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Effect of pre-exposure to vasoconstrictors on isoprenaline-induced relaxation in rat aorta: involvement of inducible nitric oxide synthase

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1 The aim of this study was to determine whether a brief (30 min) episode of contractile receptor stimulation could affect the degree of a subsequent vasorelaxation. Therefore, concentration–relaxation curves of the rat aorta to isoprenaline were compared before and after exposure of the tissue to noradrenaline (100 μM) or prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$, 100 μM).

2 Exposure to noradrenaline enhanced the second maximal relaxant effect of isoprenaline (from 20–95% relaxation). This effect was not due to significant differences in precontraction levels and was not modified by the presence of the endothelium. Treatment with $PGF_{2\alpha}$ mimicked the actions of noradrenaline on subsequent vasorelaxation to isoprenaline.

3 Before exposure to noradrenaline (100 μM), forskolin (10 μM) did not produce any significant relaxation of the rat aorta. After exposure to noradrenaline, forskolin caused a concentration-dependent relaxation with a maximal effect of more than 90% in rings with and without endothelium suggesting that the change in vasorelaxation to isoprenaline occured downstream from the β-adrenoceptor.

4 The increase in relaxation due to exposure to noradrenaline was markedly attenuated by treatment with a protein synthase inhibitor (cycloheximide), a nitric oxide (NO) synthase inhibitor (L-N^G-nitroarginine methyl ester, L-NAME) and an inhibitor of the activation of soluble guanylyl cyclase (methylene blue).

5 Western blot analysis showed an increase of inducible NO synthase (iNOS) in aortic rings exposed to noradrenaline or $PGF_{2\alpha}$.

6 Together, these findings suggest that pretreatment of rat aorta with noradrenaline or $PGF_{2\alpha}$ could induce vascular NOS which would in turn result in an increase in isoprenaline-induced vasorelaxation, this increase occuring downstream from receptor activation. Such a mechanism might participate in cardioprotection during preconditioning induced by noradrenaline.

Keywords: Pre-exposure; noradrenaline; $PGF_{2\alpha}$; isoprenaline; forskolin; relaxation; induction; nitric oxide synthase; rat aorta endothelium

Abbreviations: ECL, enhanced chemiluminescence; HRP, horseradish peroxidase; L-NAME, L-N^G-nitroarginine methyl ester; L-NIL, L-N^6-(1-iminoethyl)-lysine; LPS, lipopolysaccharide; SDS, sodium dodecyl sulphate

Introduction

Conflicting results have been reported concerning the role of the endothelium on vascular relaxation induced by β-adrenoceptor stimulation. Relaxation elicited by β-adrenoceptor agonists was found to be dependent (Gray & Marshall, 1992; Graves & Poston, 1993) or independent of the endothelium (Béa *et al*., 1994). While this controversy has not been totally resolved to date, some of these discrepancies could be explained by differences in experimental conditions especially concerning the level of precontraction. Indeed, spontaneous release of endothelium-derived nitric oxide (NO) decreases the contractile response in precontracted aortic rings (Eglème *et al*., 1984; Martin *et al*., 1986) and consequently affects the degree of relaxation. We have demonstrated that the relaxation induced by isoprenaline can be indirectly enhanced by the endothelium as a consequence of its ability to reduce the precontraction level of the aorta (Eckly *et al*., 1994).

Although these findings emphasize the importance of the precontraction level on immediate relaxation, it remains to be determined whether vasoconstrictors could also modify smooth muscle relaxation in a delayed manner. In this context, it has been demonstrated that vascular smooth muscle retained the 'memory' of contractile receptor activation, lasting over 2 h after cessation of the stimulus (Ratz, 1995). It was reported that previous α-adrenoceptor activation caused temporary modulation of KCl-induced contraction in rabbit femoral arterial muscle (Ratz, 1995).

The present study was designed to determine whether or not a brief episode (30 min) of contractile receptor stimulation could affect subsequent relaxation to isoprenaline. To this end, we have compared the isoprenaline-induced relaxation before and after exposure to noradrenaline or prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) in rat aortic rings with and without endothelium. The data show that exposure to noradrenaline or $PGF_{2\alpha}$ resulted in a marked increase in subsequent relaxation to isoprenaline. We also attempted to identify the underlying mechanism(s) involved in the delayed effect of vasoconstrictors on subsequent relaxation. The respective influences of the endothelium and of the NO/cyclic GMP pathway were assessed: (1) pharmacologically using L-NAME (a NO synthase inhibitor), methylene blue (an inhibitor of the activation of guanylyl cyclase) and cycloheximide (an inhibitor

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of protein synthesis) and (2) biochemically by Western blot analysis of the inducible NO synthase (iNOS) content in aortic rings removed at the end of contraction experiments.

