Effects of Endothelin Family on ANP Secretion

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ABSTRACT

The endothelins (ET) peptide family consists of ET-1, ET-2, ET-3, and sarafotoxin (s6C, a snake venom) and their actions appears to be different among isoforms. The aim of this study was to compare the secretagogue effect of ET-1 on atrial natriuretic peptide (ANP) secretion with ET-3 and evaluate its physiological meaning. Isolated nonbeating atria from male Sprague-Dawley rats were used to evaluate stretch-activated ANP secretion in response to ET-1, ET-2, ET-3, and s6C. Changes in mean blood pressure (MAP) were measured during acute injection of ET-1 and ET-3 with and without natriuretic peptide receptor-A antagonist (A71915) in anesthetized rats. Changes in atrial volume induced by increased atrial pressure from 0 to 1, 2, 4, or 6 cmH₂O caused proportional increases in mechanically-stimulated extracellular fluid (ECF) translocation and stretch-activated ANP secretion. ET-1 (10 nM) augmented basal and stretch-activated ANP secretion in terms of ECF translocation, which was blocked by the pretreatment with ET_A receptor antagonist (BQ123, 1 μM) but not by ET_B receptor antagonist (BQ788, 1 μM). ET_A receptor antagonist itself suppressed stretch-activated ANP secretion. As compared to ET-1-induced ANP secretion (3.2-fold by 10 nM), the secretagogue effects of ANP secretion by ET-2 was similar (2.8-fold by 10 nM) and ET-3 and s6C were less potent (1.7-fold and 1.5-fold by 100 nM, respectively). Acute injection of ET-1 or ET-3 increased mean blood pressure (MAP), which was augmented in the presence of natriuretic peptide receptor-A antagonist. Therefore, we suggest that the order of secretagogue effect of ET family on ANP secretion was ET-1 ≥ ET-2 >> ET-3 > s6C and ET-1-induced ANP secretion negatively regulates the pressor effect of ET-1.

Keywords: atrial natriuretic peptide, endothelin, stretch, atrium, receptor, blood pressure

1.Introduction

The endothelins (ET) peptide family consists of 21 amino acid isoforms, ET-1, ET-2, ET-3, and sarafotoxin (a snake venom). ET-2 is very similar to ET-1, while ET-3 and sarafotoxin differ from ET-1 by six and seven amino acids, respectively. ET-1 is the most abundant isoform and secreted mainly by endothelial cells. ET-2 is found in the kidney and intestine, whereas ET-3 is found in the central nervous system. ET-1 has a wide range of biological actions including vasoconstriction [14, 31], positive inotropic effect [17, 18], cellular proliferation, and control of water and sodium excretion [1, 2]. The potency of mitogenesis [25], renovascular constriction [14], and sodium excretion [6] are reported to be different among isoforms.

On the other hands, atrial natriuretic peptide (ANP) is a cardiac peptide hormone which is involved in the regulation of blood volume and pressure [7, 8]. The synthesis and secretion of ANP is regulated by developmental, hormonal, and hemodynamic factors [15, 29]. Atrial myocytes constitute major site of synthesis and the secretion of ANP is stimulated by atrial wall stretch [5, 9, 22]. ET-1 is well-known as one of the most potent chemicals for ANP secretion [10]. ET-1 increases ANP secretion in isolated rat cardiac myocytes [11, 12], in isolated rat atria [16, 28], and in conscious rats [13, 26]. Based on their biological functions in cellular proliferation and vascular smooth muscle, ET-1 is an antagonistic hormone to ANP. Eventhough many studies for ET-1-induced ANP secretion have been done, there are a few reports about the potency of secretagogue effect of ET-3 and sarafotoxin on ANP secretion as compared to ET-1. Therefore, the aim of the present study was to compare the secretagogue effect of ET-1 on ANP secretion with ET-3 and sarafotoxin (s6C) in isolated perfused nonbeating atria and evaluated its physiological meaning in anesthetized rats.

2. Materials and methods

2.1. Animals

Male Sprague-Dawley rats, weighing between 230 and 250 g, were obtained from Daehanbiolink (Eumsung, Korea) and housed in a temperature-controlled room with a 12:12-h light-dark cycle. Animals were provided free access to standard laboratory chow (5L79 Purina rat & mouse 18% chow, Charles River Laboratories Inc., Wilmington, MA) and water. All of experimental protocols conformed to the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH publication No. 85-23, revised 1996) and were approved by our institution.

