

Nitric Oxide (NO) Primarily Accounts for Endothelium-Dependent Component of β -Adrenoceptor-Activated Smooth Muscle Relaxation of Mouse Aorta in Response to Isoprenaline

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Abstract

Isoprenaline is known to produce vascular relaxation through activation of β -adrenoceptors. In recent years, β -adrenoceptor-activated vascular relaxation has been the focus of pharmacological study in terms of both the receptor subtypes and the intracellular signaling mechanisms which trigger smooth muscle mechanical functions. In addition, the possible contribution of the endothelium to β -adrenoceptor-activated relaxation of vascular beds has provoked considerable discussion, with consensus still to be established. In the present study, we examined the effects of isoprenaline on isolated mouse aortic smooth muscles to determine whether the presence of the endothelium plays a substantial role in the relaxation it produces. A possible role for nitric oxide (NO) as a primary endothelium-derived factor released in response to isoprenaline was also elucidated pharmaco-mechanically. In isolated thoracic and abdominal aortae pre-contracted with phenylephrine (3×10^{-7} – 10^{-6} M), isoprenaline elicited relaxation in a concentration-dependent fashion (10^{-9} – 10^{-5} M). In endothelium-denuded preparations, isoprenaline-elicited relaxation was reduced to 40–50% of the response obtained in endothelium-intact preparations. In the preparations treated with *N*^G-nitro-L-arginine methyl ester (L-NAME, 3×10^{-4} M; an NO synthase inhibitor) or 1*H*[1,2,4]-oxadiazolo[4,3-*a*]-quinoxalin-1-one (ODQ, 10^{-5} M; a soluble guanylyl cyclase inhibitor), isoprenaline-elicited relaxation was attenuated almost to the same degree as the response in endothelium-denuded preparations. The degree of endothelium-dependency in isoprenaline-elicited relaxation was largely diminished when treated with propranolol (3×10^{-6} M). The present findings indicate that isoprenaline substantially relaxes the mouse aorta with both endothelium-dependent and -independent mechanisms. The endothelium-dependent component seems to correspond to about 50% of the isoprenaline-elicited relaxation, and is almost entirely due to endothelium-derived NO. Activation of propranolol (3×10^{-6} M)-inhibitible β -adrenoceptors seems to be primarily responsible for the NO-mediated endothelium-dependent pathway in isoprenaline-elicited

relaxant response of mouse aorta.

Key words: β -adrenoceptor, endothelium, isoprenaline, mouse aorta, nitric oxide (NO)

Introduction

A wide variety of chemicals elicit relaxation of vascular beds via release of relaxant substances from endothelial cells. Endothelium-derived relaxant substances include prostacyclin (PGI₂) (Moncada *et al.*, 1977), nitric oxide (NO) (Furchgott, 1984; Ignarro *et al.*, 1987; Moncada *et al.*, 1991), and non-PGI₂, non-NO substance(s) tentatively termed endothelium-derived hyperpolarizing factor(s) (EDHF(s)) (Chen *et al.*, 1988; Garland *et al.*, 1995; Mombouli and Vanhoutte, 1997; Taylor and Weston, 1988). The extent of the contribution of these endothelium-derived factors varies considerably depending on the region of the vascular bed, the species and the type of stimulant used in the study. However, the vasorelaxations induced by these endothelium-mediated mechanisms seem to be produced through corresponding specific intracellular signaling pathways, with various cytosolic and membrane components involved in the initiation of vascular relaxation. For instance, NO produced in endothelial cells activates a soluble guanylyl cyclase and elevates intracellular cyclic GMP levels. This in turn enhances cyclic GMP-dependent protein kinase (PKG) and causes phosphorylation of further downstream cellular components (Ignarro and Kadowitz, 1985; Waldman and Murad, 1987). Such physiological coupling between the endothelium and underlying vascular smooth muscle functions to modulate the mechanical activity of the vascular bed by suppressing excess excitability and contractility.

Chemical stimulants which exhibit endothelium-dependent relaxation include vascular smooth muscle constrictors as well as vasorelaxants. In the case of the noradrenaline-elicited vascular response, this catecholamine releases NO from the endothelium through activating endothelial α_2 -adrenoceptors and thus counteracts smooth muscle α -adrenoceptor-mediated contraction (Cocks and Angus, 1983; Kaneko and Sunano, 1993; Yamaki *et al.*, 2000). The endothelium may also play a substantial role in β -adrenoceptor-activated vascular relaxation, although this issue is controversial and needs to be resolved. For instance, in the rat aorta, β -adrenoceptor-activated relaxation was reported to be totally dependent on the presence of an intact endothelium (Gray and Marshall, 1992). In contrast, other investigators have shown the endothelium to be only partially responsible (Brawley *et al.*, 2000; Satake *et al.*, 1997; Trochu *et al.*, 1999, van der Zyppe *et al.*, 2000), or not to be a prerequisite (Eckly *et al.*, 1994) for the generation of β -adrenoceptor-activated relaxation in the rat aorta. Possible explanations for these inconsistent findings have been suggested such as the different pre-contraction levels between endothelium-intact and -denuded preparations used in the studies (Eckly *et al.*, 1994), the different ages of the rats used (van der Zyppe *et al.*, 2000) as well as technical problems with the incomplete removal of endothelial cells (Gray and Marshall, 1992). Furthermore, in mouse vascular beds, it is still unclear whether activation of β -adrenoceptors elicits smooth muscle contraction or relaxation (Chan and Fiscus, 2001; Russell and Watts, 2000).