Methods

Preparation of rat aortic rings

Male Wistar rats (400–450 g) were sacrificed by cervical dislocation and the thoracic aorta was quickly dissected, cleaned off fat and connective tissues and cut into rings of about 3 mm in length. Care was taken to avoid abrasion of the intimal surface of the rings to maintain the integrity of the endothelial layer. The rings were mounted under 0.5 g of resting tension in organ baths containing Krebs solution of the following composition (mM): NaCl 118; KCl 4.7; $CaCl_2$ 1.25; KH_2PO_4 1.14; $MgSO_4$ 1.1; glucose 10; $NaHCO_3$ 25, at 37°C and gassed with 95% O_2 and 5% CO_2, pH = 7.4. All experiments were carried out in the presence of 10 μM indomethacin in order to inhibit cyclo-oxygenase activity. Tissues were allowed to equilibrate for 75 min with periodic washes before starting experiments. Tension was measured with an isometric force transducer (DSC-6BE4-110, Euro-Sensor) and recorded by an home made software computer.

From some aortic rings the endothelium was removed by gently rubbing the intimal surface with fine wires. Removal of functional endothelium was checked by the lack of any relaxation to acetylcholine (1 μM) in rings pre-contracted with noradrenaline (0.1 μM). Aortic preparations with functional endothelium exhibited at least 80% relaxation under identical conditions.

Measurement of aortic relaxation

Since it is well known that the higher the level of precontraction, the lower will be the relaxing effect induced by vasodilators, aortic rings with and without endothelium were contracted to the same level with a dose of noradrenaline corresponding to the respective EC_{80} values (2.3 and 0.5 μM, respectively). When the contractile response reached a steady state, cumulative concentration-tension curves with isoprenaline (0.01–100 μM) were established. The tissues were then equilibrated for 75 min with periodic washes before exposure to noradrenaline (100 μM) or $PGF_{2\alpha}$ (100 μM). After the contractile response had stabilized for 20–30 min, the tissues were washed again for at least 90 min until the resting tension was restored. Then the second isoprenaline-induced relaxation using exactly the same protocol as for the first relaxation was performed. In control tissue, the exposure to vasoconstrictor was omitted but the same time course between the two isoprenaline-induced series of relaxations was conserved. The experimental time (from mounting aortic rings to the end of the second dose-response curve of isoprenaline) was 7–8 h.

To investigate the mechanism underlying the effect of noradrenaline on isoprenaline-mediated aortic relaxation, cycloheximide, L-NAME and methylene blue were used. L-NAME (100 μM), L-NIL (25 μM) and methylene blue (3 μM) were added 30 min before the second isoprenaline-induced relaxation. Cycloheximide (355 μM) was directly incubated in Krebs solution and was present throughout the experiment. In some experiments, forskolin was used (0.001–10 μM) instead of isoprenaline. Relaxation produced by each concentration of isoprenaline/forskolin was measured after 5 min incubation and results are expressed as percentage relaxation of tone induced by noradrenaline. The concentration of relaxant drug giving 50% of relaxation (EC_{50}) was estimated by non-linear regression of each log concentration-effect curve. Relaxant responses are expressed as percentage relaxation of tone induced by noradrenaline.

At the end of contraction-relaxation experiments, aortic rings were removed from the organ bath and subjected to quick-freezing using a clamp precooled in liquid N_2 and stored at −80°C for Western blot analysis.

Western blot analysis

Aortic rings (2 mg) were homogenized individually with a glass homogenizer at 4°C manually for 2 × 1 min in 150 μl of the following buffer: (in mM) Tris-HCl (pH 7.5) 25, NaCl 250, EDTA (pH 8.0) 5, Pefabloc® 1, aprotinin 2 mg l^{-1}, leupeptin 2 mg l^{-1}, pepstatin 2 mg l^{-1}, SDS 1% v v^{-1}. The tissue homogenate was centrifuged at 12,000 × g (2 × 10 min at 4°C) and the 12,000 × g supernatant was used for Western blot analysis. Proteins were quantified by spectrophotometric absorbance at 280 nm using BSA as standard.

