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Angiotensin II enhances endothelin-1-induced vasoconstriction through upregulating endothelin type A receptor

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ABSTRACT

Endothelin-1 (ET-1) is the most potent vasoconstrictor by binding to endothelin receptors (ET_AR) in vascular smooth muscle cells (VSMCs). The complex of angiotensin II (Ang II) and Ang II type one receptor (AT_1R) acts as a transient constrictor of VSMCs. The synergistic effect of ET-1 and Ang II on blood pressure has been observed in rats; however, the underlying mechanism remains unclear. We hypothesize that Ang II leads to enhancing ET-1-mediated vasoconstriction through the activation of endothelin receptor in VSMCs. The ET-1-induced vasoconstriction, ET-1 binding, and endothelin receptor expression were explored in the isolated endothelium-denuded aortae and A-10 VSMCs. Ang II pretreatment enhanced ET-1-induced vasoconstriction and ET-1 binding to the aorta. Ang II enhanced ET_AR expression, but not ET_BR, in aorta and increased ET-1 binding, mainly to ET_AR in A-10 VSMCs. Moreover, Ang II-enhanced ET_AR expression was blunted and ET-1 binding was reduced by AT_1R antagonism or by inhibitors of PKC or ERK individually. In conclusion, Ang II enhances ET-1-induced vasoconstriction by upregulating ET_AR expression and ET-1/ET_AR binding, which may be because of the AngII/Ang II receptor pathways and the activation of PKC or ERK. These findings suggest the synergistic effect of Ang II and ET-1 on the pathogenic development of hypertension.

1. Introduction

Endothelin-1 (ET-1), known as the most potent vasoconstrictor [1], is secreted mainly from vascular endothelial cells (ECs) and has multiple effects on vascular tone regulation through the endothelin type A receptor (ET_AR) in vascular smooth muscle cells (VSMCs) and through the endothelin type B receptor (ET_BR) in ECs [2]. The ET_AR predominates in VSMCs and mediates vasoconstriction and cellular proliferation [3,4], whereas ET_BR predominates in ECs or and induces vasodilation [5]. We have previously demonstrated the overexpression of vascular ET_AR in the fructose-fed hypertensive rat model [6] and ET-1-dependent hypertension in the hyperinsulinemia rat model [7], which further supports the role of ET-1 secretion and ET_AR overexpression in hypertension development [8].

Angiotensin II (Ang II) is a regulatory peptide hormone that stimulates the constriction of VSMCs [9]. Two isoforms of Ang II receptor have been identified in VSMCs [10,11]. Ang II type 1 receptor (AT_1R) is predominant in VSMCs [12], and Ang II type 2 receptor (AT_2R) is mainly expressed in fetal tissues and vascular adventitia [12,13]. AT_1R and AT_2R usually act oppositely in the cardiovascular system [12–14].

When ET-1 and Ang II are combined and administered at subpressor doses, they synergistically affect on blood pressure [15]. In addition, studies have demonstrated that Ang II increases expression or secretion of ET-1 in vitro [16,17]; however, the underlying mechanisms of the interactions between Ang II and endothelin receptors have not been fully understood.

We hypothesized that Ang II may increase the vasoconstriction by upregulating endothelin receptors or ET-1 binding. The aim of

Abbreviations: Ang II, angiotensin II; ET-1, endothelin-1; ET_AR, endothelin type A receptor; ET_BR, endothelin type B receptor; AT_1R, angiotensin II type 1 receptor; AT_2R, angiotensin II type 2 receptor; PKC, protein kinase C; ERK, extracellular signal-regulated kinase.

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this study was to investigate the regulatory mechanisms of Ang II on ET-1-induced vasoconstriction and endothelin receptor using the endothelium-denuded aortae and A-10 VSMCs.

2. Materials and methods

2.1. Animals

Six-week-old male Sprague–Dawley (SD) rats weighing 200–250 g were purchased from the Animal Center of National Yang-Ming University and housed in a temperature- and light-controlled room (20 ± 5 °C; 12-h light/dark cycle). The animals had access to regular rat chow and water ad libitum. All animal procedures were conformed to the guidelines and approved by the Animal Welfare Committee of Taipei Veterans General Hospital. In the ex vivo study, aortic isolation was performed under full anesthesia with urethane (1.4 g/kg, ip, Sigma–Aldrich, St. Louis, MO, USA) plus α-cholorase (100 mg/mL, Sigma–Aldrich, St. Louis, MO, USA).

