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The role of nitric oxide synthase in reduced vasocontractile responsiveness induced by prolonged α_1-adrenergic receptor stimulation in rat thoracic aorta

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1 Prolonged exposure (6–12 h) of rat aorta to alpha1-adrenergic receptor (α_1AR) agonist phenylephrine (Phe) leads to a decrease in α_1AR-mediated vasoconstriction. This reduced responsiveness to α_1AR stimulation was strongly dependent on the intactness of the endothelium.

2 We examined the effect of Phe on nitric oxide synthase (NOS) activity by measuring the conversion of [^{3}H]L-arginine to [^{3}H]L-citrulline in rat aorta or in endothelial cells isolated from rat aorta. Phe stimulation increased NOS activity in control aortas. This response was antagonized by prazosin. However, Phe increased neither the activity of NOS nor intracellular Ca^{2+} in the isolated endothelial cells from the control aortas, whereas acetylcholine (Ach) was able to stimulate both responses in these cells. This result suggests that Phe stimulates α_1AR on vascular smooth muscle cells and has an indirect influence on endothelial cells to increase NOS activity.

3 In Phe-exposed aortic rings, basal NOS activity was found to have increased compared to vehicle-exposed control rings. Stimulation with Phe or Ach caused a small increase over basal NOS activity in these preparations. Prolonged exposure to Phe also caused an enhancement of Ach-mediated vasorelaxation in rat aorta.

4 Immunoblot and reverse transcription–polymerase chain reaction experiments showed that prolonged exposure of rat aorta to Phe resulted in an increased expression of eNOS, but not iNOS. This increase was antagonized by nonselective antagonist prazosin. Immunohistochemical staining experiments also showed that expression of eNOS increased in endothelial cells after Phe exposure of the aortas.

5 These results, all together, showed that prolonged exposure of rat aorta to α_1AR agonist Phe enhanced the expression of eNOS and basal NOS activity, which probably causes a decreased vasocontractile response to Phe or to other agonists such as 5HT (5-hydroxytryptamine) in rat aorta.

6 This phenomenon can be considered more as a functional antagonism of vasocontractile response to agonists mediated by endothelium than a specific desensitization of α_1AR-mediated signalling in vascular smooth muscle cells.

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Abbreviations: Ach, acetylcholine; α_1AR, alpha1-adrenergic receptor; NOS, nitric oxide synthase; Phe, phenylephrine

Introduction

Alpha1-adrenergic receptor (α_1AR)-mediated vasocontractile responses of various vessels are reduced by chronic infusion of catecholamines or α_1AR agonists *in vivo* or by prolonged exposure of vessels to adrenergic agonists *in vitro* (Lurie *et al*., 1985; Maze *et al*., 1985; Hiremath *et al*., 1991; Hu *et al*., 1992a, b; 1994; Seasholtz *et al*., 1997a, b). Several studies have been performed to clarify the underlying mechanisms involved in the reduction of α_1AR-mediated responses in blood vessels. No change in α_1AR expression has been observed in such reduced vasocontractile responsiveness (Lurie *et al*., 1985; Seasholtz *et al*., 1997a, b). Our previous studies have shown that activation of Gi and Gq proteins by α_1AR or α_1AR-G protein coupling was impaired in this phenomology (Seasholtz *et al*., 1997a, b). It is possible to explain some part of this phenomenon by desensitization of α_1AR-mediated signalling to produce vasocontraction in vascular smooth muscle. On the other hand, some studies have shown the important role of the endothelium in this event (Hiremath *et al*., 1991; Hu *et al*., 1992a, b; 1994; Kamata & Makino, 1997).

The regulatory role of the endothelium in α_1AR-mediated vasocontractile responses has long been known. Removal of endothelium or inhibition of nitric oxide (NO) release enhances the vasocontractile response to α_1AR agonists (Angus & Cocks, 1983; Carrier & White, 1985; Martin *et al*., 1986; Cohen *et al*., 1988; Kaneko & Sunano, 1993; Amerini & Mantelli, 1995). NO release from endothelial cells during vasocontractile response to α_1AR agonists (or to other

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vasocontractile agents) has an inhibitory effect on vasoconstriction.

It has been shown that this reduced vasocontractile responsiveness induced by chronic *in vivo* infusion or prolonged *in vitro* incubation of blood vessels with catecholamines was mainly mediated by the endothelium (Hiremath *et al*., 1991; Hu *et al*., 1992a, b; 1994; Kamata & Makino, 1997). Removal of endothelium or inhibition of NO activity significantly decreased the level of reduction in the vasocontractile response to α_1AR agonists (Hiremath *et al*., 1991; Hu *et al*., 1992a, b; 1994; Kamata & Makino, 1997).

In this study, we examined the effect of prolonged phenylephrine (Phe) incubation on nitric oxide synthase (NOS) activity and the expression levels of eNOS and iNOS in rat aorta.

