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β-Adrenoceptor activates endothelium-dependent release of nitric oxide in rat aorta[1]

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AIM: To examine the possible role of agents elevating cAMP to release NO from aortic endothelial cells. **METHODS**: N^G-nitro-*L*-arginine methylester (*L*-NAME), an inhibitor of NO synthase, partially inhibited endothelium-dependent relaxation evoked in phenylephrine-precontracted rings by isoproterenol and abolished relaxation mediated by forskolin 0.2 μmol L^{-1}. **RESULTS**: In rings without endothelium, isoproterenol and forskolin were less effective relaxants and *L*-NAME had no effect on the responses. In methylene blue-treated rings isoproterenol- and forskolin-induced relaxation were prevented in both endothelium-intact and -denuded rings, but the inhibitory effects of methylene blue were significantly more in rings with endothelium than in those without. On the other hand, relaxation induced by sodium nitroprusside was not inhibited by *L*-NAME, but was inhibited by methylene blue in both the endothelium-intact and -denuded rings. The concentration-relaxation curves to sodium nitroprusside after methylene blue were identical for rings with and without endothelium. **CONCLUSION**: β-Adrenoceptors or any agent which raises cAMP elevate NO release from endothelial cells.

KEY WORDS cyclic AMP; methylene blue; forskolin; endothelium-derived relaxing factor; vascular endothelium; smooth muscle contraction

The role of release of nitric oxide (NO), which is formed from *L*-arginine in endothelium, and its role in the control of blood vessel relaxation has become a major focus of scientific study for more than a decade since the discovery of such a factor[1]. A recent review[2] contains over 450 references. While the direct measurement of NO formation is technically difficult, the easy availability of stereoselective inhibitor of NO synthase, such as *L*-NAME[3], and methylene blue (MB) which inhibits the target enzyme of NO, guanylate cyclase[4], have been proven useful in the study of events related to the physiological effects of NO. In vascular endothelial cells, the NO generation from the constitutive NO synthase is Ca^{2+}-dependent[5]. Elevation of cytosolic Ca^{2+} resulting from the inhibition of endoplasmic reticulum Ca^{2+}-ATPase pump also elicited biphasic change of contraction in phenylephrine-precontracted rat aorta with intact endothelium[6]. We observed an initial rapid vascular relaxation due to endothelium-derived NO formation resulting from elevation of cytosolic Ca^{2+} in endothelial cells and a subsequent contraction due to elevation of cytosolic Ca^{2+} in smooth muscle cells. β-Adrenoceptors were observed in both vascular smooth muscle cells and endothelial cells based on radioligand binding studies[7,8]. Although it is

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widely known that activation of β-adrenoceptors of vascular smooth muscle leads to relaxation, study on the functional role of β-adrenoceptors of the vascular endothelial cells is relatively meagre. In cultured bovine aortic endothelial cells, isoproterenol (Iso) and forskolin (For) augmented $[Ca^{2+}]_i$ increase caused by thromin, bradykinin or ATP[9]. The first provision of functional evidence that β-adrenoceptor may be involved in modulating the endothelial production of NO has recently been reported[10]. The objective of this study was to determine whether activation of vascular endothelial cells by β-adrenoceptor agonists mediated NO release and modulated vascular contraction in rat aorta.

MATERIALS AND METHODS

Male adult Wistar rats were killed by stunning and decapitation. The thoracic aortas were excised and placed in Krebs' solution at pH 7.4 containing: NaCl 119, KCl 5, $CaCl_2$ 2.5, $MgCl_2$ 2, $NaHCO_3$ 25, NaH_2PO_4 1, and glucose 11 mmol L^{-1}. Indomethacin 10 μmol L^{-1} was present to preclude a contribution from PGI_2. The tissues were cleared of adhering fat and connective tissue and cut into 3 − 4 mm wide rings. The endothelium was either left intact or removed by gently rubbing against the teeth of pair of forceps. These rings were mounted under 2 g resting tension in 3 mL organ-bath chambers, connected to a force transducer (Grass FTO3) and a pen recorder. The organ-bath chambers containing Krebs' solution were gassed continuously with 95 % O_2+5 % CO_2 at 37 ℃. Tissues were allowed to equilibrate for at least 1.5 h. Stimulation of rings with KCl 60 mmol L^{-1} was repeated every 30 min for 3 times until responses were stable. The integrity of endothelium was assessed by testing whether acetylocholine (ACh, 0.1 μmol L^{-1}) induced relaxation in the rings precontracted by phenylephrine (PE, 3 μmol L^{-1}). Contractions were expressed as % of the stable level of contraction to KCl 60 mmol L^{-1}. The failure of relaxation to ACh in some precontracted rings was taken as evidence for endothelial removal[11].

The experiments were carried out on sets of 2 matched rings containing and lacking endothelium from the same aorta. For relaxation studies, precontracted rings were first induced by 3 μmol L^{-1}. Control concentration-response curves and those obtained in the presence of inhibitory agents, such as MB and *L*-NAME, were determined on the same aortic rings. In most studies, responses of experimental groups were accompanied by test groups in which relaxing agents were applied from time to time during the experiments to control for any effects of time alone. Such changes were minimal.