The present study of the mouse aorta was thus carried out to answer the following questions: 1) Does isoprenaline elicit vascular relaxation or contraction? 2) If isoprenaline produces relaxation of the mouse aorta, does the endothelium substantially contribute to the relaxant response and to what extent? 3) Can NO account for the endothelium-dependent component of the isoprenaline-elicited vascular relaxation? 4) Does activation of β -adrenoceptors initiate the isoprenaline-elicited relaxation? This study with mouse aorta is also significant since little information is available on the normal vascular responses including β -adrenoceptor-activated relaxation, although the mouse is widely used as an experimental model for gene deletion and overexpression (Chan and Fiscus, 2001).

Methods

Male *ddY* mice (30–50 g) were housed under controlled conditions (temperature 21–22°C, relative air humidity 50 ± 5%). Food and water were available *ad libitum* to all animals. This study was conducted in accordance with the Guidelines for the Care and Use of Laboratory Animals of Toho University School of Pharmaceutical Sciences, accredited by The Ministry of Education, Culture, Sports, Science, and Technology (MEXT), Japan.

Preparation of aortic vascular beds

The mice were killed by decapitation, and the thoracic and abdominal portions of their aortae were removed and immersed in ice-cold Krebs-Hepes solution (mM: NaCl, 126.9; KCl, 5.9; Hepes, 10.03; CaCl₂, 2.36; MgCl₂, 1.18; and glucose, 11.8, pH = 7.4) bubbled with 100% O₂. The aortic segments were cleaned of loosely adhering fat and connective tissue under a dissecting microscope and cut into ring segments about 1 mm wide. In these procedures, special care was taken not to damage the intimal surface of the arteries. Endothelium-free preparations were made by gently rubbing the intimal surface with a small piece of dried grass stem.

Recording of isometric tension changes

The ring segments were mounted using stainless steel hooks (outer diameter, 100 μ m) and held at an optimal resting tension of 0.75 g in normal Tyrode's solution in a 5-ml organ bath (UC-5; UFER Medical Instrument, Kyoto, Japan). The optimal tension represented the resting tension which gave approximately 80% of the maximal contractile response to high-KCl (80 mM) (*n*=4, data not shown). The composition of normal Tyrode's solution in mM was: NaCl, 158.3; KCl, 4.0; CaCl₂, 2.0; MgCl₂, 1.05; NaH₂PO₄, 0.42; NaHCO₃, 10.0 and glucose, 5.6. Tension changes of the muscle preparation were isometrically recorded with a force-displacement transducer (T7-8-240; Orientec, Tokyo, Japan) connected to a minipolygraph (Signal Conditioner: Model MSC-2; Labo Support, Suita-City, Osaka, Japan). Ring preparations were equilibrated for 40–60 min prior to high-KCl-induced contraction.

After an equilibration period, the bathing fluid was replaced with isotonic 80 mM KCl Tyrode's solution (depolarizing solution) which was prepared by substituting NaCl with an equimolar amount of KCl; the composition of isotonic high-KCl (80 mM) Tyrode's solution in mM was: NaCl, 82.3; KCl, 80.0; CaCl₂, 2.0; MgCl₂, 1.05; NaH₂PO₄, 0.42; NaHCO₃, 10.0 and

glucose, 5.6. The artery preparations were depolarized with this solution to produce an almost maximum contraction. After this procedure, the presence or absence of endothelium was confirmed by the extent of relaxation in response to acetylcholine (ACh: 10^{-5} M) in preparations which were pre-contracted with phenylephrine or noradrenaline (3×10^{-7} or 10^{-6} M). Experiments were started after a 30-min equilibration period following the washout of phenylephrine (or noradrenaline) plus ACh. Normal and 80 mM KCl Tyrode's solutions were continuously bubbled with 95% O₂-5% CO₂, and kept at $36.5 \pm 0.5^\circ\text{C}$ (pH=7.35).

Cumulative addition of isoprenaline and (\pm)-(*E*)-4-ethyl-2-[(*E*)-hydroxyimino]-5-nitro-3-hexenamide (NOR 3, an NO donor) to the bath solution in which aortic segments were pre-contracted with phenylephrine (3×10^{-7} or 10^{-6} M) enabled the determination of the concentration-response relationship for the relaxation they produced. Inhibitors of NO synthase (*N*^G-nitro-L-arginine methyl ester, L-NAME) and soluble guanylyl cyclase (1*H*-[1,2,4]-oxadiazolo-[4,3-*a*]-quinoxalin-1-one, ODQ) were applied to the bath solution 20 min before the cumulative application of isoprenaline or NOR 3.

