Angiotensin II Activates Endothelial Constitutive Nitric Oxide Synthase via AT₁ Receptors

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To determine whether angiotensin (ANG) II, a vasoconstrictor hormone, activates constitutive nitric oxide synthase (cNOS) in endothelial cells (ECs), we investigated the cellular mechanism by which ANG II induces nitric oxide (NO) formation in cultured bovine ECs. ANG II rapidly (within 1 min) and dose-dependently (10^{-9} – 10^{-6} M) increased nitrate/nitrite (NOx) production. This effect of ANG II was abolished by a NOS inhibitor, N^G-monomethyl-L-arginine. An ANG II type 1 (AT₁) receptor antagonist (DuP 753), but not an ANG II type 2 (AT₂) receptor antagonist (PD 123177), dose-dependently inhibited ANG II-induced NOx production. A Ca²⁺-channel blocker (barnidipine) failed to affect ANG II-induced NOx production, whereas an intracellular Ca²⁺ chelator (BAPTA) and a calmodulin inhibitor (W-7) abolished NOx production induced by ANG II. A protein kinase C (PKC) inhibitor (H-7) and down-regulation of endogenous PKC after pretreatment with phorbol ester decreased NOx production stimulated by ANG II. ANG II transiently stimulated inositol 1,4,5-trisphosphate (IP₃) formation, and increased cytosolic free Ca²⁺ concentrations; these effects were blocked by DuP 753. Our data demonstrate that ANG II stimulates NO release by activation of Ca²⁺/calmodulin-dependent cNOS via AT₁ receptors in bovine ECs. (*Hypertens Res* 1996; 19: 201–206)

Key Words: angiotensin II, endothelial cells, nitric oxide (NO), constitutive NO synthase (cNOS)

Nitric oxide (NO), a potent vasodilator with antiplatelet aggregation properties, is synthesized from the guanidino nitrogen atom of L-arginine by the catalytic action of an enzyme termed NO synthase (NOS) (1), and is released from a variety of cells. These include endothelial cells (ECs), vascular smooth muscle cells (VSMC), macrophages, and neuronal cells. There exist at least three distinct NOS isoforms (2). Inducible NOS (iNOS), a Ca²⁺/calmodulin-independent enzyme, is activated by bacterial lipopolysaccharides and several cytokines, and is predominantly expressed in many cells, including macrophages, hepatocytes, and VSMC. In contrast, constitutive NO synthases (cNOS), Ca²⁺/calmodulin-dependent enzymes, consist of two distinct isozymes, namely endothelial cNOS and neuronal cNOS, respectively. NO generated by endothelial cNOS accounts for the major biological activity of endothelium-derived relaxing factor (EDRF) (3), and leads to the stimulation of soluble guanylate cyclase in VSMC to increase cyclic GMP (cGMP) concentrations, which act as an intracellular mediator in vasorelaxation.

It has been shown that several vasoconstrictive substances, such as acetylcholine (4), serotonin (5), bradykinin (6) and endothelin (ET)-1 (7), stimulate the production and release of EDRF/NO to counteract vasoconstriction. Therefore, EDRF/NO regulates vascular tonus.

Angiotensin (ANG) II is a potent vasoconstrictor hormone that induces vasoconstriction via type 1 (AT₁) receptors in VSMC (8). It has been reported that NO regulates ANG II receptors through a cGMP-independent mechanism (9), and that NO inhibits ANG II-induced growth and DNA synthesis of rat VSMC (10, 11). Recently, it has been reported that vasoconstriction evoked by ANG II is impaired in the presence of endothelium in rabbit aorta (12) and rat carotid artery (13), suggesting the involvement of NO. However, there is no evidence that ANG II directly activates endothelial cNOS to generate NO. Therefore, the present study was designed to examine whether ANG II stimulates the synthesis and release of NO from cultured bovine ECs, and to elucidate the cellular mechanism by which ANG II activates endothelial cNOS.