The % relaxation data were expressed as $\bar{x} \pm s$ and compared using *t* test.

RESULTS

Effects of MB on relaxation induced by sodium nitroprusside (SNP), Iso and For SNP, Iso and For induced endothelium-independent relaxation. MB 3 μmol L^{-1} slightly increased ($P > 0.05$) basal tension in both endothelium-intact (23 ± 20 %) and -denuded (15 ± 18 %) aortic rings. However, MB slightly increased ($P < 0.05$) the contraction to PE 3 μmol L^{-1} when endothelium was present (from 92 ± 9 to 119 ± 18 %) and when endothelium was absent (from 92 ± 15 to 107 ± 6 %) (Fig 1).

Pretreatment of endothelium-containing rings with MB 3 μmol L^{-1} for 10 min inhibited ($P < 0.05$) the relaxation induced by SNP (0.5 nmol L^{-1} − 1 μmol L^{-1}) (Fig 1A). This inhibitory action of MB was less than that produced by MB against ACh-induced relaxation[6]. On the other hand, the responses to SNP in rings without endothelium were similar to those in rings with endothelium.

Iso induced relaxations in both intact rings and those lacking endothelium and these were inhibited by MB 3 μmol L^{-1}. The inhibitory effect of MB was much more in endothelium-containing (Fig 1B) than in denuded rings (Fig 1C). The maximal relaxation was attenuated by MB and this inhibition by

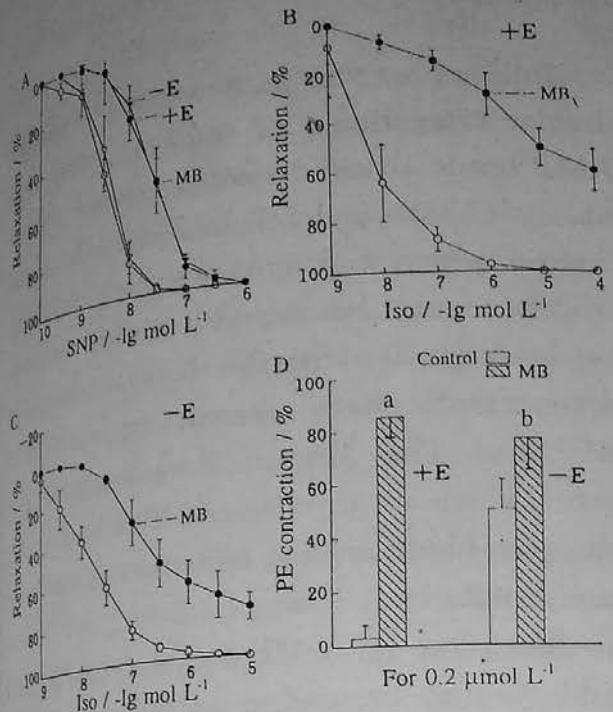

Fig 1. Effects of methylene blue (MB, 3 μmol L^{-1}) on (A) sodium nitroprusside (SNP)-, (B) and (C) isoproterenol (Iso)- and (D) forskolin (For)-induced relaxations in endothelium-intact (+E) and -denuded (−E) rings. (A)−(C), MB-treated rings are indicated as filled symbols and control rings are indicated as open symbols. The relaxation induced with SNP and Iso is expressed as % relaxation of PE (3 μmol L^{-1})-induced contraction. In (D), data are expressed as the % contraction to PE 3 μmol L^{-1}. $^aP<0.01$, $^bP<0.05$. Each point is the mean for 5 and 6 (SNP, +E and −E), 4 and 6 (Iso, +E and −E), 6 (For +E and −E) pairs of rings.

MB was not overcome by higher concentrations of Iso (Fig 1B).

Similarly, MB 3 μmol L^{-1} nearly abolished relaxation to For 0.2 μmol L^{-1} in rings with endothelium and reduced partially that to For in rings without endothelium when they were precontracted by PE 3 μmol L^{-1} (Fig 1D). In the control experiments, the maximal relaxation induced with For 0.2 μmol L^{-1} was larger in endothelium-containing than that in denuded rings. The maximal relaxations expressed as % of PE-induced contraction were 97±5 % and 50±19 %, respectively (Fig 1D). *L*-NAME 100 μmol L^{-1} had no significant effects on basal tone of endothelium-intact or -denuded rings, but did slightly increase the response to PE 3 μmol L^{-1} when endothelium was present (from 92±11 to 118 ±18 %).

Effects of *L*-NAME on relaxation induced by SNP, Iso and For We have prevously demonstrated[6] that *L*-NAME (at 100 μmol L^{-1} for 20 min) abolished endothelium-dependent relaxation induced by ACh. This inhibition of *L*-NAME appeared to be irreversible at least 80 min following extensive washout. *L*-arginine, on the other hand, restored ACh-induced relaxation in *L*-NAME-treated rings following extensive washout for 30 min, but *D*-arginine had no such reversing effect (Tab 1).

