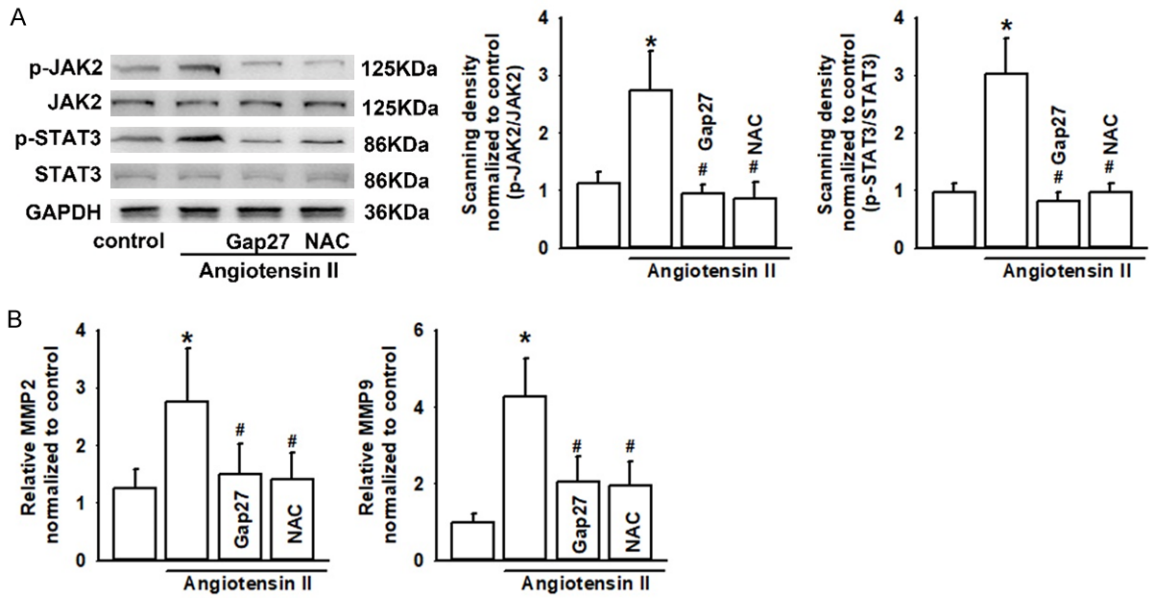

Figure 3. Gap27 and NAC attenuated angiotensin II-induced JAK2/STAT3 signaling pathway activation and its downstream MMP2 and MMP9. A. Gap27 (300 μM, 1 hour before angiotensin II exposure) and NAC (10 mM, 1 hour before angiotensin II exposure) attenuated angiotensin II-induced JAK2/STAT3 signaling pathway activation (data are shown as mean ± SEM; n=4, **P* < 0.05 *vs* control; #*P* < 0.05 *vs* angiotensin II group); GAPDH is used as a loading control. B. Gap27 (300 μM, 1 hour before angiotensin II exposure) and NAC (10 mM, 1 hour before angiotensin II exposure) attenuated angiotensin II-induced MMP2 and MMP9 (data are shown as mean ± SEM; n=5, **P* < 0.05 *vs* control; #*P* < 0.05 *vs* angiotensin II group).

the activity of JAK2/STAT3 signaling pathway (**Figure 3B**). From this, we concluded that Cx43 regulated the level of ROS in HUVECs, and ROS affected MMP2 and MMP9 expression through