2.2. Isolated perfused nonbeating atrial preparation

Rats were sacrificed by decapitation and an isolated perfused atrial preparation was made by the method described previously [19]. Briefly, the heart was rapidly removed, placed in oxygenated saline and both atria were separately dissected from the heart. A Tygon cannula containing three small catheters sealed within it was inserted into the atrium and secured by ligatures. The cannulated atria were transferred, fitted into the organ chamber containing buffer solution (36.5°C) and fixed with water-tight silicone rubber cap. The atrium was immediately perfused with oxygenated HEPES buffer solution at a rate of 0.4 ml/min with peristaltic pump. The composition of the buffer solution was as follows: NaCl 118, KCl 4.7, CaCl₂ 2.5, MgSO₄ 1.2, NaHCO₃ 25, HEPES 10, glucose 10 mM and bovine serum albumin (BSA) 0.1%. The pericardial buffer solution which contained [³H]-inulin to measure the translocation of extracellular fluid (ECF) was also oxygenated by silicone tubing coils located inside the organ chamber. The pericardial space of organ chamber was sealed and connected with a calibrated microcapillary tube, by which changes in atrial volume were

monitored. The atrium was perfused for 30 min to stabilize the secretion of ANP and to maintain the [^3H]-inulin level in the extracellular space at a steady-state. The perfusate was collected every 2 min interval at 4°C. After two collection periods, atrial distension was induced for 2 min by elevating the position of out-flow catheter tip to 1 cmH₂O and atrial contraction was induced by lowering the position of the catheter tip to the basal level. Atrial pressure was subsequently increased from 0 to 1, 2, 4, and 6 cmH₂O for 2 min at every 8 min. After finish experiments, atrial wet weight was measured.

2.3. Experimental groups

Experiments were conducted with 11 groups. Group 1 included control atria perfused with HEPES buffer (n = 7). Group 2 included ET-1 (human, porcine, dog, rat form) -perfused atria (Bachem AG, Bubendorf, Switzerland; 10 nM, n = 8) [20]. Group 3 and 4 included ET-1-perfused atria in the presence of ET_A receptor competitive inhibitor, cyclo (D-Asp-Pro-D-Val-Leu-D-Trp, (BQ123; Bachem AG; 1 μM, n = 8) and ET_B receptor antagonist, BQ788 (Bachem AG ; 1 μM, n = 8), respectively. In group 5 and 6, atria were perfused with BQ123 (1 μM, n = 7) or BQ788 (1 μM, n = 7) alone, respectively. In group 7 and 8, atria were perfused with HEPES buffer containing low Ca²⁺ (25 μM) in the absence (n = 6) and presence of ET-1 (10 nM, n = 8), respectively. Group 9 included ET-2 -perfused atria (Bachem AG ; 10 nM, n = 6). Group 10 included ET-3 -perfused atria (Bachem AG; 10 nM, 100 nM, n = 7 each). Group 11 included s6C-perfused atria (Sigma-Aldrich, St. Louis, MO; 100 nM, n = 7).

ET-1, ET-2, ET-3, or s6C was perfused into atria at 5 min before sample collection. Atria were pretreated with BQ123 or BQ788 15 min before sample collection and perfused simultaneously with ET-1 at 5 min before sample collection.

2.4. Acute infusion of ET-1 or ET-3 with and without NPR-A antagonist

In order to evaluate the physiological role of ANP in ET-induced pressor effect, an increase in arterial blood pressure (ABP) in response to intravenous injection of ET-1 and ET-3 was compared in the absence and presence of natriuretic peptide receptor-A (NPR-A) antagonist, A71915. Male Sprague-Dawley rats, weighing 230-250 g, were anesthetized by intraperitoneal injection of a mixture of ketamine and xylazine (9:1, 2 ml/kg). Body temperature was maintained at 37°C using a heating pad. Following midline incision in the neck, the jugular vein and carotid artery were carefully dissected, cannulated with a polyethylene tube (PE-50), and secured with ligation. The cannulus in the jugular vein was connected to a peristaltic pump (Minipuls 2 Gilson, Villiers le Bel, France) for infusion of vehicle (0.9% NaCl) or ET and the cannula in the carotid artery were connected to the pressure transducer (Statham P23Db). ABP and heart rate (HR) were recorded using a Power lab (ML-820, ADInstruments Pvt. Ltd.) via the pressure transducer. After stabilization for 5 min, one ml/kg of ET-1 or ET-3 solution, at a concentration of 0.1 nM or 30 nM, respectively, was infused for 20 sec through the jugular vein and ABP and HR were continuously recorded for 15 min. After recovery of BP and HR to control level, 250 µl of NPR-A receptor antagonist (Bachem AG; A71915, 3 µM) was injected at 5 min before ET-1 or ET-3 injection followed by the infusion for 20 min at a rate of 100 µl/min. ET-1 or ET-3 was injected in the presence of A71915 and BP and HR were continuously recorded (n = 5 for each group).