Protein samples (60 μg) were heated for 5 min at 95°C in Laemmli buffer (50 mM Tris-HCl, 0.005% w v^{-1} bromophenol blue, 5% v v^{-1} glycerol, 1% v v^{-1} SDS, 2.5% w v^{-1} β-mercaptoethanol) and electrophoresed along with a positive control (C+; 1 μg LPS-stimulated macrophage lysate protein) on SDS-8% polyacrylamide gel. A human endothelial cell lysate (2 μg) which expresses endothelial eNOS was used as a negative control (C−) to ensure the specificity of the antibody towards iNOS. Proteins were electro-transferred onto nitrocellulose membrane, and processed for immunoblotting with affinity purified polyclonal anti-iNOS antibody (1/5,000 dilution). Anti-rabbit IgG HRP conjugate was used as secondary antibody (1/5,000 dilution). The immobilized antigens were detected using an ECL™ assay kit.

Data analysis

Western blot data were densitometrically analysed using a Starwise software from Imstar (Paris, France). The signal intensity is expressed in arbitrary units as mean ± s.e.mean of three experiments. Relaxation data are presented as mean ± s.e.mean of n experiments.

Statistical comparisons were performed using Student's t-test for unpaired data. Statistical significance was assumed when $^*P<0.05$; $^{**}P$ or $++$ $P<0.01$, $^{***}P<0.001$.

Drugs and reagents

Noradrenaline bitartrate (Sigma Chemical Co.) was stored as a 10 mM stock solution in buffer containing Na_2SO_3 7.9 mM and HCl 34 mM and was diluted as required. Indomethacin (Sigma Chemical Co.) was dissolved in 0.5% w v^{-1} Na_2CO_3. Methylene blue (Sigma Chemical Co.) was dissolved in water and stored frozen as a 10 mM stock solution and diluted as required. L-N^6-(1-iminoethyl)-lysine (L-NIL) was from Alexis, Isoprenaline, cycloheximide, L-N^G-nitroarginine methyl ester (L-NAME) and forskolin were from Sigma Chemical Co., and were diluted in Krebs solution. These solutions were freshly prepared before each experiment. Aprotinin, leupeptin, 4-(2-aminoethyl)-benzenesulphonyl fluoride hydrochloride (Pefabloc®) were from Boehringer Mannheim Biochemicals. Bovine serum albumin (BSA), bromophenol blue, glycerol, β-mercaptoethanol, pepstatin A were from Sigma. Ethylenediaminetetraacetic acid (EDTA), NaCl, sodium dodecyl sulphate (SDS), (hydroxymethyl)-aminomethane (Tris) were from Bioprobe® Systems. Enhanced chemiluminescence (ECL™)