Drugs

The following drugs were used in the present study: (–)-isoproterenol hydrochloride (isoprenaline), phenylephrine hydrochloride, prazosin hydrochloride, papaverine hydrochloride (Sigma, St. Louis, MO, USA); *l*-noradrenaline bitartrate (Wako, Osaka, Japan); (\pm)-propranolol hydrochloride (Zeneca Chemical, Osaka-City, Japan); *N*^G-nitro-L-arginine methyl ester hydrochloride (L-NAME), (\pm)-(*E*)-4-ethyl-2-[(*E*)-hydroxyimino]-5-nitro-3-hexenamide (NOR 3) (Dojindo Laboratories, Kumamoto, Japan); 1*H*-[1,2,4]-oxadiazolo-[4,3-*a*]-quinoxalin-1-one (ODQ) (Biomol Research Laboratories, Inc., Plymouth Meeting, PA, USA). All other chemicals used in the present study were of reagent grade and commercially available. ODQ and NOR 3 were dissolved in 100% dimethyl sulfoxide (DMSO) to give stock solutions of 10^{-2} M and 10^{-3} M, respectively. All other drugs were prepared as aqueous solution and diluted with distilled water. Final DMSO concentrations in the bath medium did not exceed 0.1%. Drugs were added directly to the organ bath and expressed in molar concentration (M) in the bath medium.

Analysis and statistics

The percentage relaxation was calculated by considering the maximum tension level attained with either phenylephrine (or noradrenaline) alone or with phenylephrine (or noradrenaline) plus inhibitors of NO-cyclic GMP systems just before addition of vasorelaxants (isoprenaline or NOR 3) to be 0% relaxation, while full recovery to the basal tension before the application of phenylephrine or noradrenaline (3×10^{-7} or 10^{-6} M) was considered to be 100% relaxation. Data were plotted as a function of the drug concentrations and fitted to the equation:

$$E = E_{\max} \times A^{n_H} / (EC_{50}^{n_H} + A^{n_H}) \quad (1)$$

where *E* is the % relaxant response at a given drug concentration, *E*_{max} is the maximum relaxant response, *A* is the concentration of the drug, *n*_H is the slope function and *EC*₅₀ is the effective drug concentration that produces a 50% response. Curve fitting was performed using GraphPad Prism™ (version 2.01; GraphPad Software, San Diego, CA, USA).

Data are expressed as mean values \pm standard error of the mean (S.E.M.) and n refers to the number of experiments (preparations). The significance of the difference between mean values was evaluated with GraphPad Prism™ using paired and unpaired Student's t -test or unpaired Student's t -test with Welch's correction if necessary. P values of less than 0.05 were considered to be statistically significant.

Results

Effects of endothelium on isoprenaline-elicited relaxation in mouse aortae

In endothelium-intact thoracic aorta, isoprenaline elicited relaxation in a concentration-dependent fashion at the concentration range from 10^{-9} to 3×10^{-6} – 10^{-5} M (Fig. 1A, open circles). In endothelium-denuded preparations, isoprenaline also produced a concentration-dependent relaxation at concentrations until 3×10^{-6} – 10^{-5} M (Fig. 1A, filled circles). However, in this case, relaxant effect of isoprenaline was strongly diminished when compared to its effect on endothelium-intact preparations. For instance, the relaxation induced by 10^{-5} M isoprenaline was $68.3 \pm 5.5\%$ in endothelium-intact preparations *vs.* $29.3 \pm 3.4\%$ in endothelium-denuded preparations ($P < 0.01$, $n = 6$ for each). Thus, in endothelium-denuded preparations, the isoprenaline-elicited relaxation was reduced to $\approx 40\%$ (42.9%) of the response obtained with endothelium-intact preparations, and about 60% of the response was estimated to be mediated via an endothelium-dependent mechanism. In endothelium-denuded preparations, the pre-contraction level obtained with phenylephrine (3×10^{-7} M) was higher ($P < 0.075$) than that in endothelium-intact preparations, and ACh (10^{-5} M)-induced relaxation was almost abolished (Fig. 1B). At concentrations over 10^{-5} – 3×10^{-5} M, isoprenaline induced a contraction instead of a relaxation in both endothelium-intact and -denuded preparations (Fig. 1A). Isoprenaline-elicited tension development was also observed in both endothelium-intact and -denuded preparations that had not been pre-contracted with phenylephrine (data not shown). In abdominal aortic segments, almost similar findings were obtained with isoprenaline (Fig. 1C, D). The phenylephrine (3×10^{-7} M)-induced tension development remaining in the presence of isoprenaline was abolished by prazosin (3×10^{-6} M) or papaverine (10^{-4} M) (data not shown). In subsequent studies, the endothelium-dependent component of the isoprenaline-elicited relaxant response was pharmacologically analyzed in segments of the thoracic aorta.