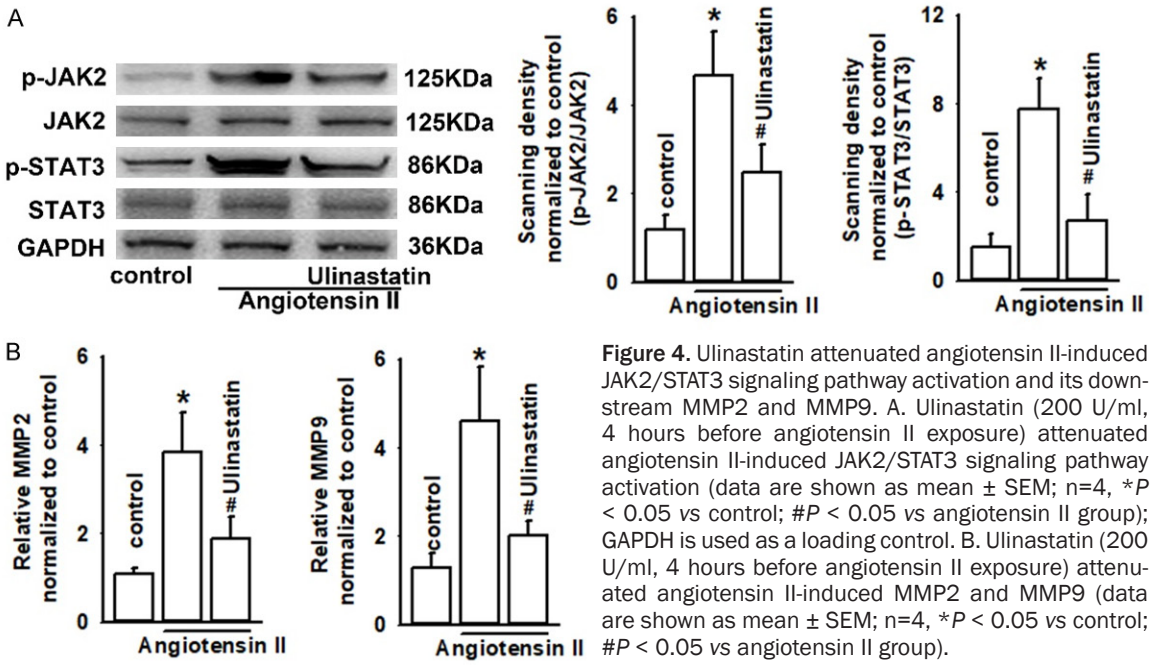

Figure 4. Ulinastatin attenuated angiotensin II-induced JAK2/STAT3 signaling pathway activation and its downstream MMP2 and MMP9. A. Ulinastatin (200 U/ml, 4 hours before angiotensin II exposure) attenuated angiotensin II-induced JAK2/STAT3 signaling pathway activation (data are shown as mean ± SEM; n=4, **P* < 0.05 *vs* control; #*P* < 0.05 *vs* angiotensin II group); GAPDH is used as a loading control. B. Ulinastatin (200 U/ml, 4 hours before angiotensin II exposure) attenuated angiotensin II-induced MMP2 and MMP9 (data are shown as mean ± SEM; n=4, **P* < 0.05 *vs* control; #*P* < 0.05 *vs* angiotensin II group).

regulating JAK2/STAT3 signaling pathway. Inhibition of Cx43 function by ulinastatin had been confirmed in **Figure 1C**. Therefore, we could see that ulinastatin could inhibit the JAK2/STAT3 signaling pathway activation and its downstream MMP2 and MMP9 expression (**Figure 4A** and **4B**).

Inhibition of MMP2 and MMP9 with SB-3CT attenuates angiotensin II-induced U937-HUVECs adhesion, VCAM-1, ICAM-1, sVCAM-1 and sICAM-1

In **Figure 5**, we used the specific inhibitor of MMP2 and MMP9, SB-3CT to inhibit the function of MMP2 and MMP9 [16]. Angiotensin II-induced U937-HUVECs adhesion was suppressed significantly. As gelatinases, both MMP2 and MMP9 are essential for the formation of VCAM-1 and ICAM-1, which are responsible for monocyte-endothelial adhesion [17]. Thus, we explored the effects of MMP2 and MMP9 on VCAM-1 and ICAM-1 expression, as well as the contents of soluble VCAM-1 and ICAM-1 (sVCAM-1 and sICAM-1). **Figure 6** showed that when HUVECs were pretreated with SB-3CT, angiotensin II-induced VCAM-1, ICAM-1, sVCAM-1 and sICAM-1 were all decreased obviously, which clarified that inhibiting MMP2 and MMP9 could attenuate the formation of adhesion molecules. All of Gap27 (inhibiting Cx43 function), NAC (scavenging ROS) and ulinastatin could inhibit the levels of VCAM-1, ICAM-1, sVCAM-1 and sICAM-1. Combining with all of the results, we believed that ulinastatin attenuated U937-HUVECs adhesion, mechanism of which was that ulinastatin blocked the ROS transmission between the neighboring cells, resulting in the down-regulation of JAK2/STAT3 signaling pathway and its downstream MMP2 and MMP9 expression. Ultimately, the formation of important adhesion molecules, such as VCAM-1, ICAM-1, sVCAM-1 and sICAM-1, were all reduced.