Methods

Materials

The compounds used in the experiments were ANG II (Peptide Institute Inc., Osaka, Japan), 1, 5-isoquinolinylnyl-sulfonyl 2-methylpiperazine (H-7), 12-O-tetradecanoylphorbol-13-acetate (TPA), N-(6-aminohexyl)-6-chloro-1-naphthalenesulfonamide (W-7), L-arginine (Sigma Chemical Co., St. Louis, MO), 1, 2-bis (2-aminophenoxy) ethane N,N,N',N'-tetraacetic acid (BAPTA), ethylene glycolbis (β-

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Received January 24, 1996; accepted in revised form February 28, 1996.

aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA), fura-2 acetoxymethyl ester (Dojin Chemical, Kumamoto, Japan), *N*^G-monomethyl-L-arginine (LNMMMA) (Calbiochem Co., La Jolla, CA), DuP 753 (Banyu Pharmaceutical Co., Tsukuba, Japan), PD 123177 (Parke-Davis Pharmaceutical Research, Ann Arbor, MI), and barnidipine (Yamanouchi Pharmaceutical Co., Tokyo, Japan).

Cell Culture

ECs from bovine carotid artery were prepared by digestion with collagenase and were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum and antibiotics (penicillin, 100 U/ml; streptomycin, 100 mg/ml) at 37°C in a humidified atmosphere of 95% air-5% CO₂ as previously reported (14). Subcultured ECs (5-10th passages) were used in the experiments.

Determination of NOx (NO²⁻/NO³⁻)

Confluent ECs (5×10^5 cells) in 6-well dishes were usually incubated in 1-ml Hanks' medium without phenol red at 37°C for 1 min as reported previously (6). Concentrations of NOx in culture media were determined by the Griess method using an autoanalyzer (TCI-NOx 1000; Tokyo Kogyo Co., Tokyo, Japan) as reported previously (15).

Determination of Inositol 1,4,5-Trisphosphate (IP₃)

Confluent ECs were incubated with ANG II at 37°C in Hanks' medium containing 10 mM LiCl for the indicated times. Incubation was terminated by the rapid removal of medium and the addition of ice-cold 15% trichloroacetic acid, and IP₃ levels were determined by a competitive protein binding assay kit (Amersham, Tokyo, Japan) as described elsewhere (7).

Measurement of Cytosolic Free Ca²⁺ Concentration ([Ca²⁺]_i)

[Ca²⁺]_i was measured by the Ca²⁺-fura-2 fluorescence method as described previously (6). In brief, after incubation in serum-free DMEM for 24 h, ECs were trypsinized and incubated with 4 μM fura-2-acetoxymethyl ester at 37°C for 20 min in HEPES-buffered physiological salt solution. The Ca²⁺-fura-2 fluorescence of the suspended cells (5×10^6 cells/ml) was measured with a spectrofluorometer (CAF-100; JASCO Co., Tokyo, Japan) using excitation at 340 and 380 nm and emission at 500 nm. Values of [Ca²⁺]_i were calculated according to the method of Grynkiewicz *et al.* (16).

Statistical Analysis

Data are expressed as mean ± SE. Statistical analysis was performed by ANOVA analysis, followed by appropriate *post hoc* tests (Fisher PLSD) for comparison between groups. A *p* value less than 0.05 was considered to indicate statistical significance.

Results

ANG II (10^{-7} M) increased NOx production (about 2-fold) as early as 1 min after the start of incuba-

Fig. 1. Dose-response effects of ANG II on NOx production in cultured bovine ECs. Confluent ECs (5×10^6 cells) were incubated with various doses (10^{-9} - 10^{-6} M) of ANG II at 37°C for 1 min; production of NOx was measured. Each point is the mean of 6-well dishes; bars show SE. **p* < 0.05 vs. control.

tion. NOx production then remained constant for at least 20 min after the initiation of incubation. As shown in Fig. 1, ANG II dose-dependently (10^{-9} - 10^{-6} M) stimulated NOx production; the approximate EC₅₀ value was 10^{-8} M. To determine whether ANG II activates endothelial cNOS, the effect of a NOS inhibitor, LNMMMA, was studied. The stimulatory effect of ANG II (10^{-7} M) on NOx production was completely abolished by LNMMMA (4×10^{-4} M). This effect was reversed by the simultaneous addition of excess L-arginine (10^{-2} M) (Fig. 2).