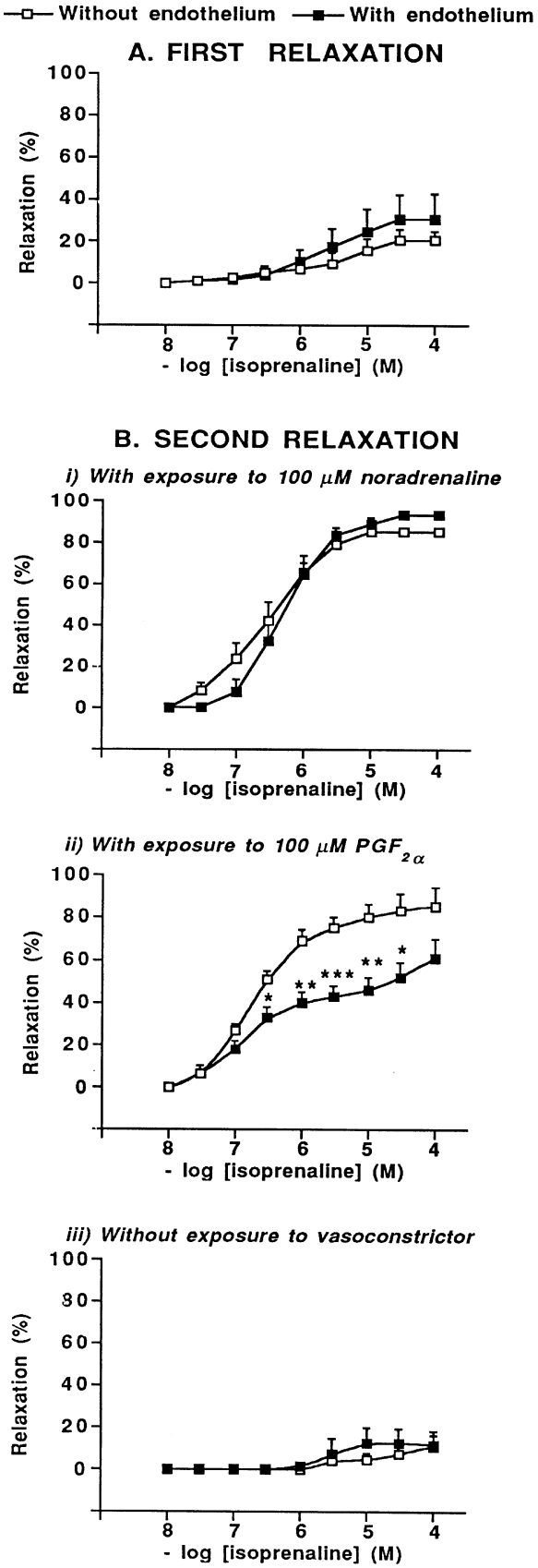

Figure 1 Effect of previous contractile stimulation on subsequent relaxation to isoprenaline-induced relaxations in rat aortic rings with or without endothelium. Relaxation before (A) and after (B) exposure to contractile agent are shown: (i) after exposure to 100 μM noradrenaline, (ii) after exposure to 100 μM $PGF_{2\alpha}$, (iii) without exposure to vasoconstrictor. For the first relaxation, the precontraction levels of rings with and without endothelium were 2.5 ± 0.2 g ($n=23$) and 2.03 ± 0.23 g ($n=24$), respectively. For the second relaxation, the precontraction levels in rings with and without endothelium were respectively: 1.8 ± 0.14 g with $P<0.05$ and 1.4 ± 0.17 g with $P<0.05$ compared to first relaxation (mean$\pm$s.e. mean of 10–15 rings) for (i); 2.5 ± 0.3 g and 2.7 ± 0.4 g with $P<0.05$ compared to first relaxation (mean$\pm$s.e. mean of 7–8 rings) for (ii); and 1.7 ± 0.3 and 1.8 ± 0.2 g, (mean$\pm$s.e. mean of 8–11 rings) for (iii).

assay kit, Hybond™ ECL™ nitrocellulose membrane were from Amersham. Precast 8% Tris-glycine gels were from Novex. Prestained SDS-polyacrylamide gel electrophoresis (PAGE) molecular weight standards were from Bio Rad. Na_2HPO_4, NaH_2PO_4 were from Merck. Affinity purified polyclonal anti-iNOS antibody, mouse macrophage lysate positive control (macrophages cells stimulated with IFN-γ and LPS for 12 h) and human endothelial cell lysate negative control from aortic endothelium cell line expressing endothelial NOS (eNOS) were from Transduction Laboratories. Anti-rabbit IgG horseradish peroxidase (HRP) conjugate was from Promega.

Results

Effects of vasoconstrictor on isoprenaline-induced relaxation

To investigate whether noradrenaline or $PGF_{2\alpha}$ could influence the subsequent relaxation to isoprenaline, we compared isoprenaline-induced relaxation before (first relaxation, Figure 1A) and after (second relaxation, Figure 1B) vasoconstrictor exposure. Exposure to noradrenaline (100 μM), significantly increased the magnitude of the second relaxation in comparison with the first with no change in the EC_{50} values. The second maximal relaxing responses obtained with 100 μM isoprenaline in rings with or without endothelium were 3.1 and 4.6 fold higher than the first, respectively (Figure 1B, panel i). Exposure to $PGF_{2\alpha}$ (100 μM) between both relaxations also significantly increased the second relaxing effect of isoprenaline in rings with and without endothelium (Figure 1B, panel ii). In the absence of exposure to vasoconstrictor, but with the same time course between the two sets of relaxations, the first and the second relaxation-response curves to isoprenaline were not significantly different and both maximal relaxant responses were reduced (Figure 1B, panel iii).