Methods

Vasocontrictile responses and desensitization

Thoracic aortas were obtained from male Wistar rats (200–300 g) that were exsanguinated under thiopental (35 mg kg^{-1} i.v.) anesthesia. Surrounding tissues were removed and the vessels were placed in cold Krebs–Henseleit solution. The vessels were cut into rings (approximately 3-mm width). In some experiments, the endothelium was removed by passing a cannula through the arterial lumen. The functional integrity of the endothelium was tested by observing acetylcholine (Ach)-mediated vasorelaxation. The rings were opened by a single cut and then fixed with stainless steel clips at both ends in organ baths of 5 ml volume containing oxygenated (5% CO_2, 95% O_2) and warmed (37°C) Krebs solution (pH = 7.4) with the following composition (in mM): NaCl 112, KCl 5, $NaHCO_3$ 25, NaH_2PO_4 1, $MgCl_2$ 0.5, $CaCl_2$ 2.5 and glucose 11.5. Isometric contractions were measured using force–displacement transducers (Grass FT.03) and a general-purpose amplifier (MayCom, Ankara) connected to a personal computer. All preparations were given an initial tension of 1–1.5 g and were allowed to equilibrate for 1 h by changing the bath buffer every 10 min. Following the equilibration period, the vessels were contracted by using 10 μM of Phe and quickly washed three times. The preparations were then allowed to equilibrate for another hour under the conditions mentioned above. Concentration–response curves were obtained by using cumulatively increasing concentrations of Phe (in 1/2 Log steps). After the 1 h washing and equilibration period, some of the vessels were incubated with 10 μM of Phe for 6 h (incubation time and concentration of Phe were determined by preliminary experiments). After the incubation, the rings were washed and allowed to equilibrate for another hour by changing the buffer every 10 min. Then, the Phe concentration–response curve was obtained. In separate experiments, the endothelium was removed before or after the incubation with Phe. In a different set of experiments, Phe- or vehicle-incubated aortas were contracted with 80 mM KCl including Krebs solution and Ach-mediated vasodilatation response were obtained. In another set of experiments, aortas were incubated with dexamethasone (100 μM) for 1 h before Phe incubation and during the incubation. The integrity of agonist responses in serial experiments was tested in parallel controls. We also incubated aortic rings with Phe (10 μM) for 12 h in Dulbecco's modified Eagle's medium (DMEM) with 250 U ml^{-1} penicillin/streptomycin in a 37°C incubator containing 5% CO_2. Parameters of concentration–response curves were estimated by means of nonlinear regression of a three-parameter logistic function.

NOS activity in aorta

[^{3}H]L-arginine to [^{3}H]L-citrulline conversion in aortas and isolated endothelial cells was measured by a modification of the method described previously (Brown *et al*., 1996; Ferro *et al*., 1999). The 5 mm rings were transferred to 24-well plates containing 2 ml of DMEM, 2 μCi ml^{-1} of [^{3}H]L-arginine (56 Ci mmol^{-1} Amersham, Vienna, Austria) and placed in a 37°C incubator containing 95% air, 5% CO_2 and incubated for 1 h. Labelled rings were washed two times with oxygenated HEPES buffer of the following composition (mM): NaCl 125, KCl 5.4, $NaHCO_3$ 16.2, $MgSO_4$ 0.8, $CaCl_2$ 1.8, glucose 5.5, HEPES 15, pH 7.4 at 37°C and placed in individual tubes and equilibrated for 30 min with or without antagonist. Rings were incubated with an agonist for 30 min. Termination of reaction was carried out by adding 300 μl of ice-cold 15% trichloroacetic acid. The tubes were then left on ice for 60 min. They were then centrifuged (1500 × *g*, 10 min) and the supernatant was taken and mixed with 125 μl of 10 mM EDTA and 500 μl of 1 : 1 Freon tri-*n*-octylamine in 1.5 ml microcentrifuge tubes. The samples were vortexed and allowed to stand for 10 min before centrifugation (12,000 × *g*, 10 min), and 300 μl of aqueous phase was taken and mixed with 700 μl 20 mM HEPES (pH 6.0). Samples were loaded on 1 ml column of Dowex (Na^+ form). [^{3}H]L-citrulline was eluted with 4 ml distilled H_2O and [^{3}H]L-arginine with 3 ml of 0.1 M NaOH. Radioactivity was measured by liquid scintillation spectrometry.

NOS activity and intracellular Ca^{2+} level in isolated endothelial cells

Thoracic aortas (from three to four rats) were obtained as described above and incubated with DMEM with 1 mg ml^{-1} collagenase (type II, Sigma) at 37°C in an incubator containing 95% air, 5% CO_2 for 20 min. Following incubation, the aortas were cannuled and massaged and flashed through with 30 ml DMEM. The endothelial cells including 30 ml DMEM were centrifuged (400 × *g*, 5 min) and suspended with DMEM. Cells were loaded on Petri dishes and incubated for 2 h in a 37°C incubator containing 95% air, 5% CO_2. The cells were incubated with 4 μM fura-2AM for 45 min at room temperature. The cells were then washed with DMEM and left at room temperature for 15 min. After characterization of endothelial cells under microscopy by typical morphology, Fura-2 fluorescence was recorded using a PTI Ratiomaster microspectrophotometer and FELIX software (Photon Technology International, Inc., NJ, U.S.A.). Cells were excited at 340/380 nm and emission was measured at 510 nm. The ratio of fluorescence at 340 nm to the fluorescence at 380 nm was calculated and used as an indicator of Ca^{2+}. In separate experiments, endothelial cells were incubated with DMEM including 2 μCi ml^{-1} of [^{3}H]L-arginine (56 Ci mmol^{-1} Amersham, Vienna, Austria) and placed in a 37°C incubator containing 95% air, 5% CO_2 and incubated for 1 h. Labelled

cells were washed two times by centrifugation ($400 \times g$, 5 min) and suspended in HEPES buffer. The cells were separated into individual tubes and experiments were carried out as described above.