Effects of L-NAME and ODQ on isoprenaline-elicited relaxation in endothelium-intact thoracic aorta

In this series of experiments, the possible involvement of the NO-cyclic GMP system was pharmacologically investigated in the endothelium-dependent component of the isoprenaline-elicited relaxation. For this purpose, the effects of an NO synthase inhibitor (L-NAME) and a soluble guanylyl cyclase inhibitor (ODQ) on isoprenaline-elicited relaxation were examined in endothelium-intact preparations. As shown in Fig. 2, the isoprenaline-elicited relaxation was strongly diminished by treatment with L-NAME (3×10^{-4} M) (Fig. 2A) and ODQ (10^{-5} M) (Fig. 2C). When L-NAME (3×10^{-4} M) and ODQ (10^{-5} M) were added after establishment of the isoprenaline (10^{-5} M)-elicited relaxation, they also counteracted the relaxation (data not shown). Inhibited relaxant responses to isoprenaline in the presence of L-NAME and ODQ were shown

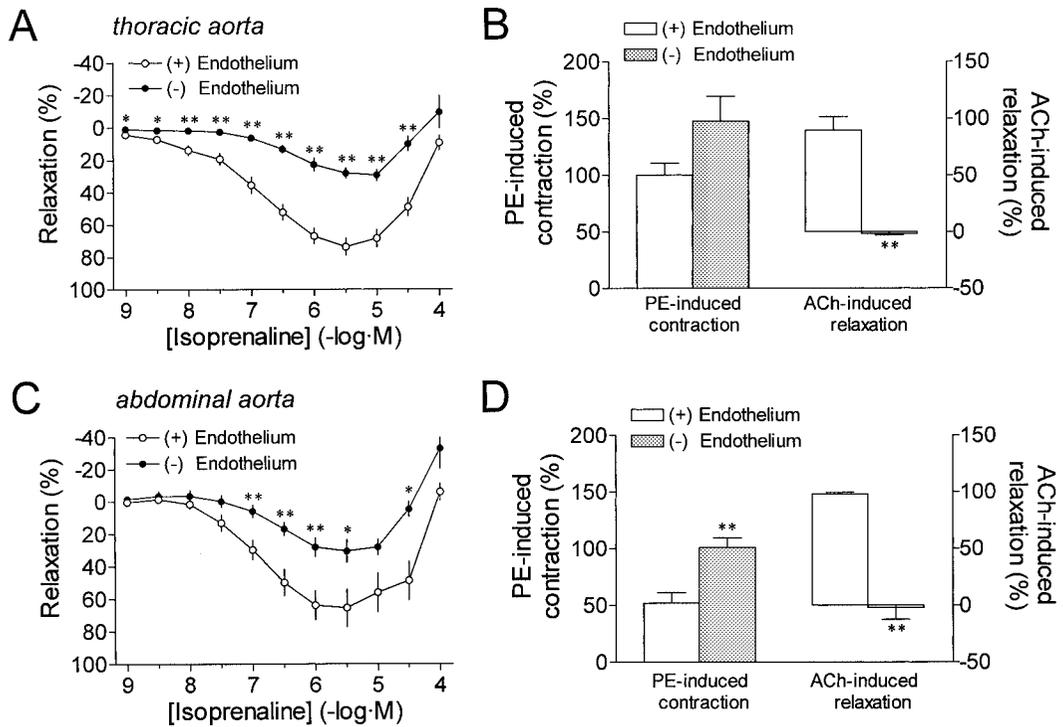


Fig. 1. Isoprenaline relaxation of mouse aortic segments via both endothelium-dependent and -independent pathways. A, C: Concentration-response relationships for isoprenaline-elicited tension changes of mouse thoracic (A) or abdominal (C) aortic segments with and without endothelium. Segments were pre-contracted with phenylephrine (3×10^{-7} or 10^{-6} M). When this pre-contraction reached a steady-state level, isoprenaline was cumulatively applied to the bath medium. Vascular relaxation is expressed as a percentage of the pre-contraction level immediately before isoprenaline application. Data are expressed as mean \pm S.E.M. ($n=5$ or 6); significant differences between the two groups at each concentration are denoted by * ($P<0.05$) or ** ($P<0.01$). B, D: Phenylephrine (PE)-induced pre-contraction and acetylcholine (ACh)-induced relaxation in endothelium-intact and -denuded preparations of mouse thoracic (B) or abdominal (D) aorta. Phenylephrine (3×10^{-7} or 10^{-6} M)-induced pre-contraction is normalized with respect to the high-KCl (80 mM)-induced contraction obtained at the beginning of experiments. High-KCl contractions were: thoracic aorta, 0.42 ± 0.03 g (with endothelium) and 0.25 ± 0.04 g (without endothelium) (both $n=6$); abdominal aorta, 0.50 ± 0.06 g ($n=5$, with endothelium) and 0.45 ± 0.06 g ($n=6$, without endothelium). ACh-induced relaxation is expressed as a percentage of the pre-contraction level with either phenylephrine (3×10^{-7} M) or noradrenaline (3×10^{-7} M) immediately before application of 10^{-5} M ACh. Data are expressed as mean \pm S.E.M. ($n=5$ or 6); significant differences between the two groups are denoted by ** ($P<0.01$).