To study which ANG II receptor subtype is involved in NOx production, the effects of an AT₁ receptor antagonist (DuP 753) and an AT₂ receptor antagonist (PD 123177) were examined. DuP 753 dose-dependently (10^{-9} - 10^{-6} M) inhibited ANG II (10^{-7} M)-induced NOx production (Fig. 3), whereas PD 123177 had no effect even at a maximal dose (10^{-6} M); neither DuP 753 nor PD 123177 affected basal NOx production (data not shown).

To determine whether protein kinase C (PKC) is involved in ANG II-induced NOx production, the effects of a PKC inhibitor (H-7) and down-regulation of endogenous PKC on ANG II-induced NOx production were examined (Fig. 4). Addition of H-7 (10^{-7} M) as well as pretreatment with TPA (10^{-7} M) for 24 h significantly (*p* < 0.05) diminished ANG II (10^{-7} M)-induced NOx production.

To study whether the increment of NOx production by ANG II involves a Ca²⁺/calmodulin-dependent mechanism, the effects of an intracellular Ca²⁺ chelator (BAPTA), a Ca²⁺-channel blocker (barnidipine), and a selective calmodulin inhibitor (W-7) were examined (Table 1). ANG II-induced NOx production was inhibited by BAPTA (10^{-8} M) and

Fig. 2. Effect of N^G-monomethyl L-arginine (LNMMA) on ANG II-induced NOx production in cultured bovine ECs. Confluent ECs were incubated with ANG II (10⁻⁷ M) in the presence and absence of LNMMA (4 × 10⁻⁴ M) and L-arginine (10⁻² M) at 37°C for 1 min; production of NOx was measured. Each column is the mean of 6-well dishes; bars show SE. *p<0.05 vs. control.

Fig. 3. Effect of AT₁ receptor antagonist on ANG II-induced NOx production in cultured bovine ECs. Confluent ECs were incubated with (●) or without (○) ANG II (10⁻⁷ M) in the presence of various doses (10⁻⁹-10⁻⁶ M) of DuP 753 at 37°C for 1 min; production of NOx was measured. Each point is the mean of 6-well dishes; bars show SE. *p<0.05 vs. control.

by W-7 (10⁻³ M), but not by barnidipine (10⁻⁵ M); neither BAPTA, W-7, nor barnidipine affected basal NOx production.

To determine whether ANG II stimulates phosphoinositide breakdown in bovine ECs, the effect of ANG II on IP₃ production was studied (Fig. 5). ANG II rapidly (within 15 s) stimulated IP₃ produc-

Fig. 4. Effect of H-7 and TPA pretreatment on ANG II-induced NOx production in cultured bovine ECs. Confluent ECs were incubated with or without ANG II (10⁻⁷M) in the presence of H-7 (10⁻⁷ M) and after pretreatment TPA (10⁻⁷ M) for 24 h; production of NOx was measured. Each column is the mean of 6-well dishes; bars show SE. *p<0.05 vs. control.

Table 1. Effects of BAPTA, W-7 and Barnidipine on ANG II-induced NOx Production in Cultured Bovine ECs

Drugs (concentration)	NOx released (nmol/min/10 ⁵ cells)
Control	2.33±0.20
ANG II (10 ⁻⁷ M)	5.48±0.64*
BAPTA (10 ⁻⁸ M)	3.50±0.20
BAPTA (10 ⁻⁸ M)+ANG II (10 ⁻⁷ M)	2.83±0.07
W-7 (10 ⁻³ M)	2.87±0.23
W-7 (10 ⁻³ M)+ANG II (10 ⁻⁷ M)	3.33±0.19
Barnidipine (10 ⁻⁵ M)	2.93±0.10
Barnidipine (10 ⁻⁵ M)+ANG II (10 ⁻⁷ M)	4.60±0.33*

Confluent ECs were incubated with ANG II (10⁻⁷ M) in the presence and absence of BAPTA (10⁻⁸ M), W-7 (10⁻³ M) and barnidipine (10⁻⁵ M) at 37°C for 1 min; production of NOx was determined. Each value is the mean of 6-well dishes ±SE (n=4).