Immunoblotting experiments

After incubation with Phe, aortas were homogenized with a motor-driven glass to glass homogenizer in cold Tris–HCl buffer (20 mM Tris, 16 mM (3-[(3-cholamidopropyl) dimethylammonio]-1-propane-sulfonate (CHAPS), 0.5 mM DL-dithiothreitol, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, pH:7.4) and centrifuged at $500 \times g$ for 20 min at 4°C. The protein amount of the supernatant was measured (Bradford, 1976). Samples (20–30 μg protein) were subjected to 10% SDS–PAGE and then transferred electrophoretically to nitrocellulose membrane (Laemmli, 1970). Immunoblotting was performed using antiserum against iNOS (RBI, MA, U.S.A.), eNOS (RBI, MA, U.S.A.), (dilutions 1/2 : 500) and enhanced chemoluminescence (ECL). Briefly, nitrocellulose membranes were incubated overnight at 4°C in PBS (phosphate buffered saline: 20 mM NaH_2PO_4–Na_2HPO_4 (pH 7.6) containing 154 mM NaCl, 3% bovine serum albumin (BSA) and 8% nonfat dry milk). The blots were washed several times with PBS containing 0.1% tween, and then incubated with antiserum at room temperature for 1–2 h by shaking. They were then washed several times with PBS, incubated with horseradish peroxidase-labelled anti-goat IgG (Santa Cruz, CA, U.S.A.) (dilutions $1 : 10{,}000^{-1}$ $-1 : 12{,}000^{-1}$) for 1 h at room temperature. The blots were washed several times with PBS and then incubated with ECL Western blotting reagent (Amersham, Vienna, Austria) for 1 min and exposed to X-ray film for 45–90 s.

Reverse transcriptase–polymerase chain reaction (RT–PCR)

The expression of mRNA in the aorta was analyzed by RT–PCR. The expression of glyceraldhyde-3-phosphate dehydrogenase (GAPDH) mRNA was determined as a control sample. Total RNA was extracted using the method based on the acid guanidium thiocyanate–phenol–choloroform method. The RNA concentration and purity was determined by u.v. spectrometry at absorption at 260 and 280 nm. DNAse treatment of RNA extracts was performed before RT–PCR experiments. DNAse was eliminated by phenol/chloroform extraction or by incubation for 10 min at 99°C. In an RT–PCR experiment, 0.5–1.5 μg total RNA was incubated with 0.4 U μl^{-1} eAMV-RT, 0.05 U μl^{-1} JumpStart AccuTag LA DNA polymerase, 0.2 mM of each dNTP, 0.2 μM mRNA specific primers, 0.4 U μl^{-1} RNAase inhibitor in a final volume of 50 μl RT–PCR buffer with 3 mM $MgCl_2$ (Sigma Enhanced Avian HS RT–PCR Kit, MO, U.S.A.). PCR cycles were performed in a Gene Amp system 9700 (Applied Byosystems) by a denaturing step for 5 min at 99°C, 35 cycles each consisting of 0.30 min at 60°C, 1 min at 72°C and final step extension for 5 min at 72°C. A 10 μl aliquot of PCR reaction was analyzed by electrophoretic separation on 10% agarose gel containing 0.5 $\mu g\,ml^{-1}$ of ethidium bromide. Based on the sequences of the rat eNOS and GADPH, the following sets of primers were used in the RT–PCR experiments; eNOS sense 5′-CTG-GCA-AGA-CCG-ATT-ACA-CGA-3′, eNOS antisense 5′-CGC-AAT-GTG-AGT-CCG-AAA-ATG-3′, GADPH sense 5′-TCC-ACC-ACC-CTG-TTG-CTG-TA-3′, GADPH antisense 5′-ACC-ACA-GTC-CAT-GCC-ATC-AC-3′.

Sectioning, immunostaining and microscopic detection

Rats were anesthetized and perfused with saline solution by transthoracic cannulation of the left ventricle. After 2 min of saline perfusion, the rats were perfused with 3.5% paraformaldehyde (PFA) for 10 min at a pressure of 120 cm H_2O. Then, thoracic aortas were removed and immediately processed for further fixation and cryosectioning. The aorta segments were immersed in 3.5% PFA in 0.1 M phosphate buffer solution for 4 h. Prior to cryosectioning, the tissue blocks were immersed in 1.2 M sucrose solution containing 0.5% PFA as a cyroprotectant, and then 8 μm-thick serial frozen transverse sections were cut. Few sections were initially stained by routine hematoxylin–eosin (H&E) procedure. Prior to immunolabelling, the sections were washed three times in a blocking and aldehyde-reducing solution (BS) composed of PBS containing 2% BSA, 2% powdered milk, 2% normal goat serum, 0.1 M glycine, and 0.01% Triton X-100, and were then stored at 4°C until they were processed further (Can *et al*., 2003). For immunofluorescent labelling, sections were incubated with a rabbit polyclonal anti-eNOS antibody (RBI and Santa Cruz MA, U.S.A.) diluted 1 : 100 in PBS for 2 h at 37°C. This step was followed by incubation in Cy3 goat anti-rabbit IgG (Jackson ImmunoResearch, U.S.A.), diluted 1 : 200 in PBS, for 90 min at 37°C. Primary antibody omission incubations with either BS or PBS were carried out to test the specificity of the antibodies used. Finally, sections were mounted in glycerol/PBS (1 : 1) medium containing 25 $mg\,ml^{-1}$ sodium azide as an antifading reagent (Can *et al*., 2003) and Hoechst 33258 (1 $mg\,ml^{-1}$) for nuclear counterstaining.