together with the response obtained in endothelium-denuded preparation (Fig. 2E). The pre-contraction level obtained with phenylephrine (3×10^{-7} M) was substantially enhanced with L-NAME (3×10^{-4} M) (Fig. 2B) or with ODQ (10^{-5} M) (Fig. 2D) as observed in endothelium-denuded preparations (Fig. 1B, D).

Effects of L-NAME on the relaxation elicited by an NO donor (NOR 3)

The effects of L-NAME on the relaxation produced by an endothelium-independent smooth muscle relaxant (NOR 3) were examined to determine whether the pre-contraction level affects

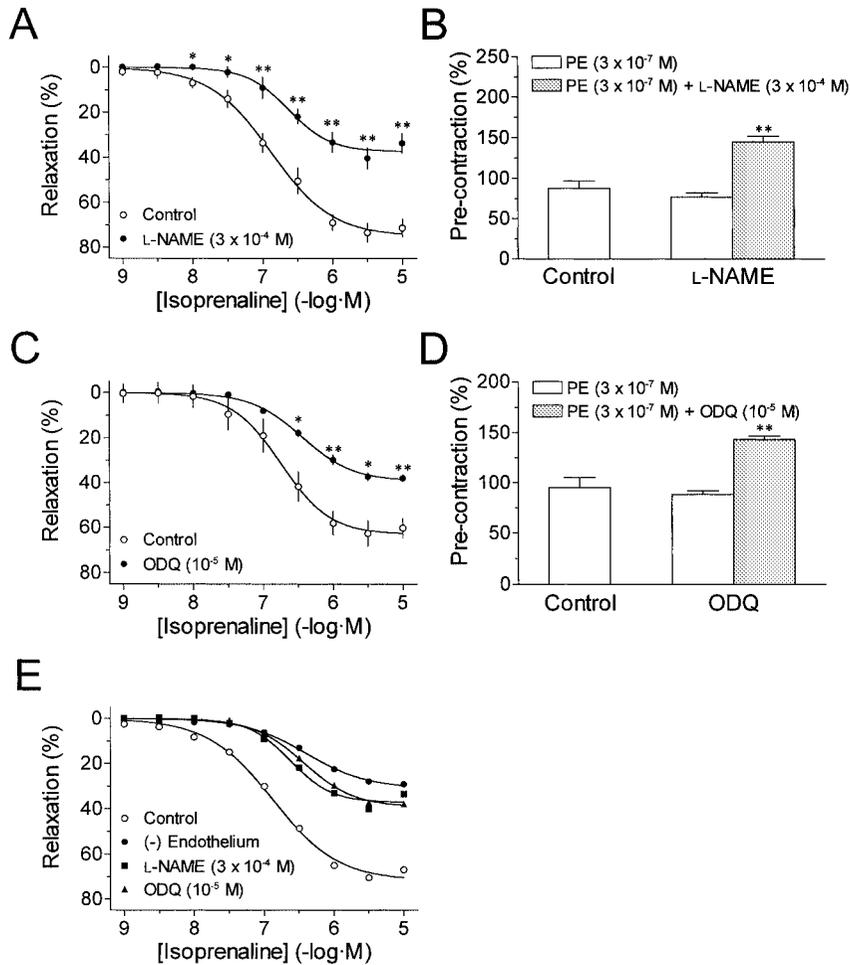


Fig. 2. Evidence for the predominant role of endothelium-derived NO in the endothelium-dependent component of the isoprenaline-elicited relaxation in the mouse thoracic aorta. A, C: Effects of inhibitors of NO-cyclic GMP systems on the concentration-response relationship for isoprenaline-elicited relaxation in endothelium-intact mouse thoracic aorta. Segments of thoracic aorta with endothelium were pre-contracted with phenylephrine (3×10^{-7} M). Subsequently, L-NAME (3×10^{-4} M) (A) or ODQ (10^{-5} M) (C) was applied to the bath medium. When the muscle tension elevated with phenylephrine alone, or phenylephrine plus L-NAME or ODQ reached a steady-state level, isoprenaline was cumulatively applied at concentrations of up to 10^{-5} M. Vascular relaxation is expressed as a percentage of the maximum contraction with phenylephrine alone or with phenylephrine plus L-NAME or ODQ. Data are expressed as mean \pm S.E.M. ($n=4$); significant differences between the two groups denoted as * ($P<0.05$) or ** ($P<0.01$). B, D: Effects of L-NAME (B) or ODQ (D) on pre-contraction before the cumulative application of isoprenaline. The pre-contraction elicited with phenylephrine (PE, 3×10^{-7} M) alone, or with PE plus L-NAME (3×10^{-4} M) (B) or ODQ (10^{-5} M) (D) is normalized with respect to the high-KCl (80 mM)-induced muscle tension obtained at the beginning of experiments. Data are expressed as mean \pm S.E.M. ($n=4$); significant differences denoted as ** ($P<0.01$). E: Summarized results showing the effects of endothelial removal and treatment with NO-cyclic GMP system inhibitors. Control response (endothelium-intact) represents the pooled data used in Fig. 1A and Fig. 2A, C. Note that the concentration-response relationships for isoprenaline-elicited relaxations in each case (endothelium removed, or with either L-NAME or ODQ) are almost identical. Data points are average responses.