Mechanism underlying the enhancing effect of noradrenaline on isoprenaline-induced relaxation

It is known that the level of precontraction can strongly influence the extent of vasorelaxation. Thus, we compared the precontraction level between the first and the second relaxation (see legend of Figure 1). The precontraction level between the first and the second relaxation were not significantly different except for noradrenaline on rings without endothelium ($P<0.05$).

To investigate the possibility that noradrenaline exposure may desensitize β-adrenoceptors, we also studied the effect of noradrenaline (100 μM) on the relaxation induced by a direct activator of adenylyl cyclase, namely forskolin (Figure 2). Before exposure to noradrenaline, forskolin did not produce any significant relaxation at 10 μM (2 ± 1 and $8.7\pm6.6\%$, $n=5-8$, in presence or absence of endothelium, respectively). After exposure to noradrenaline, this drug caused a concentration-dependent relaxation with a maximal effect of more than 90% in rings with and without endothelium. Considering the

first dose-response curves, the relaxant ability of forskolin was not significantly influenced by the presence or the absence of a functional endothelium. Considering the second dose-response curves in absence of endothelium, the relaxation was decreased without significant change either in the EC_{50} value or in the relaxation to 100 μM forskolin. Our experiments span a long time (7–8 h). Thus, the possibility of an involvement of *de novo* protein synthesis was investigated. Cycloheximide did not significantly modify the first relaxation (data not shown). The effects of cycloheximide on the second isoprenaline-induced relaxation on rat aortic rings with and without endothelium are shown in Table 1. In presence of cycloheximide, the second isoprenaline-induced relaxation was shifted significantly to the right (EC_{50} values were 10 fold higher in treated rings in comparison with control rings), with a significant decrease in the maximal relaxing effect obtained with 100 μM isoprenaline in endothelium-denuded rings.

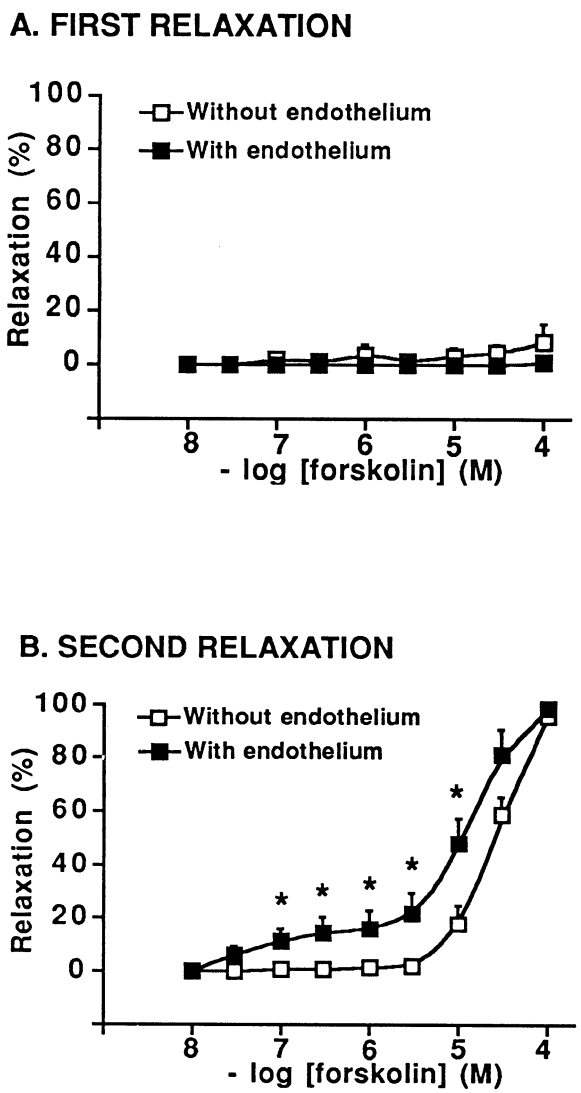

Figure 2 Forskolin-induced relaxations in rat aortic rings with or without endothelium, before (A) and after (B) exposure to 100 μM noradrenaline. Results are expressed as percentage relaxation of tone induced by noradrenaline. The precontractions were performed with noradrenaline at 2.3 and 0.5 μM in rings with and without endothelium, respectively. Results are mean ± s.e. mean of 5–8 rings. For the first relaxation, the precontraction levels of rings with and without endothelium were 1.8 ± 0.17 g (n = 5) and 1.8 ± 0.3 g (n = 8), respectively. For the second relaxation, the precontraction levels in rings with and without endothelium were respectively 1.5 ± 0.2 g and 1.7 ± 0.2 g (mean ± s.e. mean of 5–8 rings).