*Statistically significant difference from control (p<0.05).

tion, peaking at 30 s, followed by a gradual decline by 1 min after the start of incubation (Fig. 5A). In the presence of DuP 753 (10⁻⁶ M), ANG II (10⁻⁷ M)-induced IP₃ formation was completely inhibited (Fig. 5B).

To determine whether ANG II increases [Ca²⁺]_i in bovine ECs, fura-2-fluorescence was measured (Fig. 6). ANG II (10⁻⁷ M) transiently (15-30 s) increased [Ca²⁺]_i, followed by a fall to basal level by

Fig. 5. Effect of ANG II on IP₃ production and NO_x production in cultured bovine ECs. (A) Confluent ECs were incubated with ANG II (10⁻⁷ M) for the indicated times; generation of IP₃ was determined. Each point is the mean of 6-well dishes; bars show SE. *p<0.05 vs. control. (B) Confluent ECs were incubated with ANG II (10⁻⁷ M) for 30 s in the presence and absence of DuP 753 (10⁻⁶ M); IP₃ generation was determined. Each column is the mean of 6-well dishes; bars show SE. *p<0.05 vs. control.

Fig. 6. Effects of DuP 753, EGTA and barnidipine on ANG II-induced [Ca²⁺]_i increase in cultured bovine ECs. Cell suspensions loaded with fura-2 were challenged with ANG II (10⁻⁷ M) pretreated without (A) and with (B) DuP 753 (10⁻⁶ M), (C) EGTA (3 × 10⁻³ M), and (D) barnidipine (10⁻⁵ M). Calculated values for [Ca²⁺]_i are shown on the ordinates.

1 min after the initiation of incubation (Fig. 6A). The ANG II-induced transient increase in [Ca²⁺]_i was inhibited by prior treatment with DuP 753 (10⁻⁶ M) (Fig. 6B). Removal of extracellular Ca²⁺ by EGTA (3 × 10⁻³ M) (Fig. 6C) or blockade of voltage-dependent Ca²⁺-channels by barnidipine (10⁻⁵ M) (Fig. 6D) had no effect on the ANG II-

induced transient increase in [Ca²⁺]_i.

Discussion

ANG II has diverse biological actions in the cardiovascular system, including vasoconstriction, stimulation of aldosterone release, facilitation of peripheral adrenergic outflow, and cellular proliferation and growth (8). Although ANG II causes remarkable vasoconstriction in many vasculature, its vasoconstrictor responses show marked differences among blood vessels, depending on the type of preparation, and the region and species of the blood vessels studied (17, 18). Such heterogeneous responses to ANG II may also be due to the presence or absence of vascular endothelium. Recently, it has been shown that ANG II-induced vasoconstriction is impaired by the presence of endothelium (12, 13). These data suggest that endothelial NO is responsible for the endothelium-dependent inhibition of constriction induced by ANG II. However, it is not certain whether ANG II directly stimulates the synthesis and release of NO by ECs.

The present study clearly demonstrated that ANG II rapidly (within 1 min) and dose-dependently (10⁻⁹-10⁻⁷ M) stimulated NO_x production in cultured bovine ECs. This effect was abolished by a stereospecific NOS inhibitor, LNMMA, and reversed by L-arginine. In contrast to the activation of Ca²⁺/calmodulin-independent iNOS, which requires several hours for de novo synthesis after stimulation with bacterial LPS and cytokines, Ca²⁺/calmodulin-dependent cNOS is activated immediately after [Ca²⁺]_i increases. Thus, our data suggest that ANG II activates endothelial cNOS.