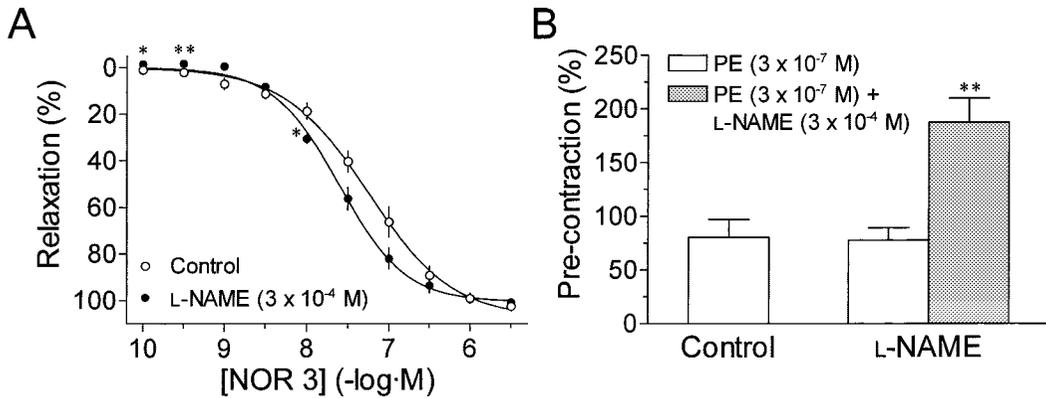


Fig. 3. The lack of effect of L-NAME on the endothelium-independent relaxation elicited by an NO donor. A: Concentration-response relationships showing the effect of L-NAME on the NOR 3-elicited relaxation of endothelium-intact mouse thoracic aorta. These segments were pre-contracted with phenylephrine (3×10^{-7} M). Subsequently, L-NAME (3×10^{-4} M) was applied to the bath medium. When the muscle tension elevated with phenylephrine or phenylephrine plus L-NAME reached a steady-state level, NOR 3 was cumulatively applied to the bath medium. Vascular relaxation is expressed as a percentage of the pre-contraction with phenylephrine or phenylephrine plus L-NAME. Data are expressed as mean \pm S.E.M. ($n=4$); significant differences between the two groups denoted as * ($P<0.05$) or ** ($P<0.01$). B: Effects of L-NAME on the pre-contraction before cumulative application of NOR 3. Pre-contraction elicited with phenylephrine (PE, 3×10^{-7} M) alone, or PE plus L-NAME (3×10^{-4} M) is normalized with respect to the high-KCl (80 mM)-induced muscle tension obtained at the beginning of experiments. Data are expressed as mean \pm S.E.M. ($n=4$); significant differences denoted as ** ($P<0.01$).

vascular smooth muscle NO-cyclic GMP systems (Fig. 3A). The presence of L-NAME (3×10^{-4} M), which also again elevated the pre-contraction level obtained with phenylephrine (3×10^{-7} M) (Fig. 3B), did not diminish NOR 3-elicited relaxation of mouse aorta.

Effects of propranolol (3×10^{-6} M) on isoprenaline-elicited relaxation

To determine whether β -adrenoceptors mediate the endothelium-dependent component of the isoprenaline-elicited relaxation, the effects of propranolol, a non-selective β -adrenoceptor antagonist, were examined on the relaxant response to isoprenaline (Fig. 4). In the presence of 3×10^{-6} M propranolol, isoprenaline-elicited relaxation was largely diminished in both endothelium-intact and -denuded preparations (Fig. 4, solid lines *vs.* dotted lines). Furthermore, in the presence of propranolol (3×10^{-6} M), isoprenaline elicited vascular relaxation to a similar extent in both endothelium-intact and -denuded preparations although the relaxation in endothelium-intact preparations was greater than that in endothelium-denuded preparations at higher concentrations (Fig. 4, open circles *vs.* filled circles).