2.5. Radioimmunoassay of ANP

The concentration of immunoreactive ANP in atrial perfusates and plasma was measured using specific radioimmunoassay (RIA) as described previously [5]. RIA was performed in

Tris-acetate buffer (0.1 M, pH 7.4) containing neomycin (0.2%), ethylenediamine tetraacetic acid (1 mM), soybean trypsin inhibitor (50 benzoyl arginine ethyl ester units/ml), aprotinin (200 KIU/ml), phenylmethylsulfonylfluoride (0.4 mg%), sodium azide (0.02%) and BSA (1%). Standard and samples were incubated with anti-ANP antibody and [125 I]-ANP for 24 h at 4°C. Bound forms were separated from free forms using charcoal suspension for ANP in perfusate. RIA for ANP was done on the day of experiments and all samples in an experiment were analyzed in a single assay. The intra- and inter-assay coefficient of variation were 6.3% (n=9) and 7.8% (n=11), respectively.

The secreted amount of ANP was expressed as ng ANP per min per g of tissue wet weight. The molar concentration of ANP release was calculated as follows [3]:

$$\text{ANP released } (\mu\text{M}) = \text{ANP (pg/min/g)} / \text{ECF translocation } (\mu\text{l/min/g}) / 3060$$

The denominator 3060 refers to the molecular mass for ANP₍₁₋₂₈₎ (Da) since the ANP secreted was found to be mainly the processed ANP [3, 4].

2.6. Measurement of ECF translocation

The ECF translocated from atria was measured as described previously [3]. At the start of atrial perfusion, pericardial buffer solution contained [^3H]-inulin. Radioactivities in atrial perfusate and pericardial buffer solution were measured with a liquid scintillation counter and the amounts of ECF translocated through atrial wall were calculated as follows:

$$\text{ECF translocation } (\mu\text{l/min/g}) = \frac{\text{total radioactivity in perfusate (cpm/min)}}{\text{radioactivity in pericardial reservoir (cpm}/\mu\text{l)} / \text{atrial wet weight} \times 1000}$$

2.7. Statistical analysis

The results were given as means $\pm$ SEM. Statistical significance of differences was performed by one-way or two-way repeated measures analysis of variance followed by the Bonferroni

test. The linear regression analysis was also used (Prism 5, GraphPad Software, Inc., La Jolla, CA).

3.Results

3.1. Effect of ET-1 on ANP secretion from isolated atria

Figure 1 shows the effect of atrial distension on volume change (Fig. 1B), ANP secretion (Fig. 1C), and ECF translocation (Fig. 1D) in left atria of normal rats. By increasing atrial pressure from basal level (0 cmH₂O) to 1, 2, 4, or 6 cmH₂O for 2 min, the atrium was distended and then, the atrial size was reduced after return of atrial pressure to basal level. Changes in atrial volume induced by atrial distension and reduction (DRV) were proportionately increased in response to atrial pressure (Fig. 1B). ET-1 tended to decrease atrial volume changes in response to increased atrial pressure without significance.

When atrial distension induced by increasing the pressure was reduced to the basal level, the secretion of ANP was significantly increased proportionately to DRV. ET-1 (10 nM) stimulated both basal and stretch-activated ANP secretion (Fig. 1C). We have already shown that the ANP secretion is regulated by two step-sequential mechanism, one is the release of ANP from atrial myocytes into the extracellular space and then the translocation of ECF with the released ANP into the atrial lumen by atrial contraction [3]. Therefore, the translocation of ECF using [³H]-inulin clearance was measured simultaneously with the secretion of ANP. Increases in DRV also caused proportional increases in mechanically-stimulated ECF translocation, which were attenuated by ET-1 (Fig. 1D). Therefore, the stretch-activated ANP secretion from atria into the atrial lumen in relation to the translocation of ECF (ANP concentration in the interstitium) was increased. ET-1 augmented basal and stretch-activated ANP concentration (Fig. 1E).