2.2. Preparation of endothelium-denuded aortic constriction assay

A thoracic descending aorta isolated from SD rats was endothelium denuded by gently rubbing its surface and cutting it into 4 mm long pieces. All aortic rings and aortae used in this study were referred to endothelium-denuded unless specified otherwise. Aortic rings were mounted in organ bathes containing 5 mL of a Krebs–Henseleit (KH) buffer at 37 °C and aerated with 95% O_2 and 5% CO_2. The aortic constriction activity was adjusted with 60 mM KCl. The aortic rings were divided into four groups and treated for 12 h by using an RP-1 digital peristaltic pump (Rainin Instruments, Oakland, CA, USA). Before the ET-1-stimulated constriction assay, the aortic rings were washed and rested in the KH buffer for 1 h. The aortic constriction in response to ET-1 (from 10^{-9} to 10^{-7} M) was recorded using a transducer. The data were collected using Xction View 2.0 and analyzed using Charter Five software 5.5.6, and the endothelium-denuded aortic proteins was confirmed using immunoblotting.

2.3. Cell culture

A-10 cells possessing the characteristics of VSMCs were purchased from the Bioresource Collection and Research Center, Taiwan, and cultured in Dulbecco's Modified Eagle Medium (DMEM) (Gibco BRL, Gaithersburg, MD, USA) containing 10% fetal bovine serum (Biological Industries, Kebbutz Beit Haemek, Israel) plus 100 U/mL penicillin, 100 μg/mL streptomycin, and 25 mM HEPES (pH 7.4) at 37 °C in a humidified incubator with 5% CO_2. Cells were grown to 80% confluence and split into 12-well plates for the binding assay, 6-cm dishes for protein or mRNA harvest, and 6-well pates for siRNA treatment. Cells were serum free for 6 h before treatment with Ang II, and some cells were subjected to 30 min pretreatment by using BQ610, BQ788 (Phoenix Pharmaceuticals, Burlingame, CA, USA), losartan (Sigma–Aldrich, St. Louis, MO, USA), PD123319 (Sigma–Aldrich, St. Louis, MO, USA), or kinase inhibitors SB203580, SP600125, PD98059, H7 (Biomol International, Farmingdale, PA, USA) individually.

2.4. ^{125}I-ET-1 specific binding

The ET-1 binding was performed in the binding buffer (DMEM with 25 mM HEPES, pH 7.4, 0.1% bovine serum albumin, and 5.5 mM glucose) containing 20 pM ^{125}I-ET-1 (specific activity, 5 mCi/mmol; Perkin-Elmer-New England Nuclear, Boston, MA, USA), and it was then incubated for 1 h at 37 °C. A-10 VSMCs were then washed twice with an ice-cold phosphate buffer saline and solubilized through incubation in 1 N NaOH for 3 h at room temperature. The bound ^{125}I-ET-1 was measured with a γ-counter. Nonspecific binding determined in the presence of 1×10^{-6} M unlabeled ET-1 was subtracted from the total binding to give the specific binding.

2.5. Total membrane protein extraction and binding assay

Total membranes from A-10 VSMCs or an endothelium-denuded aortic ring were harvested in a homogenizing buffer containing 20 mM $NaHCO_3$ and 0.1 mM phenylmethylsulphonyl fluoride. The protein concentration was assayed and adjusted to a final concentration of 0.2 mg/mL. ^{125}I-ET-1 binding was performed at 37 °C for 1 h with a final ^{125}I-ET-1 concentration of 20 pM. Nonspecific binding was defined as the binding in the presence of 1×10^{-6} M ET-1. Bound and free ^{125}I-ET were separated through rapid vacuum filtration across 2.5 μm GF/C Whatman filters after dilution with 3.5 mL in an ice-cold 10 mM Tris buffer containing 6.6% polyethyleneglycol (PEG) 6000 (pH 7.4). After two additional washes with Tris–PEG buffer, the radioactivity of the filters was determined using the γ-counter.

2.6. Transfection of siRNA

Fifty nanomole of a nontarget control (siControl2#, Dharmacon, Thermo Scientific, Tewksbury, MA, USA) or siRNA of Agtr 1α (si Agtr1α SMART pool, Dharmacon, Thermo Scientific, Tewksbury, MA, USA) was added to Opti-MEM (Gibco Life Technologies, Grand Island, NY, USA) to obtain a final volume of 200 μL. Five hundred nanoliters of Dharma FECT 2 siRNA transfection reagent (Dharmacon, Thermo Scientific, Tewksbury, MA, USA) was blended thoroughly for 5 min with 200 μL of DMEM. The above two solutions were mixed and incubated for 20 min. The culture media were replaced with the mixed solution plus 1.6 mL of DMEM. Experiments were performed 48 h after transfection, and knock down of proteins was confirmed using immunoblotting.

2.7. Total RNA extraction

Total RNA was extracted using the Tri Reagent kit (Molecular Research Center, Cincinnati, OH, USA) by following the manufacturer protocol. The integrity of the extracted total RNA was examined using 1% agarose gel electrophoresis, and the RNA concentration was determined by considering ultraviolet light absorption at 260 nm. The RNA samples were incubated for 30 min at 37 °C with RNase-free DNase I and then for 10 min at 70 °C, before performing real-time PCR.