There are two major isoforms of ANG II recep-

tor (AT₁ and AT₂), both of which have seven transmembrane domains common to the G protein-coupled receptor superfamily (19-22). AT₁ receptors are predominantly expressed in VSMC, adrenal glomerulosa, and lung. The activation of AT₁ receptors stimulates phosphatidylinositol-specific phospholipase C, which results in the generation of second messengers, IP₃ and diacylglycerol, thereby leading to intracellular Ca²⁺ mobilization and PKC activation respectively. AT₂ receptors, on the other hand, are expressed predominantly in rat fetal mesenchymal tissues, brain, adrenal medulla, heart, and myometrium, although the signaling mechanisms involved remains elusive. It has recently been shown that cultured rat ECs express both AT₁ and AT₂ receptors, as demonstrated by binding studies and by the presence of their respective mRNA through RT-PCR (23); the ratio of AT₁/AT₂ receptors was about 80%/20%. In the present study, the AT₁ selective antagonist (DuP 753), but not the AT₂ selective antagonist (PD 123177), dose-dependently inhibited ANG II-induced NOx production in bovine ECs. Recent studies have shown that ANG II decreases the contractions of rat carotid artery in the presence of vascular endothelium and increases cyclic GMP concentrations; these effects were blocked by DuP 753, but not by PD 123177 (13). Taken together, these data indicate that ANG II-induced NOx production is mediated via AT₁ receptor in ECs.

Our study demonstrated that neither removal of extracellular Ca²⁺ by EGTA nor blockade of voltage-dependent Ca²⁺-channels by barnidipine had any effect on ANG II-induced [Ca²⁺]_i increase and NOx production in ECs, whereas an intracellular Ca²⁺ chelator, BAPTA, completely blocked ANG II-induced NOx production. In fact, ANG II transiently and concomitantly stimulated IP₃ production and [Ca²⁺]_i increase, effects that were blocked by the AT₁ receptor antagonist (DuP 753). Furthermore, the peak time (30 s) for IP₃ formation and a transient increase in [Ca²⁺]_i elicited by ANG II preceded that of NOx production (1 min). Therefore, NOS activation by ANG II requires an IP₃-mediated increase in [Ca²⁺]_i derived from the intracellular store sites in bovine ECs. We have previously demonstrated that bradykinin (6) and ET-1 (7) stimulated receptor-mediated phosphoinositide breakdown in cultured bovine ECs, which triggers the release of intracellular free Ca²⁺, thereby activating Ca²⁺/calmodulin-dependent cNOS. Collectively, it is suggested that ANG II activates endothelial cNOS via a Ca²⁺/calmodulin-dependent pathway in bovine ECs in the same manner as bradykinin and ET-1.

The present study showed that NOx production was partially inhibited by a PKC inhibitor (H-7) and by the depletion of endogenous PKC after pretreatment with TPA. Therefore, activation of PKC by diacylglycerol resulting from the ANG II-stimulated phosphoinositide breakdown may be partly involved in the mechanism of ANG II-induced cNOS activation. However, it remains unknown whether PKC phosphorylates cNOS to activate its enzyme activ-

ity.

Our data are consistent with those of pharmacological studies showing an endothelium-derived NO inhibition of ANG II-induced contractions in isolated rabbit aorta (12) and rat carotid artery (13). The approximate EC₅₀ value (10⁻⁸ M) for stimulation of NOx production by ANG II appears to be comparable to that of the IC₅₀ (3 × 10⁻⁸ M) for endothelium-dependent inhibition of ANG II-induced contraction (13). Taken together, the results suggest that ANG II-induced NO production in ECs may regulate vascular tone by counteracting the remarkable vasoconstriction elicited by ANG II.

In conclusion, the present study demonstrated that ANG II directly stimulates NO synthesis by activation of Ca²⁺/calmodulin-dependent cNOS via AT₁ receptor-mediated phosphoinositide breakdown in bovine ECs.

Acknowledgements

This study was supported in part by Grants-in-Aid from the Ministry of Education, Science and Culture and the Ministry of Health and Welfare of Japan. We thank Merck-Banyu Co., Parke-Davis Co., and Yamanouchi Co., for providing DuP 753, PD 123177, and barnidipine, respectively.

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