3.2. Effect of ET-1 on ANP secretion with and without ET receptor blocker

Figure 2 shows the effect of ET_A receptor blocker (BQ123) on DRV, ANP secretion and ECF

translocation. BQ123 attenuated stretch-activated ANP secretion (Fig. 2C) but did not change basal ANP secretion and ECF translocation (Fig. 2D). Therefore, the ANP concentration in the interstitium was attenuated by BQ123 (Fig. 2E). However, ET_B receptor blocker (BQ788) had not significant effects on ANP secretion and ECF translocation (data not shown).

Figure 3A shows the relationships between atrial pressure, DRV, changes in mechanically-stimulated ECF translocation and stretch-activated ANP secretion with and without ET-1. When intra-atrial pressure was increased by 1, 2, 4, or 6 cmH₂O, DRV were increased proportionately to pressure (Fig. 3Aa). DRV positively correlated with ANP secretion and ECF translocation (Fig. 3, Ab and c). So, there was a close correlation between ANP secretion and ECF translocation (Fig. 3Ad). The stretch-activated ANP secretion was markedly augmented without difference in DRV by ET-1. Therefore, the relationship between ANP secretion and ECF translocation shifted to the left and upward by ET-1 (Fig. 3Ad). The stretch-activated ANP secretion was markedly attenuated by BQ123. So, BQ123 caused the relationship between ANP secretion and ECF translocation to the right and downward (Fig. 3Ba). However, BQ788 did not cause any significant changes in ANP secretion and ECF translocation. Therefore, there was no shift the relationship between these parameters by BQ788 (Fig. 3Bb).

To compare quantitatively the stretch-activated ANP secretion among groups, the stretch-activated ANP secretion in terms of ECF translocation (i.e., the slope of linear regression) was calculated (Fig. 3C). ET-1 increased the ratio of changes in ANP secretion and ECF translocation (ANP/ECF) by a 4-fold (1.68 ± 0.10 vs 0.37 ± 0.03 ng/ μ l, $p < 0.001$). BQ123 alone attenuated ANP/ECF (0.34 ± 0.02 vs 0.58 ± 0.04 ng/ μ l, $p < 0.05$) and completely blocked ET-1-induced ANP/ECF (0.44 ± 0.04 vs 0.34 ± 0.02 ng/ μ l). However, BQ788 did not affect ANP/ECF and ET-1-induced ANP/ECF. In low Ca²⁺ condition, basal and stretch-activated ANP secretions were markedly increased and ANP/ECF was higher than that in

normal Ca^{2+} condition. ET-1-stimulated ANP secretion was abolished in low Ca^{2+} condition (3.90 ± 0.18 vs 3.82 ± 0.42 ng/ μ l) (Fig. 3C).

3.3. Effect of ET-2, ET-3, and s6C on ANP secretion from isolated atria

To compare ET-1 to ET-2, ET-3 and s6C on ANP secretion, ET-2, ET-3, or s6C was perfused. ET-2 at a dose of 10 nM augmented stretch-activated ANP secretion and the relationship between these two parameters shifted to the left and upward (Fig. 4Aa). ET-3 at a dose of 10 nM and 100 nM also augmented stretch-activated ANP secretion and the relationship between these two parameters shifted to the left and upward (Fig. 4Ab). s6C also shifted the relationship between these two parameters to the left and upward (Fig. 4Ab). As a results of direct comparison of ANP/ECF among ET family, the effect of 10 nM ET-3 on ANP/ECF (1.35 ± 0.06 ng/ml) was significantly lower than those of 10 nM ET-1 (2.53 ± 0.23 ng/ml) and ET-2 (2.21 ± 0.10 ng/ml). The effect of 100 nM ET-3 on ANP/ECF (1.95 ± 0.12 ng/ml) was significantly higher than that of 100 nM s6C (0.91 ± 0.04 ng/ml) (Fig. 4B).