2.8. Real-time polymerase chain reaction (real-time PCR)

One microgram of total RNA was reverse transcribed to cDNA by using poly(dT) primers and reverse transcriptase (HT Biotechnology, Cambridge, UK) with the following protocols: 1 cycle of 42 °C for 90 min, 70 °C for 10 min, 4 °C for 60 min. Real-time PCR was performed using a Fast SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) and following the manufacturers' protocol. Reactions were performed at 95 °C for 3 min, followed by 40 cycles of 95 °C for 20 s, and 60 °C for 35 s; this was followed by observing the melting curve for 30 min. The ΔCt values were obtained by subtracting the average Ct value of the reference gene from the average Ct value of the target gene. The ΔCt values for each treatment group were further compared with those for the controls (ΔΔCt) for further relative quantification of gene expression.

2.9. Immunoblotting

Total protein lysates were prepared in a lysis buffer (10 mM Tris, pH 7.4, 100 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM NaF, 20 mM $Na_4P_2O_7$, 2 mM Na_3VO_4, 1% Triton X-100, 10% glycerol, 0.1% SDS, 0.5% deoxycholate, 1× phosphotase inhibitor, 1× protease inhibitor), and samples of 50 μg of the total protein were mixed with the sample buffer dye and resolved in 10% SDS–PAGE for 2 h at 100 mV. The proteins were then transferred to polyvinylidene difluoride membranes at 300 mA for 2 h. The membranes were first blocked for 1 h at room temperature with 5% skimmed milk in a TBS buffer, followed by incubation for 24 h at 4 °C with the primary antibody of ET_AR (Merck Millipore, Billerica, MA, USA), ET_BR (GeneTex, Irvine, CA, USA), or AT_1R (Abcam, Cambridge, MA, USA). Finally, incubation was performed for 60 min at room temperature with a horseradish peroxidase-conjugated secondary antibody for chemiluminescence detection. To detect multiple signals by using a single membrane, the membrane was stripped before reblotting with a different antibody. The internal control used was β-actin (Merck Millipore, Billerica, MA, USA).

2.10. Data analysis

The experiments were repeated at least three times and the results were expressed as mean ± SD of the number of observations. Statistical significance was assessed using one-way analysis of variance or Student's *t*-test. A p value of 0.05 was considered statistically significant.