The cyclic GMP/NO pathway is known to mediate vascular relaxation. Therefore, the possible involvement of NO in this phenomenon was checked. Treatment with L-NAME decreased the sensitivity (EC_{50}) and caused a pronounced reduction of the maximal responses (E_{max}) elicited by isoprenaline in aorta with and without endothelium (Table 1). This inhibitory effect of L-NAME was significantly greater in rings with endothelium than without endothelium. Because we previously observed that methylene blue modified the precontraction level in rings with endothelium (unpublished data), treatment with methylene blue was only performed in rings without endothelium (Table 1). Methylene blue also caused a significant rightward shift of the second isoprenaline dose-response curve with a decrease in the EC_{50} and maximal relaxant response to 100 μM isoprenaline.

Preliminary experiments (not illustrated) using 25 μM L-NIL, a specific inhibitor of purified iNOS (Moore *et al*., 1994), have revealed that this compound increased *per se* by 0.6 g and 1.3 g precontraction level of the first relaxation in aorta without and with endothelium. This treatment overcomed the relaxant effect of isoprenaline after 100 μM noradrenaline pretreatment of aorta with endothelium (100 μM isoprenaline inducing only 25% relaxation) but did not modify the response to isoprenaline in aorta without endothelium. These non expected data could be related to differences in the precontraction level and/or to an unknown action on aortic tissue.

Western blot analysis

Western blot analysis revealed iNOS signal in all samples (Figure 3). The results of densitometric analysis as illustrated in Figure 3 show that: (1) the expression in treated aortic rings (lanes 3–6) was higher than in control aortic rings (lanes 1 and 2); (2) the expression in the presence or absence of endothelium was similar in treated rings, whereas it was twice as high in the presence of endothelium for control rings (lanes 1 and 2); (3)

Table 1 Effect of cycloheximide, L-NAME and methylene blue on EC_{50} values and E_{max} from the second isoprenaline-induced relaxation

	With endothelium		*Without endothelium*	
	EC_{50} (μM)	E_{max} (%)	EC_{50} (μM)	E_{max} (%)
Control	0.3 ± 0.02 (12)++	79.4 ± 2 (12)+	1.2 ± 0.2 (11)	98.7 ± 0.8 (11)
Cycloheximide	4.9 ± 0.9 (6)***	70.3 ± 10 (6)	9.5 ± 2.1 (8)***	72.5 ± 6 (8)**
Control	0.7 ± 0.3 (12)	79.4 ± 1.9 (12)+	1.0 ± 0.3 (11)	98 ± 0.7 (11)
L-NAME	20.7 ± 3.9 (8)***	45 ± 5.8 (8)**	4.6 ± 1.1 (8)**	79 ± 4.1 (8)***
Control			7.6 ± 2.1 (8)	77.2 ± 5.4 (8)
Methylene Blue			30.3 ± 7.4 (6)***	35.2 ± 8.4 (8)**

Results are the mean ± s.e.mean of n experiments (n indicated in brackets). E_{max} represents the maximal relaxant effect obtained with 100 μM isoprenaline. **P<0.01 and ***P<0.001 in comparison with their respective control. +P<0.05 , ++P<0.01 comparison of EC_{50} values between control rings with and without endothelium.