Labelled aorta sections were initially examined by a Zeiss Axiovert 100M inverted microscope using a conventional fluorescence Cy3/UV filter set and mercury-arc lamp. Hoechst 33258 staining of nuclear material was used to check for topographic orientation. Then, a Zeiss LSM-510 Meta confocal laser scanning microscope (Germany) equipped with 63 × plan-apo objective was used for further detection of eNOS signals. The 543 nm He–Ne laser was used to excite the marker. Single optical sections (5.0 mm in thickness) in 2048×2048 pixel resolution were obtained and pseudocolored according to the original fluorochrome using the Zeiss LSM 510 v3.0 software (Germany).

Statistics

Results are presented as arithmetic means with standard error of the mean from n observations. Student's unpaired t-test was used to assess the significance of differences between mean values, maximal responses, pEC_{50} values and significance being defined by a P-value less than 0.05.

Drugs

Sources of compounds used were as follows: phenylephrine, prazosin, (Sigma, Munich, Germany); iNOS and eNOS antibody (N-200, N-201, RBI, and Santa Cruz, MA, U.S.A.).

Results

Vasocontractile studies

Prolonged exposure of the rings to Phe (6 h) reduced the vasocontractile response to Phe in endothelium-intact aortas (Figures 1 and 2a). Maximal response and potency of Phe were significantly decreased by prolonged incubation of the rings with Phe. We observed quite similar results in the rings incubated with Phe for 12 h (data not shown). Removal of endothelium before (Figure 2a) or after (Figure 2a) Phe incubation restored the response to Phe; maximal response to Phe was fully recovered, but its potency was restored partially. pEC_{50} values of Phe were 6.7 ± 0.08, *n*: 6 in control, 5.57 ± 0.09, *n*: 6 in endothelium-intact Phe-incubated, 6.22 ± 0.007, *n*: 5 in endothelium-removed (before Phe incubation) aortas and 5.8 ± 0.04, *n*: 6 in endothelium-removed (after Phe incubation) aortas. The values in Phe-incubated aortas were significantly different from the control values. All these results were in agreement with the results of the previous studies (Hiremath *et al*., 1991; Hu *et al*., 1992a, b; 1994; Kamata & Makino, 1997). Ach-mediated vasodilatation, which is mediated by NO, was also enhanced by prolonged exposure of endothelium-intact rings to Phe (Figure 2b). Ach-mediated maximal relaxation of KCl (80 mM)-induced vasocontraction in Phe-incubated aortas was significantly high ($70 \pm 7\%$ *n*: 5) compared to controls ($48 \pm 2\%$ *n*: 5). Dexamethasone, an inhibitor of iNOS induction, did not have an effect on Phe-mediated desensitization in rat aorta (Figure 2c). In this study, as it has been shown previously (Hu *et al*., 1994; Seasholtz *et al*., 1997a, b), 5HT (5-hydroxytryptamine)-mediated vasocontractile response was also reduced ($32 \pm 5\%$, *n*: 3) in Phe-exposed rings.

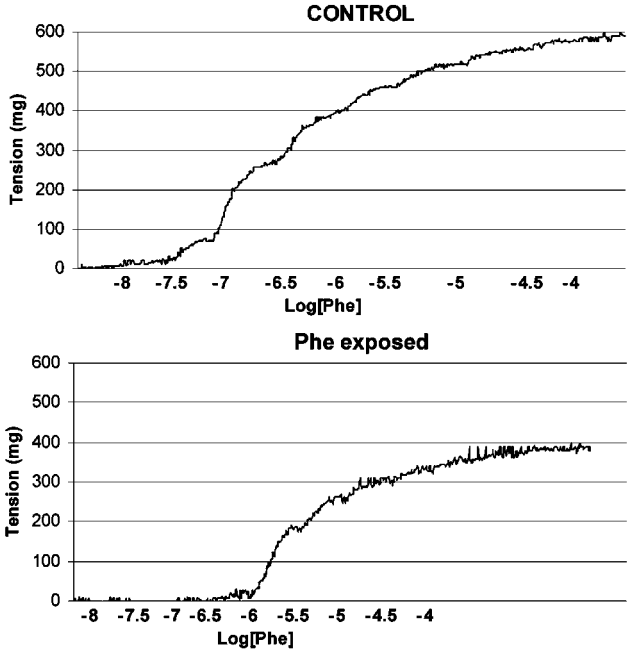

Figure 1 Representative of raw data of phenylephrine (Phe) concentration–response curves in control and Phe-exposed endothelium-intact aortic rings.