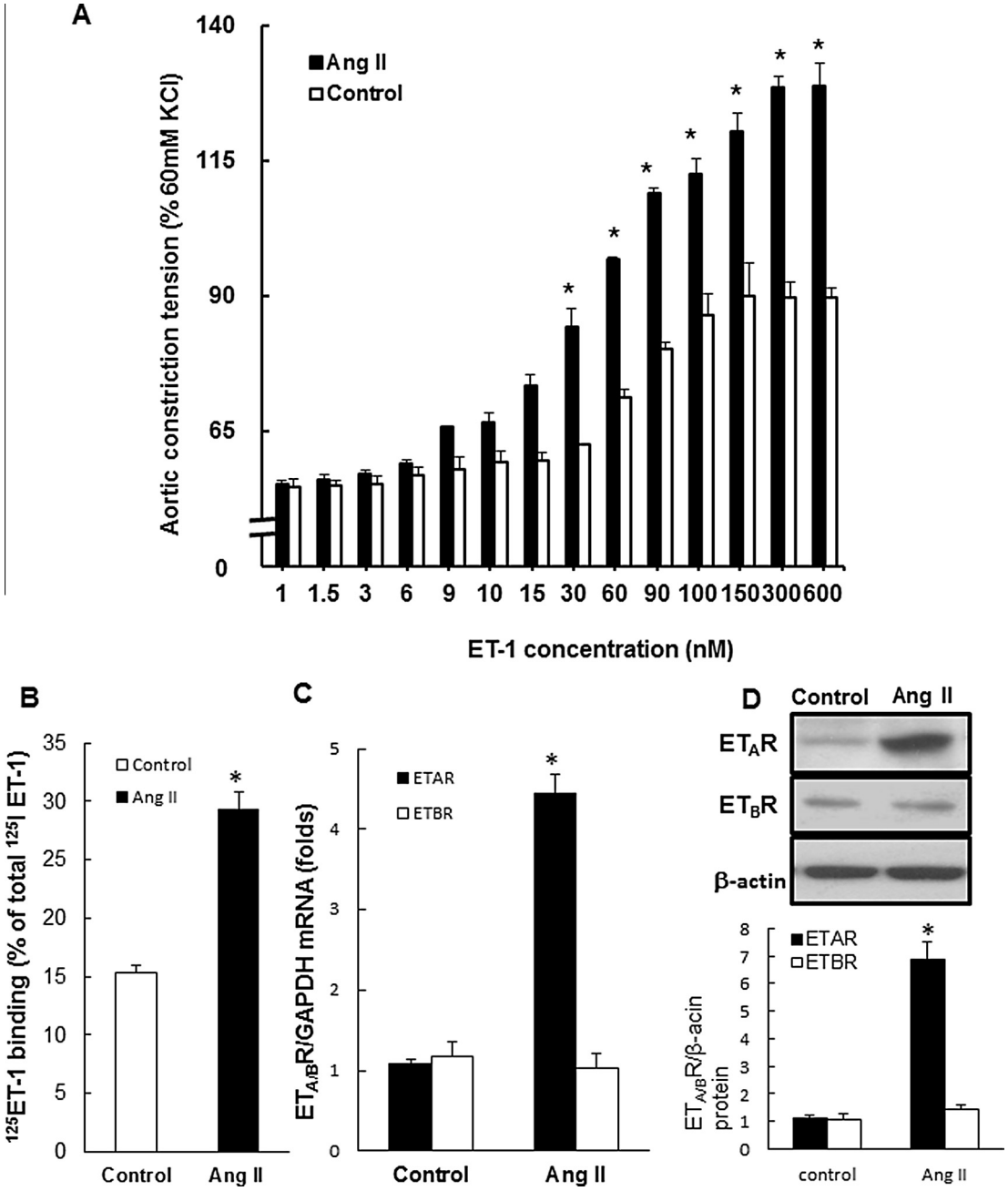
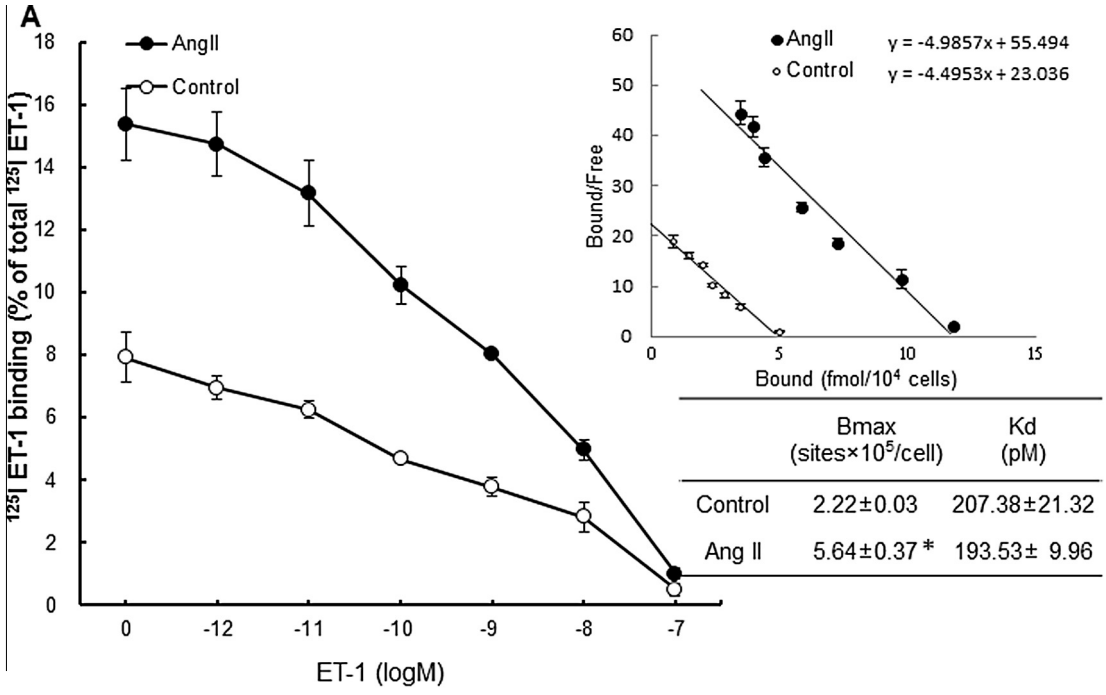


Fig. 1. Ang II enhanced ET-1-induce aortic constriction, ET-1 binding, and ET_AR expression in the endothelium-denuded aorta. An endothelium-denuded aorta was pretreated with Ang II (10^{-7} M) or a vehicle buffer in an organ bath for 12 h. (A) Ang II increased aortic constriction to the maximal level following ET-1 induction (10^{-9}–6×10^{-7} M). (B) Ang II enhanced ET-1 binding in total membrane protein. Ang II enhanced ET_AR, but not ET_BR, (C) mRNA expression and (D) protein expression. Values were mean ± SD for all four experiments. $^*p < 0.05$ vs. control.