Discussion

Monocyte-endothelial adhesion is considered to be the initial factor of atherosclerosis. Adherent monocytes transmigrate into the arterial intima, where they will propagate, mature and accumulate lipids. Ultimately monocytes transform into macrophage foam cells, the hallmark of atherosclerotic pathology [1, 12]. Therefore, inhibiting monocyte-endothelial adhesion might prevent the development of atherosclerosis in the early stage. In present study, we demonstrated that ulinastatin, commonly used in clinic, could attenuate angiotensin II-induced U937-HUVECs adhesion, mechanism of which was that ulinastatin inhibited ROS transmission between the neighboring HUVECs mediated by Cx43, resulting in the down-regulation of JAK2/STAT3 signaling pathway and its downstream MMP2 and MMP9 expression. Ultima-

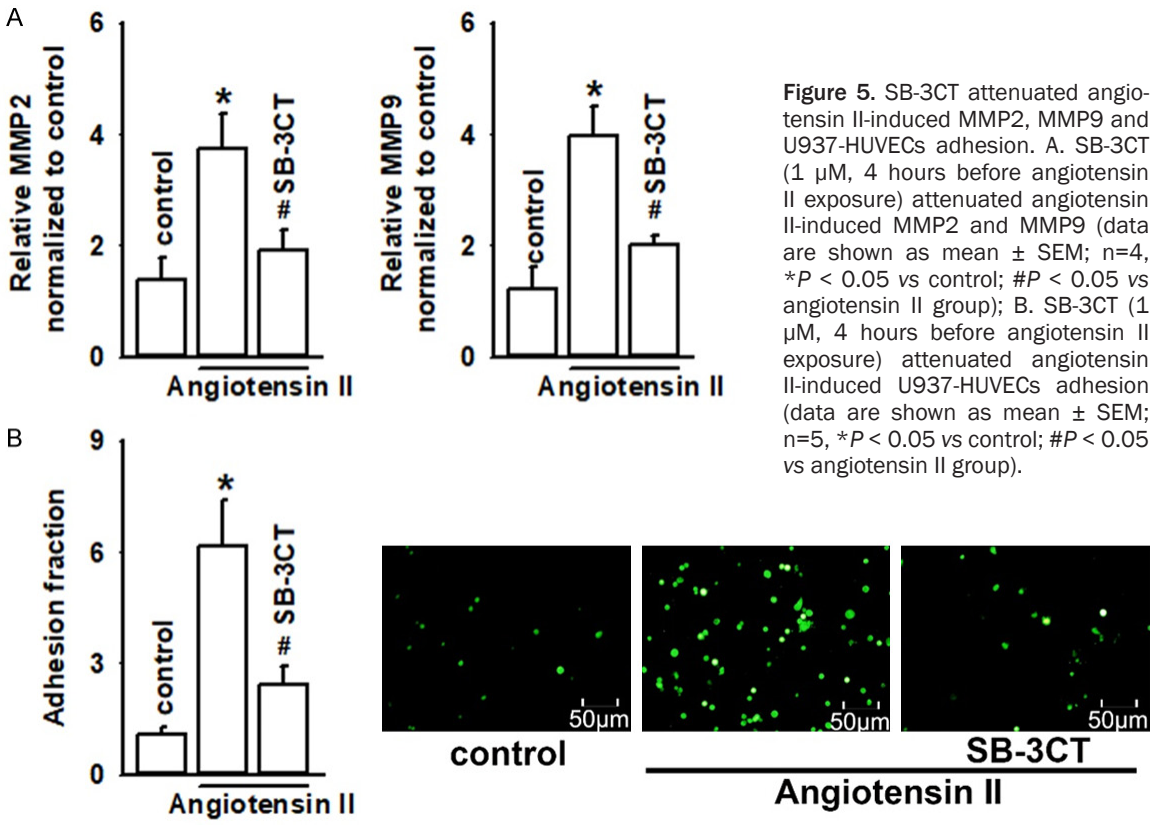

Figure 5. SB-3CT attenuated angiotensin II-induced MMP2, MMP9 and U937-HUVECs adhesion. A. SB-3CT (1 μM, 4 hours before angiotensin II exposure) attenuated angiotensin II-induced MMP2 and MMP9 (data are shown as mean ± SEM; n=4, **P* < 0.05 *vs* control; #*P* < 0.05 *vs* angiotensin II group); B. SB-3CT (1 μM, 4 hours before angiotensin II exposure) attenuated angiotensin II-induced U937-HUVECs adhesion (data are shown as mean ± SEM; n=5, **P* < 0.05 *vs* control; #*P* < 0.05 *vs* angiotensin II group).

tely, the formation of important adhesion molecules related to MMP2 and MMP9 were all reduced, such as VCAM-1, ICAM-1, sVCAM-1 and sICAM-1.

Angiotensin II is always considered to play an important role in a range of cardiovascular and cardiac metabolic diseases, such as hypertension, obesity and diabetes [18]. It initiates a ranged of inflammatory signaling cascades, resulting in the increase of ROS and cell adhesion