Discussion

The present findings show that in thoracic and abdominal aortae of the mouse, 1) isoprenaline is able to produce substantial smooth muscle relaxation through both endothelium-dependent and -independent pathways, and 2) NO almost entirely accounts for the endothelium-

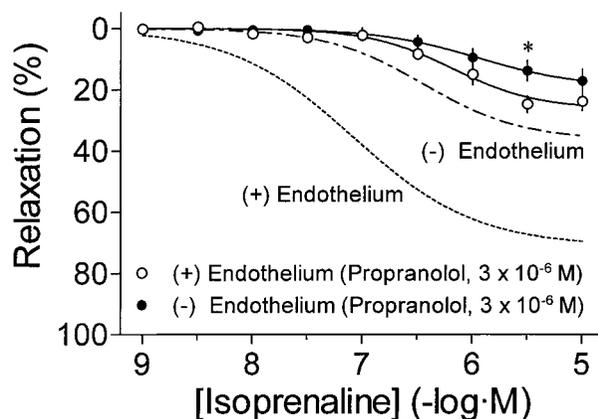


Fig. 4. Effect of propranolol on the endothelium-dependent component of the isoprenaline-elicited aortic relaxation. Segments of thoracic aorta with or without endothelium were pre-contracted with phenylephrine (3×10^{-7} M) in the presence of propranolol (3×10^{-6} M). When the pre-contraction reached a steady-state level, isoprenaline was applied cumulatively at concentrations of up to 10^{-5} M. Vascular relaxation is expressed as a percentage of the maximum pre-contraction. For comparison, the concentration-response relationships with and without endothelium obtained in the absence of propranolol (shown in Fig. 1A) are also shown as dotted lines. Data are expressed as mean \pm S.E.M. ($n=4$); significant difference between the two groups denoted as * ($P<0.05$).

dependent component of the isoprenaline-elicited relaxation.

The β -adrenoceptor-activated vasorelaxant response has been widely studied in a variety of vascular tissues isolated from different animal species. In particular, pharmacological characterization of the β -adrenoceptor subtypes and its signal transduction mechanisms which trigger vascular relaxation are currently the focus of many investigators. In those studies, the rat thoracic aorta is widely used for pharmacological analysis. However, little information is available on the pharmacological characteristics of β -adrenoceptor-activated relaxation in the mouse aorta, even though this animal species is generally used for establishing knockout and transgenic animals (Chan and Fiscus, 2001). Furthermore, it still remains to be determined whether stimulation of the mouse aorta with isoprenaline produces muscle relaxation or contraction (Chan and Fiscus, 2001; Russell and Watts, 2000). This issue needs to be resolved to delineate the physiological significance of β -adrenoceptors with regards to the mechanical function of vascular smooth muscle in this species.

The present study clearly shows that aortic segments (both thoracic and abdominal) isolated from *ddY* mice are able to relax in response to isoprenaline with its concentration ranges below 3×10^{-6} – 10^{-5} M. The relaxation elicited by isoprenaline is mediated through activation of β -adrenoceptors since this aortic response was diminished to a large extent in the presence of propranolol (3×10^{-6} M), a non-selective β -adrenoceptor antagonist. In contrast, at concentrations over 10^{-5} M, isoprenaline produced vascular contraction instead of relaxation. However, when the concentration of phenylephrine for eliciting pre-contraction level was increased to 10^{-5} M, isoprenaline elicited only relaxation even at concentrations over 10^{-5} M

(results not shown). Isoprenaline seems to produce contraction by activation of smooth muscle α (α_1)-adrenoceptors since high concentrations of isoprenaline should not activate α -adrenoceptors when they are fully activated with the high concentration of phenylephrine. In any case, these findings strongly indicate that functional β -adrenoceptors are expressed in the mouse aorta, and their activation with isoprenaline leads to smooth muscle relaxation.

Isoprenaline-elicited relaxation of the mouse aorta occurred in both endothelium-dependent and -independent pathways. The degree of endothelial contribution to isoprenaline-elicited relaxation can be estimated to be $\approx 50\%$, and this endothelium-dependent relaxant component seems to be almost entirely attributable to endothelium-derived NO. These conclusions are based on the following observations: 1) Isoprenaline-elicited relaxation was attenuated by $\approx 50\%$ in endothelium-denuded preparations as compared to the response in endothelium-intact preparations. This finding indicates that the endothelial contribution to isoprenaline-induced relaxation is substantial in the mouse aorta though the role of the endothelium in its relaxant effect is not exclusively obligatory as in the case of ACh-elicited relaxation. 2) An NO synthase inhibitor, L-NAME, and a soluble guanylyl cyclase inhibitor, ODQ, both reduced isoprenaline-elicited relaxation almost to the same degree as the response in endothelium-denuded preparations. Non-NO type factors including EDHF(s) might be released from the endothelium due to stimulation with isoprenaline, but its functional participation in the muscle relaxant response does not seem to be apparently substantial.