3. Results

3.1. Enhancement of the constriction, ET-1 binding, and ET_AR expression of aortic rings after Ang II treatment

To test the hypothesis that Ang II leads to an increase of ET-1 function, the impact of Ang II pretreatment on vasoconstriction was determined. To avoid interference of ET-1 secreted by ECs, the isolated endothelium-denuded aortic constriction system were used. Before the constrictive assay, all aortic rings were tested using L-phenylephrine (3×10^{-7} M) and acetylcholine (3×10^{-6} M) to ensure that each constriction was endothelium independent (Supplementary Fig. 1). After pretreatment with Ang II for 12 h, the ET-1-stimulated constriction increased ($p < 0.01$) (Fig. 1A). In addition, ET-1 bindings in the aortae pretreated with Ang II were enhanced (Fig. 1B). These data suggested that Ang II pretreatment increase the ET-1-induced vasoconstriction by enhancing ET-1 binding in the aortae. To investigate whether Ang II enhances the expression of endothelin receptors in aortic VSMCs, the expressions of mRNA and protein were measured using real-time PCR and immunoblotting. The results showed that both the mRNA and protein levels of ET_AR, but not ET_BR, had increased in the aortae after Ang II pretreatment (Fig. 1C and D). Our data indicated that ET-1-induced vasoconstriction and ET_AR overexpression is induced by Ang II in the aortae.



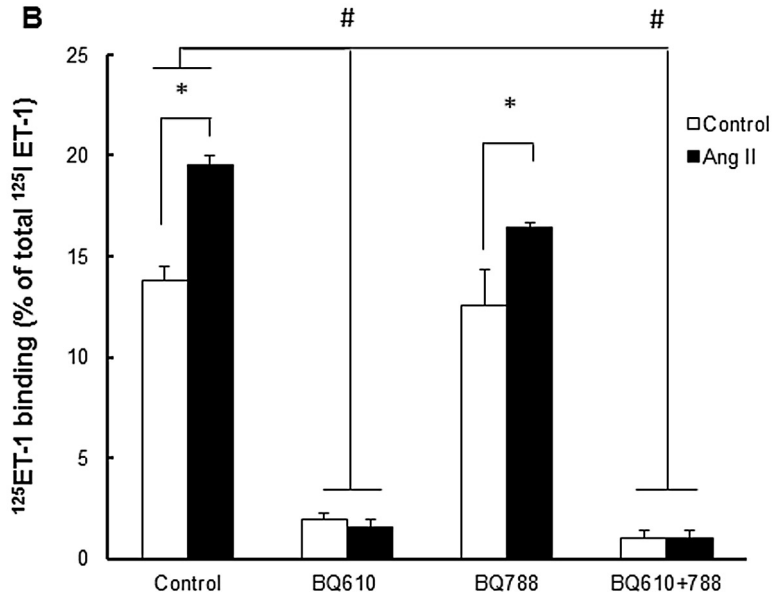


Fig. 2. Ang II enhanced ET-1 binding in A-10 VSMCs. (A) Competitive binding curve and Scatchard plot (inset) for ET-1 binding to cells with/without Ang II (10^{-7} M) pretreatment for 24 h. (B) BQ610 (ET_AR antagonist, 10^{-5} μM), but not BQ788 (ET_BR antagonist, 10^{-5} M), abolished Ang II-enhanced ET-1 binding. Values were mean ± SD for all four experiments. $^{*}p < 0.05$ vs. corresponding control, $^{\#}p < 0.05$ vs. Ang II.

3.2. Enhancement of ET-1 binding mainly through ET_AR after Ang II treatment

Examining the effect of Ang II on ET-1/endothelin receptor action required determining whether the ET-1 binding capacity or affinity in A-10 VSMCs was enhanced by Ang II. Pretreatment with Ang II (10^{-7} M) enhanced ET-1 binding dose- and time-dependently in A-10 VSMCs (Supplementary Fig. 2) and the induction trend of ET-1 binding was similar in aortic rings and A-10 VSMCs (Supplementary Fig. 3). Ang II pretreatment increased the ^{125}I-ET-1 specific binding (Fig. 2A) in A-10 VSMCs, and the Scatchard plot analysis revealed that the pretreatment with Ang II led to a 2.5-fold increase in receptor binding sites, as calculated using Bmax (Ang II $5.64 \pm 0.37 \times 10^5$ sites/cell vs. control