molecules expression [19, 20]. These responses are associated with monocyte-endothelial adhesion, contributing to the early stages of atherogenesis. The results in our present study also supported this idea. Angiotensin II not only induced cell adhesion molecules expression and U937-HUVECs adhesion, but also caused ROS generation, JAK2/STAT3 signaling pathway activation and its downstream MMP2/9 expression. All of the results demonstrated that angiotensin II is an important risk factor for monocyte-endothelial adhesion. More importantly, we noticed that angiotensin II could induce Cx43 expression on HUVECs, which had never been reported.

Cx43 is a kind of transmembrane protein that communicates signals transmission between the neighboring cells. Our previous studies clarify that the changes of Cx43 expression affected U937-HUVECs adhesion [7]. On HUVECs obtained from patients with pregnancy-induced hypertension, Cx43 expression was often elevated. Inhibition of Cx43 protein expression could attenuate U937-HUVECs adhesion significantly [1]. In this investigation, angiotensin II application could induce Cx43 expression. Thus, we believed that it might be the key early step of angiotensin II-induced U937-HUVECs adhesion. From this point, we speculate Cx43 might be one of the key targets for attenuating angiotensin II-induced monocyte-endothelial adhesion.

Ulinastatin is considered a reasonable therapy for the treatment of vascular barrier dysfunction in inflammatory disorders. It can scavenge oxygen free radicals and eliminate inflammatory cytokines [5, 21]. We also observed that ulinastatin could protect against U937-HUVECs adhesion, but it had no effects on Cx43 expression (**Figure 1A** and **1B**). It demonstrated that the influence of ulinastatin on inhibiting