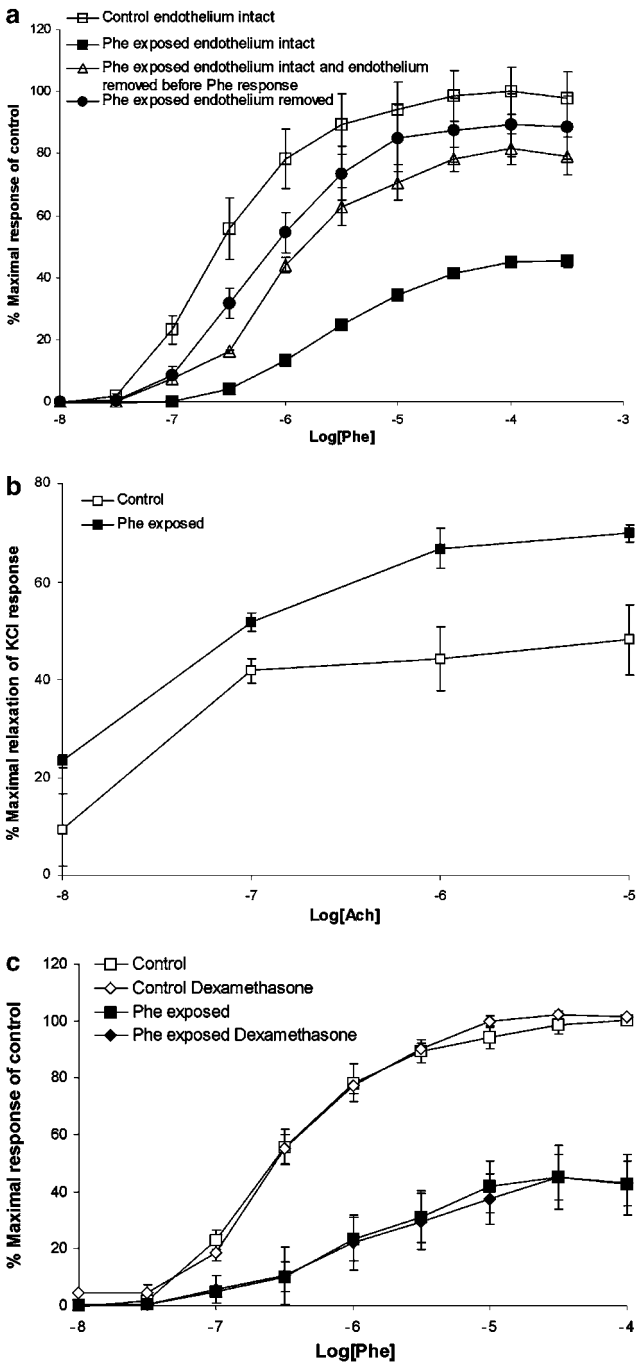

Figure 2 Phe concentration–response curves were obtained (a) in endothelium-intact aortic rings that were incubated with saline as control or Phe (6 h). The Phe responses were also obtained in endothelium-removed aortas before or after Phe incubation. Ach-mediated relaxation responses (% relaxation of 80 mM KCl-induced contraction) were obtained (b) in endothelium-intact aortic rings that were incubated with saline as control or Phe (6 h). The Phe responses were measured in endothelium-intact rings which were incubated with saline and treated with dexamethasone as controls or, those that were incubated with Phe (6 h) and treated with dexamethasone (c). Maximal response in control rings was 15 ± 2 mN. Data were obtained from five to six separate experimental groups.

NOS activity in aorta and isolated endothelial cells

Ach (10 μM) or Phe (10 μM) increased the conversion of [^{3}H]L-arginine to [^{3}H]L-citrulline, which indicates stimulation of NOS activity, in endothelium-intact control aortic rings (Figure 3). Phe-stimulated NOS activity was antagonized by prazosin. In endothelial cells isolated from control rat aortas, Ach but not Phe-stimulated NOS activity (Figure 4). Incubation with Phe (6 h) increased the basal activity of NOS in endothelium-intact aortic rings. Ach or Phe was able to further stimulate NOS activity in these rings (Figure 5).

Intracellular Ca^{2+} changes in isolated endothelial cells

In endothelial cells isolated from control rat aortas, Ach but not Phe increased intracellular Ca^{2+} (Figure 6).

eNOS and iNOS protein level

In control or Phe-incubated (6 h) rat aortas, we did not detect any iNOS protein in immunoblot experiments, whereas eNOS protein was detectable in control or Phe-incubated rat aortas. The densitometry results of four separate immunoblots showed that the expression level of eNOS significantly increased after Phe incubation in rat aorta (Figure 7). The densities in Phe-incubated (6 h) aortas were $160 \pm 15\%$ of the control aortas. This response was antagonized by prazosin. We obtained similar results in aortas incubated with Phe for 12 h (data not shown).

eNOS mRNA level

mRNA expression of eNOS and GADPH was detectable in control or Phe-incubated (6 h) rat aortas. The densitometry