3.4. Acute infusion of ET-1 or ET-3 with and without NPR-A antagonist

Figure 5A shows a representative tracing of changes in BP by ET-1 and ET-3 with and without NPR-A antagonist. MAP before ET-1 infusion in the absence or presence of A71915 infusion was not different (Fig. 5Ba). Acute injection of ET-1 (0.1 nM) caused an increased MAP by 27.5 ± 2.43 mmHg at 2 min, which gradually decreased to control level. ET-1 in the presence of NPR-A receptor blocker increased by 36.9 ± 3.09 mmHg at 3 min, which gradually decreased until 12 min after injection. An increased MAP by ET-1 with NPR-A receptor antagonist was significantly higher and its action was longer than that by ET-1 without NPR-A receptor antagonist (Fig. 5Bb). Acute injection of ET-3 (30 nM) caused an

increased MAP by 10.50 ± 1.82 mmHg at 1 min, which gradually decreased to control level. Similarly, ET-3 with NPR-A receptor antagonist caused an increased MAP by 18.13 ± 1.95 mmHg at 1 min, which was higher than that by ET-3 alone (Fig. 5Bc).

4. Discussion

The present study shows that ET-1 stimulates basal and stretch-activated ANP secretions via ET_A receptor and the order of secretagogue effect of ET family on ANP secretion was ET-1 $\geq$ ET-2 $\gg$ ET-3 > sC6. In vivo study showing an augmentation of high BP induced by ET-1 and ET-3 in the presence of NPR-A receptor blocker suggests that an increase in plasma ANP concentration by either ET-1 or ET-3 negatively inhibits the pressor effect of ET-1.

ET-1 is the main isoform of ET family which is synthesized in endothelial cells, muscular coat of arterial wall as well as in heart, kidney, and central nervous system. ET-1 raises BP, induces vascular and myocardial hypertrophy. The effects of ET family are reported to be different among isoforms. ET-1 is well known as a potent stimulator of ANP but, there are a few reports about the potency of secretagogue effect of ET family on ANP secretion. The aim of the present study was to compare the secretagogue effect of ET-1 on ANP secretion with ET-2, ET-3, and sarafotoxin in isolated perfused nonbeating atria. It is well-known that changes in atrial volume but not in pressure is an important stimulus of ANP secretion from isolated perfused atria [4, 5]. In atrial model used in this study, changes in atrial volume (DRV) induced by changing atrial pressure stimulated ANP secretion with ECF translocation [5, 19, 20]. There were positive correlations among DRV, ANP secretion, and ECF translocation. The ET family did not affect DRV and ECF translocation. ET-1 (10 nM) stimulated both basal and stretch-activated ANP secretion from atrial myocytes via ET_A receptor but not via ET_B receptor. These results are consistent with other reports [10, 16, 20, 28]. ET_A receptor antagonist but not ET_B receptor antagonist itself suppressed stretch-activated ANP secretion without change in ECF translocation. It means that ET-1 may be endogenously secreted by atrial stretch and be partly involved in the stimulation of stretch-

activated ANP secretion via ET_A receptor as a paracrine fashion. Skvorak et al showing stretch-induced ANP secretion stimulated by ET-1 and suppressed by BQ-123 using isolated perfused atria suggest that the stretch-induced release of ANP is partially regulated by ET and that the ET is locally produced and constitutes a paracrine control system [28]. Our results confirmed their previous findings [28].

Among ET family, ET-2 is very similar to ET-1 and, ET-3 and sarafotoxin differ from ET-1 by six and seven amino acids, respectively. All ET family stimulated stretch-activated ANP secretion but the potency was different. ET-2 increased ANP/ECF similarly to ET-1 whereas ET-3 increased ANP/ECF but less than ET-1. The order of secretagogue effect of ET family on ANP secretion was $ET-1 \geq ET-2 \gg ET-3 > s6C$.

It has been reported that high plasma level of ET-1 in cardiac hypertrophy and pulmonary hypertension [23, 27] increased plasma ANP secretion due to augmentation of ET-1-induced ANP secretion from hypertrophied atria [20] and ventricle [30], and a decrease in metabolism [21]. The maintenance of high level of plasma ANP in cardiac hypertrophied rats can be explained as a compensatory mechanism to reduce cardiac workload by a decreased ECF volume and vasodilation. In the present study, we tested acute compensation of ANP on ET-induced pressor effect in anesthetized rats. ET-1 increased MAP more than ET-3 and pretreatment with a NPR-A receptor antagonist potentiated the MAP response to ET-1 and ET-3 and increased the duration of action. These data suggest that elevations in plasma ANP concentration in response to ET-1 or ET-3 limits the blood pressure response to ET-1. We did not measure plasma ANP level because frequent blood collection for the measurement of plasma ANP may interfere or mask the compensatory effect of endogenous ANP. In addition, it has been reported that an infusion of ET-1 results in an increase in plasma ANP levels, which may also contribute to ET-1-induced natriuresis [24]. Taken together, ET potentially interacts with ANP in the regulation of BP and sodium balance.