The pre-contraction level of these vascular preparations may be a factor which influences the muscle relaxant potency of isoprenaline as the relaxation elicited by isoprenaline has been shown to decrease with increasing pre-contraction level and to increase with lower pre-contraction level (Eckly *et al.*, 1994). Actually, treatment with L-NAME and ODQ produces substantial muscle contraction, and thus the tension level before the application of isoprenaline is elevated in smooth muscle treated with these NO-cyclic GMP system inhibitors. Accordingly, inhibition of isoprenaline-elicited relaxation in the presence of L-NAME and ODQ might be ascribed only to the enhanced pre-contraction level due to L-NAME and ODQ, but not through inhibition of NO-cyclic GMP system. However, the presence of L-NAME (3×10^{-4} M) did not inhibit the vasorelaxant potency of an NO releaser and cyclic GMP elevating agent, NOR 3 (Fig. 3). This finding rules out the possibility that elevation of pre-contraction level suppresses vascular smooth muscle NO-cyclic GMP systems and supports the view that the treatment with L-NAME suppresses isoprenaline-elicited relaxation through inhibiting endothelial NO synthesis.

The degree of endothelial-dependency in isoprenaline-elicited relaxation was largely reduced in the presence of propranolol (3×10^{-6} M), and the concentration-response relationships for the relaxation was not substantially different between either endothelium-intact or -denuded preparations (Fig. 4). Similarly to our present findings with the mouse aorta, Brawley *et al.* (2000) found that in the rat thoracic aorta, propranolol has little or no additional effect on the concentration-response relationships for isoprenaline-elicited relaxation after either endothelium removal or L-NAME and ODQ treatment. These findings with both mouse and rat aortae indicate that the endothelium-dependent component of the isoprenaline-elicited relaxation is mainly through the activation of propranolol (3×10^{-6} M)-inhibitable β -

adrenoceptors. However, there are two possible explanations for these findings (Brawley *et al.*, 2000).

One explanation is that in aortic endothelial cells, propranolol-inhibitable β -adrenoceptors are dominantly expressed, and their activation with isoprenaline enhances the synthesis and/or release of NO from endothelial cells, which subsequently produces vascular smooth muscle relaxation in a cyclic GMP-dependent manner (Gray and Marshall, 1992). An alternative explanation is that basal release of NO from endothelial cells potentiates β -adrenoceptor-activated relaxation of vascular smooth muscle. This NO elevates smooth muscle cyclic GMP levels which in turn inhibits activity of phosphodiesterase (cyclic AMP-specific, phosphodiesterase type 3), and thus subsequently potentiates β -adrenoceptor-activated relaxation with an elevation of cyclic AMP levels (Delpy *et al.*, 1996). At present, it is difficult to identify which mechanism is exclusive or superior for isoprenaline-elicited relaxation in the mouse aorta, and these putative mechanisms might be both physiologically significant (Brawley *et al.*, 2000).

The present findings confirm that β -adrenoceptor-activated vascular relaxation occurs in mouse vascular tissue. Further research is needed to elucidate the following questions: 1) Do vascular endothelial cells express β -adrenoceptors that are functionally coupled with the NO synthesis pathway? As mentioned above, the question as to whether β -agonists activate endothelial β -adrenoceptors and enhance the production of NO should be determined. In association with this issue, endothelial cells of rat thoracic aorta are reported to express a third type of β -adrenoceptor (β_3 -adrenoceptor) which is coupled to an NO synthase pathway (Trochu *et al.*, 1999). Although the information obtained with pharmaco-mechanical studies by use of β -adrenoceptor subtype-preferential antagonists and agonists would be useful to some extents, biochemical studies to identify the possible expression of endothelial β -adrenoceptors are a prerequisite. If so, the functional coupling of β -adrenoceptors to NO production needs to be elucidated. 2) What receptor subtype predominates in aortic vascular smooth muscle? It seems certain that mouse aortic vascular smooth muscle expresses β -adrenoceptors since endothelium-removal or treatment with either L-NAME or ODQ did not completely diminish the isoprenaline-elicited relaxation which was strongly inhibited by propranolol (Fig. 1A,C, Fig. 2A,C, Fig. 4). However, propranolol exhibits a non-selective characteristic against β_1 - and β_2 -adrenoceptors. Furthermore, the concentration of propranolol used in the present study (3×10^{-6} M) might interact with atypical adrenoceptors such as β_3 -adrenoceptors rather than β_1 - or β_2 -adrenoceptors. To be able to conclude definitely on this question, biochemical studies need to be made in conjunction with pharmaco-mechanical investigation using subtype-selective agonists and antagonists. 3) In addition to these β -adrenoceptor subtype studies, subtype-specific intracellular signaling pathways functionally coupled with smooth muscle relaxation should also be considered. In any case, although further studies are necessary to answer these questions, the present findings should assist future investigations.

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