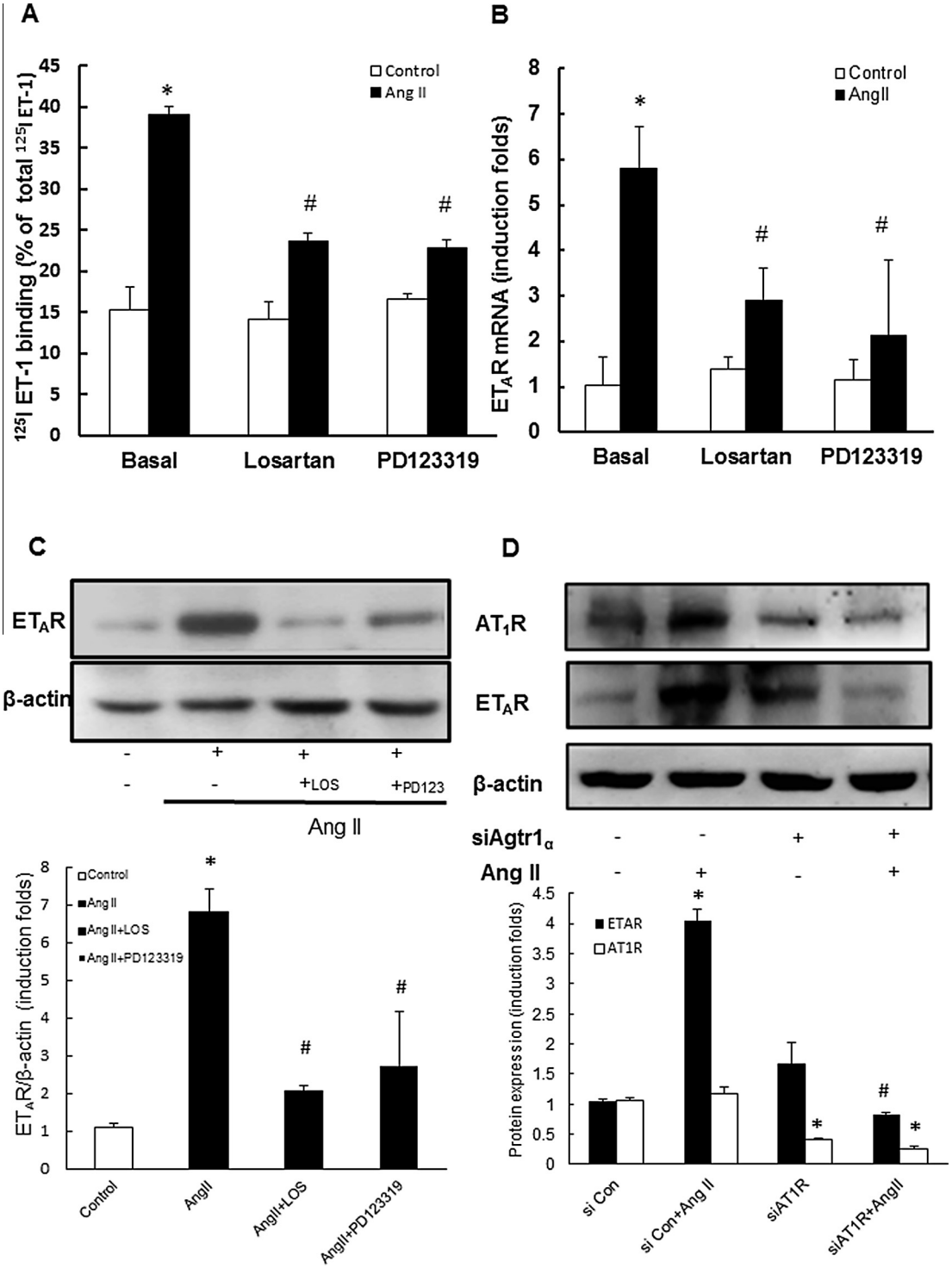


Fig. 3. Ang II-enhanced ET-1 binding, ET_AR mRNA, and ET_AR protein levels were decreased on pretreatment with AT_1R, AT_2R antagonist, or AT_1R siRNA in A-10 VSMCs. Cells were pretreated with/without losartan (10^{-5} M) or PD123319 (10^{-5} M) for 30 min and then treated with Ang II (10^{-7} M) for 24 h. Ang II-enhanced (A) ET-1 binding, (B) ET_AR mRNA, and (C) ET_AR protein were decreased on pretreatment with losartan or PD123319. (D) Cells were pretreated with siRNA of control or siAgtr1α. AT_1R protein was decreased by siAgtr1α, and Ang II-upregulated ET_AR protein was decreased by siAgtr1α. Values were mean ± SD for all four experiments. $^*p < 0.05$ vs. control, $^\#p < 0.05$ vs. Ang II.

$2.22 \pm 0.03 \times 10^5$ sites/cell, $p < 0.05$) (Fig. 2A). However, Ang II pretreatment did not change the binding affinity as calculated from the dissociation constant (k_d) ($p = 0.65$, Fig. 2A inset). To examine whether ET_AR or ET_BR is involved in the Ang II-enhanced ET-1 binding, BQ610 and BQ788, specific antagonists for ET_AR and ET_BR, respectively, were used in the binding experiments. The results demonstrated that Ang II-stimulated ET-1 binding was abolished by BQ610 (10^{-5} M), but not by BQ788 (10^{-5} M) (Fig. 2B), indicating that Ang II-enhanced ET-1 binding occurs mainly through ET_AR in A-10 VSMCs.