Therefore, we suggest that the order of secretagogue effect of ET family on ANP secretion was $ET-1 \geq ET-2 \gg ET-3 > s6C$ and ANP induced by ET participates in the homeostatic regulation of BP by inhibiting the pressor effect of ET-1.

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Figure legends

Figure 1. Changes in atrial pressure (A), distension-and-reduction volume (B, DRV), ANP

secretion (C), ECF translocation (D), and ANP concentration (conc, E) by endothelin-1 (ET-1) from left atria. Atrial volume change (DRV) was increased by the elevation of intra-atrial pressure. A step wise increase in DRV resulted in proportional increases in ANP secretion and concomitant ECF translocation. The stretch-activated ANP secretion and concentration were significantly higher in ET-1 treated atria than those in control atria. Values are the means $\pm$ SEM (n = 7 - 8). *, Significantly different from control group, $P < 0.05$; ** $P < 0.01$.

Figure 2. Changes in atrial pressure (A), DRV (B), stretch-activated ANP secretion (C), mechanically-stimulated ECF translocation (D), and ANP concentration (E) by ET_A receptor antagonist (BQ123) from left atria. The stretch-activated ANP secretion and concentration were significantly lower in BQ123-treated atria than those in control atria. Values are the means $\pm$ SEM (n = 7 for each group). *, Significantly different from control group, $P < 0.05$; ** $P < 0.01$.

Figure 3. (A) Relationships between atrial pressure, DRV, mechanically-stimulated ECF translocation, and stretch-activated ANP secretion in left atria treated with vehicle and ET-1. (B) Relationships between mechanically-stimulated ECF translocation, and stretch-activated ANP secretion in left atria treated with BQ123 and BQ788. In ET-1-treated atria, the relationships between ANP secretion and DRV, and ANP secretion and ECF translocation (Ad) shifted to the right and upward. In BQ123-treated atria, the relationships between ANP secretion and ECF translocation shifted to the left and downward (Ba). BQ788 did not cause any significant changes in ANP secretion and ECF translocation (Bb). (C) Comparison of stretch-activated ANP secretion in terms of ECF translocation by ET-1 in the presence of BQ123, BQ788, and low Ca²⁺ condition. An increase in stretch-activated ANP secretion in terms of ECF translocation was blocked by the pretreatment with BQ123 but not with BQ788. ET-1-stimulated ANP secretion

was also abolished in low Ca^{2+} condition. Values are the means $\pm$ SEM ($n = 6 - 10$). *, Significantly different from control group, $P < 0.05$, ** $P < 0.01$, *** $P < 0.005$; ##, Significantly different from BQ788-treated atria, $P < 0.01$.

Figure 4. (A) Relationships between mechanically-stimulated ECF translocation and stretch-activated ANP secretion in left atria treated with ET-2, ET-3, and s6C. (B) Comparison of stretch-activated ANP secretion in terms of ECF translocation by ET-1 with ET-2, ET-3, and s6C. Values are the means $\pm$ SEM ($n = 6 - 7$). *, Significantly different from control group, $P < 0.05$, ** $P < 0.01$.

Figure 5. (A) Representative tracing of change in arterial blood pressure (ABP) by ET-1 (0.1 nM) and ET-3 (30 nM) with and without natriuretic peptide receptor-A blocker (A71915). (B) Mean arterial pressure (MAP) before ET-1 infusion in the absence or presence of A71915 (a) and changes in mean ABP by ET-1 (b) and ET-3 (c) with and without A71915. Values are the means $\pm$ SEM ($n = 5$). *, Significantly different from ET alone group, $P < 0.05$.

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Figure 1

Fig. 1

Figure 2

Fig. 2

Figure 3

Fig. 3

Figure 4

Fig. 4

Figure 5

A

B

Fig. 5