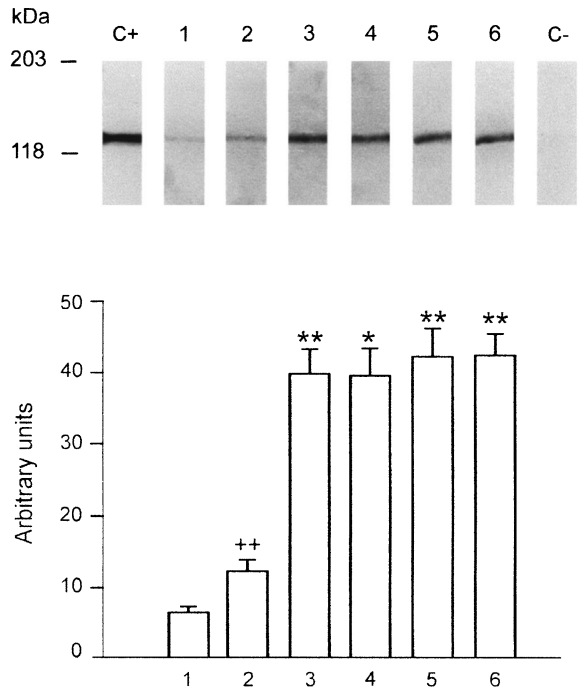

Figure 3 Western blot analysis. Protein samples (60 μg) were isolated from aortic rings after exposure to isoprenaline-induced relaxations with (lanes 1, 3 and 5) and without (lanes 2, 4, and 6) endothelium, either without exposure to vasoconstrictor (lanes 1 and 2), or with exposure to 100 μM noradrenaline (lanes 3 and 4), or with exposure to 100 μM $PGF_{2\alpha}$ (lanes 5 and 6) between the first and second relaxations. Samples were subjected to Western blot analysis using anti-iNOS antibody, along with a positive control (lane C+: 1 μg protein of stimulated-macrophage cell lysate) and a negative control (lane C−: 2 μg protein of endothelial cell lysate). The numbers on the left show the position of prestained SDS–PAGE molecular weight standards in kilo-Dalton (kDa). The iNOS signals ran as a 130 kDa protein. Western blot is representative of three experiments which were scanned and analysed by densitometry as described in Methods. The densitometric data are the mean ± s.e.mean of three experiments. $++P<0.01$: comparison with lane 1. $*P<0.05$, $**P<0.01$: comparison with the respective control in presence or absence of endothelium without exposure to vasoconstrictor.

for 100 μM noradrenaline, the expression was 6.0 and 3.2 times higher in presence and absence of endothelium respectively (lanes 3 and (4), compared to the corresponding control (lanes 1 and 2); (4) for 100 μM $PGF_{2\alpha}$, the expression was 6.4 and 3.4 times higher in presence and absence of endothelium respectively (lanes 5 and 6), compared to the corresponding control (lanes 1 and 2). The Western blot of the positive control for iNOS (lane C+) and the negative control (lane C−) clearly shows that the anti-iNOS antibody did not recognize eNOS and was specific for iNOS.

Discussion

The aim of the present study was to determine whether pre-exposure of rat aorta to vasoconstrictors could modify a subsequent vasorelaxation. The major finding is that administration of noradrenaline or $PGF_{2\alpha}$ increased the subsequent relaxant effect induced by isoprenaline or forskolin. This effect was a delayed regulation since the potentiation took place after 2–3 h and occured after complete washout of the contractile stimulus. Thus, we investigated the mechanism involved in this phenomenon.

Except for $PGF_{2\alpha}$, there was no significant difference in the precontraction level due to noadrenaline between the first and the second relaxation curves. Thus, the precontraction level could not account for the potentiating effect of exposure to vasoconstrictor on isoprenaline-induced relaxation. The enhancing effect of noradrenaline was observed in aortic rings with and without endothelium suggesting that the presence of endothelial cells is not necessary in this phenomenon. Moreover, β-adrenoceptor regulation by noradrenaline cannot explain this vasorelaxing effect because similar results were obtained for the relaxation induced by forskolin, a direct activator of adenylyl cyclase (Seamon & Daly, 1981). These results suggest that noradrenaline and $PGF_{2\alpha}$ could induce a change in the ability of the aorta to relax which would occur downstream from the receptor.

We questioned whether de novo protein synthesis could be involved in the increase in vasorelaxation. The pronounced reduction of the isoprenaline response in cycloheximide-treated aorta indicated that protein induction was involved in the phenomenon. Moreover, L-NAME and methylene blue both decreased the enhancing effect of noradrenaline on isoprenaline-induced relaxation. These results suggest a role for the inducible isoform of NOS in the potentiating effect of noradrenaline on subsequent vasorelaxation. Also, the small but not always significant decrease of the second precontraction after exposure to vasoconstrictors would be in accordance with the induction of NOS. The inhibitory effect of cycloheximide and L-NAME were observed both in the presence and in the absence of endothelium suggesting that endothelial cells are not necessary for induction of the L-Arg/NO pathway *in vitro*. However, the fact that the inhibitory effect of L-NAME is more apparent in the presence than in the absence of endothelium suggests a facilitatory role of the endothelium on the induction process. This interpretation is supported by the finding that induction of the L-Arg/NO pathway is accelerated by the presence of the endothelium (Fleming *et al*., 1993). Altogether, these results show that treatment with contractile agents could induce vascular production of NO which may be involved in the increase in subsequent relaxant response. This is in agreement with our previous results showing that an elevation of the concentration of noradrenaline significantly increased the cyclic GMP content in endothelium-denuded aortic rings (Eckly *et al*., 1994).