3.3. Inhibition of Ang II-enhanced ET-1 binding, ET_AR expression by Ang II receptor antagonisms

To clarify whether the increase in ET-1 binding and ET_AR expression was angiotensin-receptor-specific, losartan (10^{-5} M), PD123319 (10^{-5} M), specific antagonists for AT_1R and AT_2R, was used in the pretreatment of Ang II. The results demonstrated that Ang II-enhanced ET-1 binding was blunted by the AT_1R or AT_2R antagonist, however, neither antagonist per se affected ET-1 binding (Fig. 3A). Because the ET-1 binding in A-10 VSMCs occurs mainly through ET_AR, we examined ET_AR in A-10 VSMCs by using real-time PCR. The results showed that Ang II enhanced ET_AR expression, and losartan or PD123319 alone didn't affect ET_AR mRNA expression (Fig. 3B). Furthermore, we examined ET_AR protein in A-10 VSMCs by using immunoblotting. The results showed that retreatment with losartan or PD123319 inhibited the enhancement of ET_AR expression induced by Ang II (Fig. 3C). Because the AT_1R predominates in VSMCs, we further study the effect of AT_1R on ET_AR overexpression in A-10 VSMCs using siRNA of AT_1R. The results demonstrated that during AT_1R downregulation by siRNA, the enhancement of ET_AR stimulated by Ang II was significantly blunted (Fig. 3D). These data indicated that Ang II enhanced ET-1 binding and ET_AR expression through AT_1R pathway in the A-10 VSMCs.

3.4. Blocking of Ang II-enhanced ET_AR expression and ET-1 binding by PKC and ERK inhibitors

To clarify the potential signaling involved in the mechanisms whereby Ang II signaling affected the upregulation of the ET-1 binding and ET_AR expression, inhibitors of PKC (H7), ERK (PD98059), p38 (SB203580), and JNK (SP600125) were used. The results showed that PD98059 (PD) and H7 blocked the Ang II-enhanced ET-1 binding (Fig. 4A) and ET_AR expression (Fig. 4B), whereas SB203580 or SP600125 did not change ET-1 binding or ET_AR expression enhanced by Ang II. These data indicated that Ang II increased the ET-1 binding and ET_AR expression through the PKC/ERK pathway.