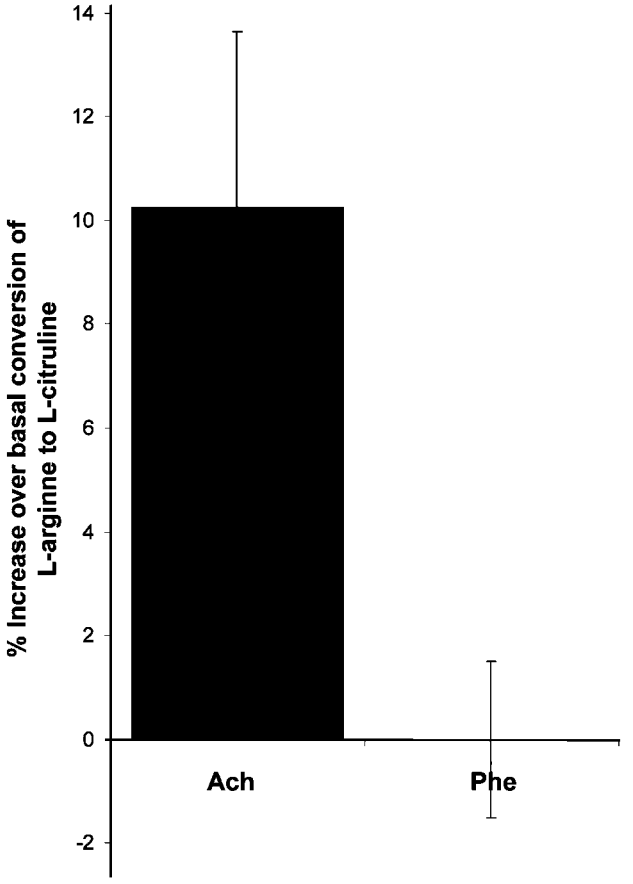

Figure 4 Ach but not Phe increased the conversion of [^{3}H]L-arginine to [^{3}H]L-citrulline in isolated endothelial cells (*$P<0.05$, *n:* 5).

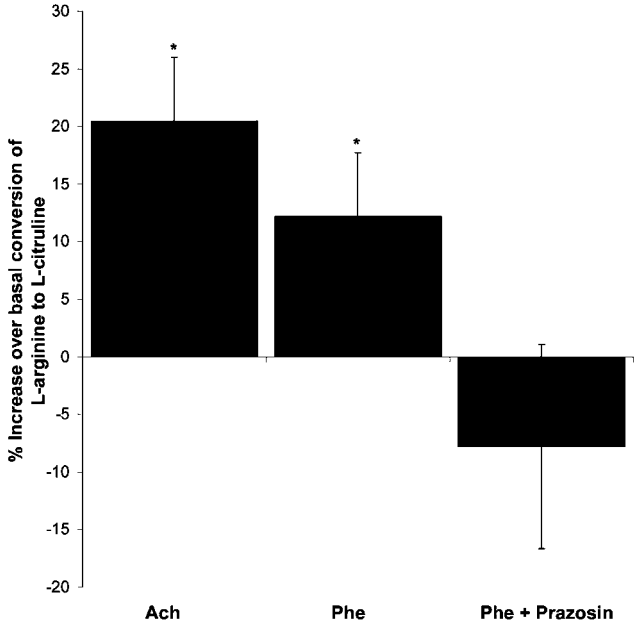

Figure 3 Phe or Ach increased the conversion of [^{3}H]L-arginine to [^{3}H]L-citrulline in aortic rings. Phe-stimulated conversion was blocked by prazosin (Pra). (* indicates the statistical difference from basal, Student's *t*, *n*: 5.)

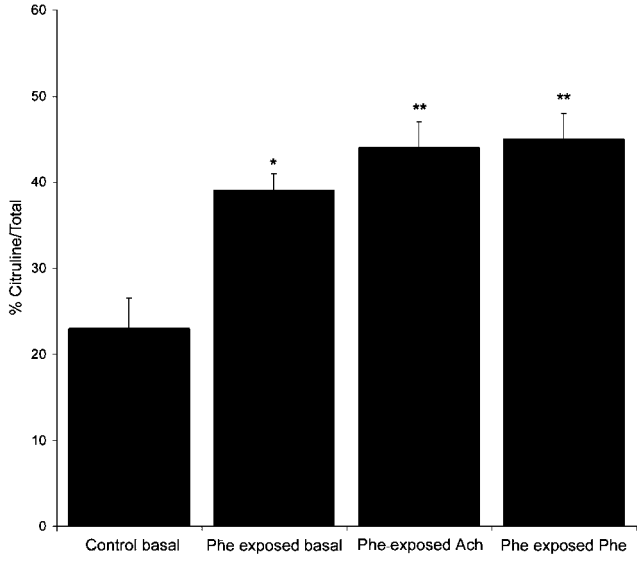

Figure 5 Phe incubation of the rings significantly increased the basal conversion of [^{3}H]L-arginine to [^{3}H]L-citrulline compared to saline incubation as control (*$P<0.05$, *n*: 7). Total count is the sum of the counts of [^{3}H]L-arginine and [^{3}H]L-citrulline. Phe or Ach also further increased the conversion over basal response in Phe-exposed ring (**$P<0.05$, *n*: 6).