Induction of the L-Arg/NO pathway has been largely studied with endotoxins such as bacterial lipopolysaccharide (LPS). The present study constitutes the first demonstration of iNOS induction by vasoconstrictors. The mechanism by which these vasoconstrictors could induce iNOS is not well understood and needs further investigation. Since the vasoconstrictor was incubated for just 20–30 min, it must be asked whether this period of time is sufficient for protein synthesis induction. It has been reported that the lag time between addition of LPS and the induction process was dependent on the total time that elapsed and not on the duration of contact with LPS (Fleming *et al*., 1993). In our experiments, the time course between addition of vasoconstrictor and induction of iNOS was about 2–3 h. It is known that the time required for *de novo* biosynthesis of iNOS is approximately 2–4 h (Fleming *et al*., 1991). To ensure that in our experimental conditions the lag time is sufficient for iNOS induction, some aortic rings were removed from the organ bath at the end of the experiment and checked for iNOS. In control rings, low signal corresponding to induction of iNOS was detected. This may be explained by spontaneous induction of iNOS consequent to the long time course of the experiment (Rees *et al*., 1990). In rings exposed to noradrenaline or $PGF_{2\alpha}$, iNOS content was significantly

increased. All these results are consistent with the idea that vasoconstrictors (noradrenaline or $PGF_{2\alpha}$) are able to induce iNOS in the rat aorta in our experiments. In agreement with contraction studies, iNOS content was not significantly different in rings with and without endothelium, suggesting that induction of iNOS could occur either in the vascular media and/or in the adventitia as recently reported (Kleschyov *et al*., 1997).

Another question is how the participation of iNOS due to the treatment with noradrenaline could cause an increase in isoprenaline-induced relaxation. It is well known that induction of iNOS by increasing NO content increases cyclic GMP level. That cyclic GMP enhances cyclic AMP-mediated relaxation *via* the inhibition of the type 3 cyclic nucleotide phosphodiesterase in vascular tissue is now widely accepted (Komas *et al*., 1991; Eckly & Lugnier, 1994). Maurice & Haslam (1990) reported that an elevation of cyclic GMP by nitrovasodilatators potentiates cyclic AMP increases induced by isoprenaline in rat aortic smooth muscle. Furthermore, rolipram which is an endothelium-dependent relaxing PDE inhibitor (Komas *et al*., 1991; Eckly & Lugnier, 1994), decreases $[Ca^{2+}]_i$ in rat arterial myocytes (Eckly-Michel *et al*., 1997).

The present investigation demonstrates that induction of iNOS is involved in the potentiating effect of noradrenaline and $PGF_{2\alpha}$ on vasorelaxation to isoprenaline. Interestingly, Ratz *et al*. (1996) showed that previous α-adrenoceptor activation induced a delay in calcium mobilization and decreased the calcium sensitivity of arterial contraction. This temporary modulation of stimulus-contraction coupling has been defined by the authors as the memory of receptor activation in vascular smooth muscle. This observation associated with the decrease in $[Ca^{2+}]$ induced by rolipram (Eckly-Michel *et al*., 1997), suggest that increased production of NO may explain the memory of receptor activation. Such a mechanism might participate in the delayed cardioprotection induced by noradrenaline (Parratt & Szekeres, 1995). This possible implication is strengthened by the delayed, enhanced NO-mediated coronary vasodilation reported in preconditioning (Kim *et al*., 1997) which is triggered by NO generation (Bolli *et al*., 1997). Furthermore inducible NOS was reported as a mediator of delayed preconditioning (Imagawa *et al*., 1999). However, this conclusion does not rule out the possibility that other mechanism(s) might be involved in this phenomenon.

In conclusion, the present findings demonstrate that treatment of rat aorta with noradrenaline or $PGF_{2\alpha}$ is associated with an increase in vasorelaxation. We present here evidence showing that treatment with constrictor agents could induce vascular iNOS which may be responsible, at least partially, for the increase in the subsequent relaxant response.

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