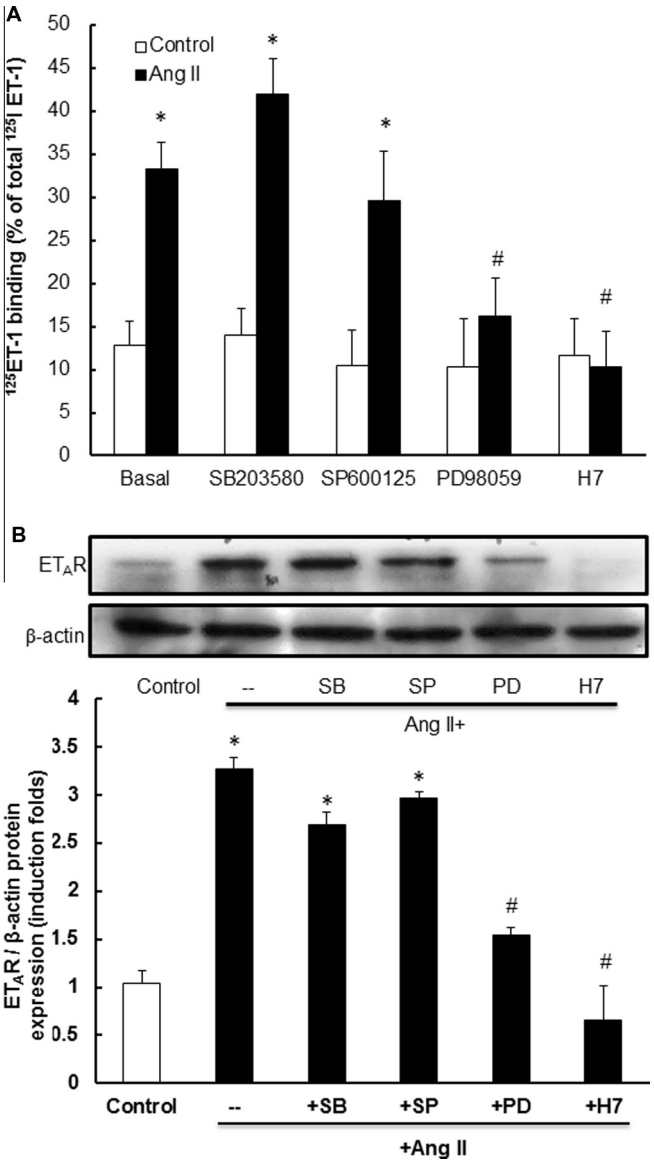


Fig. 4. Effect of inhibitors of PKC and MAP kinase on Ang II-enhanced ET-1 binding and ET_AR expression in A-10 VSMCs. Cells were pretreated for 30 min with/without mitogen-activated protein kinase (MAPK) inhibitors: SB203580 (SB, a p38 pathway inhibitor; 5 μM), SP600125 (SP, a JNK pathway inhibitor; 1 μM), PD98059 (PD, an ERK pathway inhibitor, 5 μM), or protein kinase C (PKC) inhibitor (H7, a protein kinase inhibitor, 6 μM). Then, the pretreatment was continued for an additional 24 h in the absence (open bars) or presence (filled bars) of Ang II (10^{-7} M) in the continued presence or absence of the inhibitor. Values were mean ± SD for all four experiments. $^*p < 0.05$ vs. corresponding control, $^\#p < 0.05$ vs. Ang II.

4. Discussion

We have previously reported that plasma ET-1 elevation and aortic ET_AR overexpression contribute to the insulin-infusion-induced hypertension model [6,8]. Another previous study demonstrated that Ang II and ET-1 synergistically induce blood pressure in conscious rats [15]. The key finding of the current study is that Ang II enhances the effects of ET-1 on aortic constriction. This enhancement was achieved mainly through Ang II receptor, PKC or ERK signaling, followed by ET_AR upregulation.

Previous studies have demonstrated that in hypertension, plasma ET-1 remains normal [18], but Ang II is high [19]. Ang II increases ET-1 secretion in cultured ECs in vitro but not in circulating levels in vivo [20]. Moreover, our results show that Ang II upregulated ET_AR expression and enhanced ET-1 binding in the aorta and VSMCs. Ang II may be able to enhance ET-1-induced vasoconstriction in essential hypertension subjects with normal plasma ET-1 levels. Our finding suggests that Ang II-increased ET-1 binding and ET_AR overexpression may explain the vasoconstriction observed in patients with essential hypertension.

A previous study indicates that ET-1 pretreatment causes downregulation of ET-1 binding because of lysosomal-like degradation of endothelin receptors [21]. Our results show that Ang II pretreatment increased ET_AR mRNA and protein levels in the endothelium-denuded aortae and VSMCs. Furthermore, Ang II enhanced ET-1/ET_AR binding in VSMCs, suggesting that Ang II-upregulated ET_AR indeed responds to ET-1 stimulation. Our previous study also demonstrated that insulin infusion induces hypertension in rats by enhancing ET-1 binding to the aorta [6,8]. Therefore, upregulation

of both ET-1 binding and ET_AR expression is crucial for vasoconstriction.

Transient treatment of ET-1 cannot enhance Ang II-mediated vasoconstriction in tail arteries [22]. Endothelium-derived ET-1 is believed to not be involved Ang II-mediated transient vasoconstriction in tail arteries. Here we found that Ang II pretreatment increased ET-1-induced vasoconstriction and ET_AR expression in the endothelium-denuded aorta. The data suggest a priming effect of Ang II on ET-1-induced vasoconstriction in aorta and also support the synergistic effect of Ang II and ET-1 on blood pressure [15]. Moreover, the Ang II-enhanced ET-1 binding and ET_AR overexpression were decreased by either AT_1R or AT_2R antagonism. van Esch et al. report that AT_1R deficiency inhibits the AT_2R-mediated vasodilation in the mouse heart, suggesting a cooperation between AT_1R and AT_2R through their heterodimerization [23]. As AT_2R is rarely expressed in VSMCs [12], Ang II-AT_1R may play a major role in the enhancement of ET-1 binding and ET_AR expression.

Our data showed that Ang II induce ET_AR overexpression through the PKC and ERK pathways. Previous studies indicate that the ERK and PKC pathways activate nuclear factor-κB/activator protein-1 (AP-1) promoters in rabbit cardiomyocytes [24], and that ET_AR transcription may be promoted by AP-1 [25]. It is likely that PKC and ERK pathways may also promote ET_AR transcription in Ang II-stimulated VSMCs. However, we cannot rule out the possibility that Ang II signaling enhances the ET_AR mRNA stability or protein synthesis. Nevertheless, ET_AR expression is enhanced by Ang II-induced PKC or ERK signaling in VSMCs.

In conclusion, this study provides evidences that Ang II enhances ET-1-induced vasoconstriction through the elevation of ET_AR expression and ET-1/ET_AR binding in aortic VSMCs. This would suggest that a dual antagonism of Ang II and ET_A receptors may be a therapeutic drug for hypertension, particular in early or borderline patients. This concept is required extensive experiments and further clinical proof in the future.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.07.119.

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