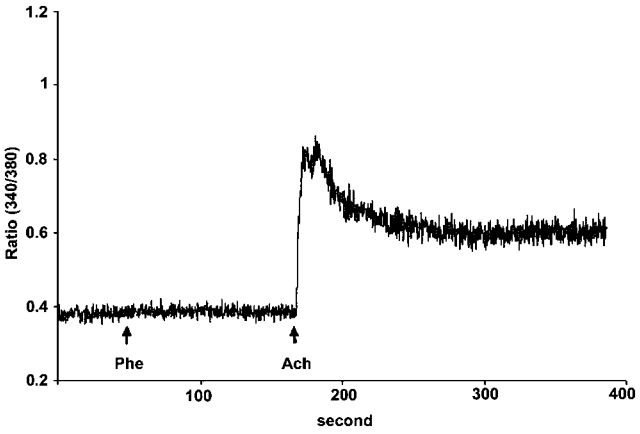

Figure 6 Ach but not Phe increased intracellular Ca^{2+} in isolated endothelial cells.

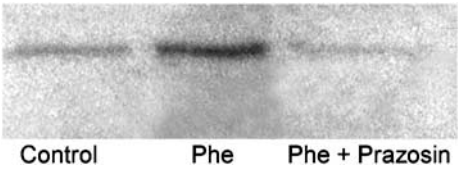

Figure 7 Immunoblot analysis of eNOS in endothelium-intact aortas, which were incubated with saline as control or Phe (6 h). Phe significantly increased the level of eNOS protein. Prazosin blocked Phe-induced increase in the level of eNOS.

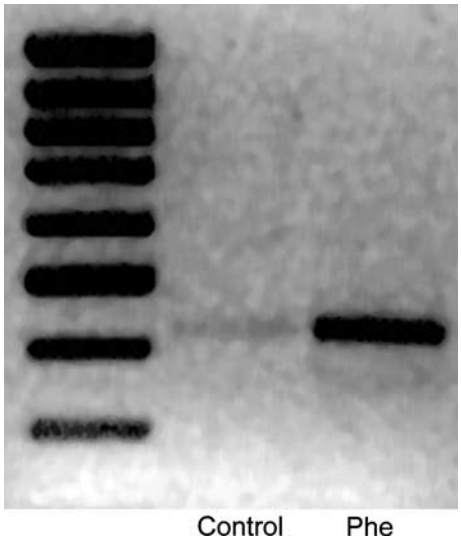

Figure 8 RT–PCR experiments in endothelium-intact aortas, which were incubated with saline as control or Phe (6 h). Phe significantly increased the level of mRNA of eNOS. This figure is representative of four different experiments.

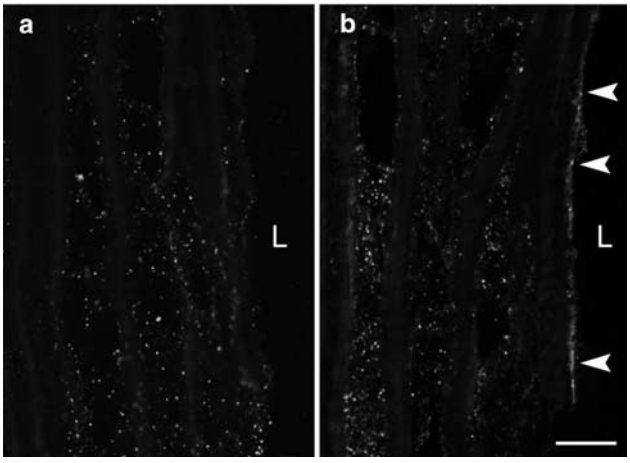

Figure 9 Immunostaining of eNOS in control (a) and Phe-incubated (b) rat aorta. A significant increase of eNOS positivity in endothelium (arrowheads) is noted in Phe-incubated rat aorta compared to controls. This figure is the representative of three different experiments. L; lumen; scale bar = 20 μm.

results showed that mRNA expression for eNOS significantly increased after Phe incubation in rat aorta (Figure 8). The densities in Phe-incubated aortas were $250 \pm 35\%$, n: 4 of the control aortas. We obtained similar results in aortas incubated with Phe for 12 h (data not shown).

Immunohistochemical staining of eNOS

We used two different antibodies for immunostaining of eNOS in aortic sections. A basal immunostaining of eNOS was observed in control aortic sections. However, in the sections from Phe-incubated rat aorta, there was a significant and enhanced eNOS positivity in the endothelium (Figure 9).

Discussion

In the present study, we confirmed that α_1AR-mediated vasoconstriction was reduced by prolonged exposure of the aorta to Phe, depending on the presence of an intact endothelium, as reported previously (Hiremath *et al*., 1991; Hu *et al*., 1992a, b; 1994; Kamata & Makino, 1997). We used this model to further investigate the underlying mechanism of the endothelium-dependent inhibition of α_1AR-mediated vasoconstriction. We examined the effect of Phe, an α_1AR agonist, on NOS activity, and found that prolonged exposure of aortic rings to Phe-enhanced Ach-mediated vasorelaxation, basal NOS activity and expression of eNOS, but not iNOS, in rat aorta.