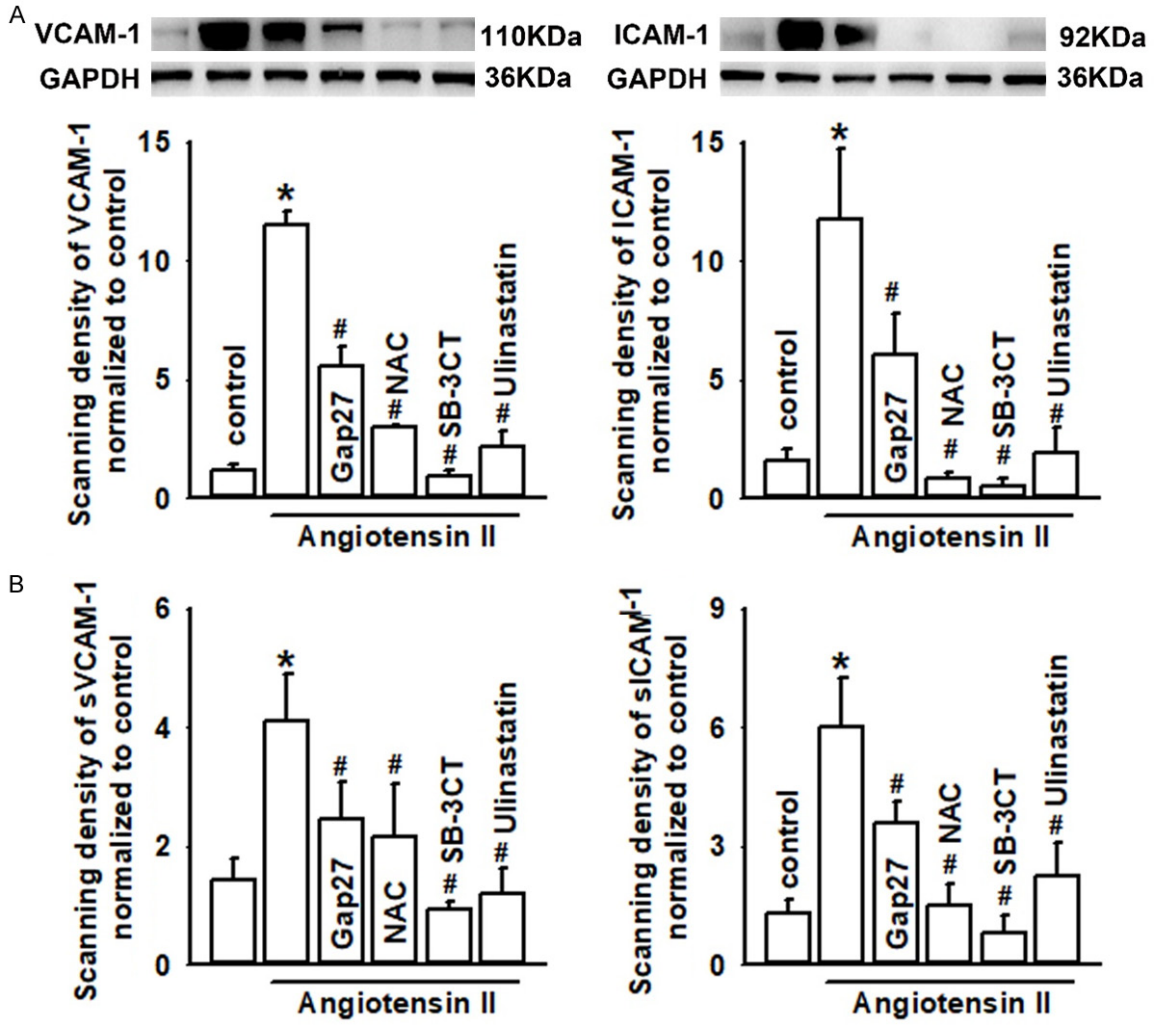

Figure 6. SB-3CT, ulinastatin, Gap27 and NAC attenuated angiotensin II-induced the increase of VCAM-1, ICAM-1, sVCAM-1 and sICAM-1. A. SB-3CT (1 μM, 4 hours before angiotensin II exposure), ulinastatin (200 U/ml, 4 hours before angiotensin II exposure), Gap27 (300 μM, 1 hour before angiotensin II exposure) and NAC (10 mM, 1 hour before angiotensin II exposure) attenuated angiotensin II-induced the increase of VCAM-1 and ICAM-1 expression on HUVECs (data are shown as mean ± SEM; n=4, *$P < 0.05$ *vs* control; #$P < 0.05$ *vs* angiotensin II group); GAPDH is used as a loading control. B. SB-3CT (1 μM, 4 hours before angiotensin II exposure), ulinastatin (200 U/ml, 4 hours before angiotensin II exposure), Gap27 (300 μM, 1 hour before angiotensin II exposure) and NAC (10 mM, 1 hour before angiotensin II exposure) attenuated angiotensin II-induced the increase of sVCAM-1 and sICAM-1 (data are shown as mean ± SEM; n=4, *$P < 0.05$ *vs* control; #$P < 0.05$ *vs* angiotensin II group).