Vascular endothelium has a regulatory effect on vasoconstriction mediated by α_1AR agonists. Removal of endothelium increases the α_1AR-mediated vasoconstriction (Angus & Cocks, 1983; Carrier & White, 1985; Martin *et al*., 1986; Cohen *et al*., 1988; Kaneko & Sunano, 1993; Amerini & Mantelli, 1995). This phenomenon can be explained, at least partly, by the release of NO from the endothelium during vasocontractile response to various agonists such as NE (noradrenaline), Phe, 5HT, etc., which in turn has an instantaneous inhibitory effect on the vasoconstriction (Angus & Cocks, 1983; Carrier & White, 1985; Martin *et al*., 1986; Cohen *et al*., 1988; Kaneko & Sunano, 1993; Amerini & Mantelli, 1995). However, the mechanism of NO release during vasocontractile response to agonists is not clear. Some studies suggest that agonist-induced vasoconstriction indirectly triggers NO release from the endothelium, without stimulating endothelial cells directly (Nakaki *et al*., 1990; Dora *et al*., 1997; Fleming *et al*., 1999; Sekiguchi *et al*., 2001;

Budel *et al*., 2001). Intracellular free Ca^{2+} is the most important determinant of vascular smooth muscle tonus. Likewise, increase in cytosolic free Ca^{2+} in endothelial cells activates NOS and causes vasodilatation. There are indications in literature that agonist-stimulated increase of cytosolic free Ca^{2+} in vascular smooth muscles can also increase Ca^{2+} in endothelial cells, by the diffusion of Ca^{2+} from smooth muscle cells into the endothelial cells (Dora *et al*., 1997; Budel *et al*., 2001), which in turn regulates the production of NO. Fleming *et al*. (1999), on the other hand, suggested that contraction-mediated mechanical stress on endothelial cells could increase Ca^{2+} in endothelial cells (Fleming *et al*., 1999). As a third alternative, agonists such as Ach can directly stimulate endothelial cells and increase intracellular Ca^{2+}, which leads to NOS activation and the release of NO. In our study, Phe did not increase intracellular calcium and did not stimulate NOS activity in isolated endothelial cells. However, Phe increased the NOS activity in the endothelium-intact aorta *via* stimulation of α_1AR. This result suggests that Phe stimulates α_1AR on vascular smooth muscle cells and has an indirect influence on endothelial cells to increase NOS activity. The possible mechanism about this indirect activation of NOS in endothelial cells might be contraction-induced mechanical stress on endothelial cells and/or the diffusion of Ca^{2+} from smooth muscle cells into the endothelial cells after Phe stimulation of vascular smooth muscle cells of aorta.

The present results showed that prolonged stimulation of α_1AR increased the expression of eNOS in endothelium-intact rat aorta. The induction of eNOS expression has been observed under shear stress, hemodynamic changes, mechanical stress on endothelial cells, stimulation with fibroblast growth factor, treatment with protein kinase C inhibitors etc. (Ohara *et al*., 1995; Ranjan *et al*., 1995; Xiao *et al*., 1997; Fleming *et al*., 1999; Fleming & Busse, 2003; Jin *et al*., 2003). Some of these factors, especially the mechanical stress on endothelial cells or Ca^{2+} diffusion to endothelial cells from smooth muscle cells produced by prolonged Phe stimulation, may have a role in the induction of eNOS expression. Further studies are necessary to clarify the underlying mechanisms for the induction of eNOS expression in the chronic stimulation of vascular smooth muscle by Phe.

Removing of the endothelium before Phe exposure greatly but not completely restored the decreased vasocontractile response to α_1AR agonist. This result indicates that there is also an endothelium-independent component in the decreased vasocontractile response to α_1AR-mediated signalling in vascular smooth muscle. It is possible to explain this component by desensitization of α_1AR-mediated signalling to produce vasocontraction in vascular smooth muscle. The reduction in α_1AR-mediated activation of G protein in vascular smooth muscle cells could be responsible for this endothelium-independent part of the reduced vasocontractile responsiveness. There is some evidence showing impairment in α_1AR-mediated activation of Gq or Gi proteins in this phenomenon (Seasholtz *et al*., 1997a, b).

Prolonged exposure of vessels to catecholamines also leads to a decreased vasocontractile response to other vasoconstrictor agonists, implying a heterologous desensitization (Hu *et al*., 1994; Seasholtz *et al*., 1997a, b). Concordant with these results, in this study and previous ones, a decreased vasocontractile response to 5HT, endotelin and angiotensin in α_1AR agonist-exposed vessels has been shown (Hu *et al*., 1994; Seasholtz *et al*., 1997a, b). Increased basal NOS activity observed in this study could also cause a decreased responsiveness to other agonists and could be an underlying mechanism for apparent heterologous desensitization, or in other words, heterologous reduced vasocontractile responsiveness.

Many studies indicated that prolonged exposure of the vessels to catecholamines upregulated endothelium-mediated inhibitory mechanism(s) on vasoconstriction. The present results show that α_1AR agonist-induced enhancement of basal NOS activity and expression of eNOS could be one of the underlying mechanisms in this inhibition. In a physiological aspect, this phenomenon could be one of the defense mechanisms against overstimulation of vascular smooth muscle to produce vasocontraction and to increase vascular tonus by catecholamines or by other vasoconstrictors. Finally, this phenomenon has two components. The major one is the endothelium-dependent functional antagonism of vasocontractile response to agonists and the other one is the desensitization of α_1AR-mediated signalling in vascular smooth muscle cells. Therefore, this phenomenon can be considered more as a functional antagonism of vasocontractile response to agonists mediated by the endothelium than a specific desensitization of α_1AR-mediated signalling in vascular smooth muscle cells.

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