U937-HUVECs adhesion was not achieved by affecting the expression of Cx43 on HUVECs. Interestingly, ulinastatin application inhibited Cx43 function obviously (**Figure 1C**). This might be another important form that Cx43 inhibited angiotensin II-induced U937-HUVECs adhesion. The following experiments also proved this view. **Figures 1C** and **2A** demonstrated that ulinastatin attenuated ROS transmission between the neighboring HUVECs via inhibiting Cx43, the effect of which was equivalent to NAC, the scavenger of ROS.

GJs composed of Cx43 mediate direct intercellular molecules transmission between the neighboring cells, which results in cell damage deterioration and magnification. This effect is called “bystander effect” [8, 22-24]. ROS is one of the few substances that can be transmitted through GJs. It not only damages the neighboring cells directly, but also activates other important signal pathways, resulting in cytotoxicity enhancement indirectly [8]. Vascular endothelial cell injury is exactly an important factor leading to the increase of monocyte-endotheli-

al adhesion [1]. In our present study, we showed that scavenging ROS could eliminate the activation of JAK2/STAT3 signaling pathway and its downstream MMP2 and MMP9 expression induced by angiotensin II (**Figure 3**). Meanwhile, inhibiting Cx43 function with Gap27 or ulinastatin could also achieve the same effect (**Figures 3** and **4**). The results demonstrated that ulinastatin could inhibit the activation of JAK2/STAT3 signaling pathway and its downstream MMP2 and MMP9 expression induced by angiotensin II via attenuating Cx43-meidating ROS transmission between the neighboring HUVECs, that might be the underlying mechanism of ulinastatin protecting against U937-HUVECs adhesion.

MMP2 and MMP9 have been extensively studied in a series of vascular diseases [25]. Multiple studies have reported increased circulating MMP2 and MMP9 levels in patients with atherosclerosis [9]. Previous researches showed that MMPs always played a major role in fibrous cap formation, collagen deposition, and calcification, which were responsible for atherosclerosis progression and plaque instability [26, 27]. However, in our present investigation, we firstly found that inhibiting MMP2 or MMP9 with SB-3CT could attenuate U937-HUVECs adhesion significantly (**Figure 5**), that might be a new discovery of MMP2 or MMP9 functioning in atherosclerosis. Now that alternation of MMP2 or MMP9 expression could affect U937-HUVECs adhesion, we tested their influence on important adhesion molecules. To our surprised, SB-3CT application, the inhibitor of MMP2 or MMP9, suppressed the expression of VCAM-1, ICAM-1, sVCAM-1 and sICAM-1. This viewpoint had never been reported. Matrix metalloproteinases (MMPs) are zinc-dependent endopeptidases involving in the breakdown of matrix proteins and degradation of extracellular proteoglycans [28]. Studies have confirmed that metalloproteinases, including MMP2 and MMP9, play an important role in the atherosclerosis formation and the invasion process of various solid tumors by digesting the extracellular matrix [29, 30]. It has been reported that the main constituent of extracellular matrix, such as heparan sulfate, could increase VCAM-1 and ICAM-1 expression via PI3K/AKT signaling pathway [17]. Thus, we speculated that MMPs degraded extracellular matrix, and the degradation products, such as heparan sulfate, could further stimulate vascular endothelial cells, resulting in the deterioration of inflammatory damage, the upregulation of VCAM-1 and ICAM-1 expression, and ultimately monocyte-endothelial adhesion increase. We believed that this was an important link in the process of MMPs-induced atherosclerosis, especially monocyte-endothelial adhesion. Certainly, this hypothesis needs to be confirmed in future research.

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Disclosure of conflict of interest

None.

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