Tab 1. Effects of *L*- and *D*-arginine (100 μmol L^{-1}) on endothelium-dependent relaxation induced by ACh, Iso and For in *L*-NAME (100 μmol L^{-1})-treated rings precontracted by phenylephrine 3 μmol L^{-1} following extensive washout for 30 min (number of rats). $^aP>0.05$, $^bP<0.05$, $^cP<0.01$ *vs* *L*-NAME.

	Relaxation/%		
	ACh	Iso	For
	0.1 μmol L^{-1}	0.1 μmol L^{-1}	0.1 μmol L^{-1}
L-NAME	1±8 (6)	37±15 (6)	35±13 (6)
L-Arginine	63±12^c(6)	84±6^b	100±0^c(4)
D-Arginine	5±1^a(3)	38±11^a	68±18^a(3)

In aortic rings precontracted with PE 3 μmol L^{-1}, *L*-NAME did not inhibit significantly relaxation induced by SNP in rings with intact endothelium (Fig 2). In contrast, *L*-NAME obviously decreased relaxing responses to Iso (Fig 2), and completely abolished those to For 0.2 μmol L^{-1} (Fig 3) in tissues with endothelium, but did not attenuate those to SNP, Iso, and For in rubbed rings (Fig 2, 3).

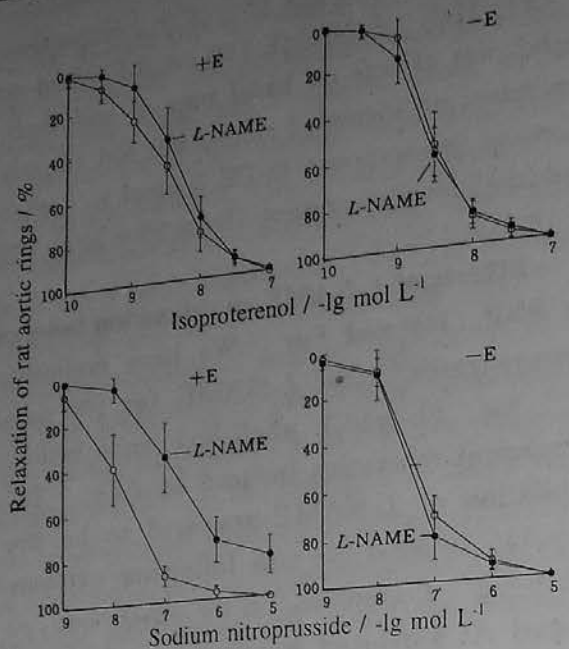

Fig 2. Effects of *L*-NAME 100 μmol L^{-1} on SNP- or Iso-induced relaxation in the presence or absence of endothelium. Data are expressed as % relaxation of PE 3 μmol L^{-1} contraction in 6 pairs of aortic rings.

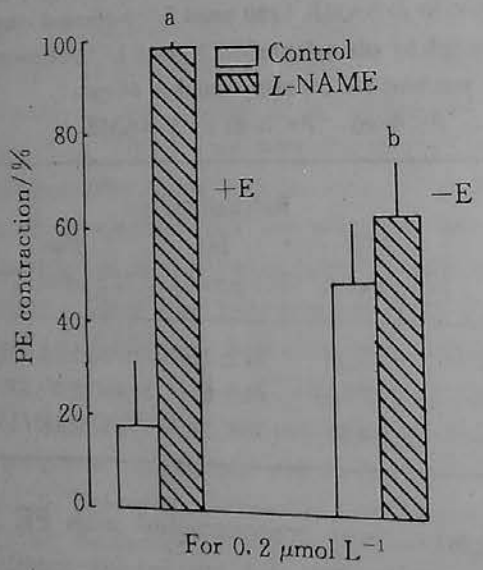

Fig 3. Effects of *L*-NAME 100 μmol L^{-1} on the relaxation induced by For 0.2 μmol L^{-1} of rings with intact or denuded endothelium in 6 pairs of aortic rings. Data are expressed as % contraction to PE 3 μmol L^{-1}. $^{a}P<0.01$, $^{b}P>0.05$ compared to the control.

L-Arginine (100 μmol L^{-1}), but not *D*-arginine, attenuated the effects of *L*-NAME on Iso and For-induced relaxation (Tab 1).

DISCUSSION

Role of cAMP pathway in NO-induced vascular relaxation Iso and For, which raise cAMP levels, caused a concentration-dependent, *L*-NAME- and MB-inhibited relaxation. This relaxation was, in major part, endothelium-dependent, thus supporting the contention that binding sites with the characteristics of β-adrenoceptors were present in endothelial cells[7,8]. The present data suggest that agents which act to raise cAMP in endothelial cells are able to release NO and relax arterial smooth muscle.

Elevation of cGMP in rat fetal lung fibroblasts may be used as an index of NO release after exposure to medium in which bovine aortic endothelial cells were cultured[11]. Bradykinin and a Ca^{2+} ionophore added to endothelial cells produced a substance which elevated cGMP in fibroblasts and inhibitors of NO-synthase prevented the production of this substance. MB and haemoglobin prevented its effects on fibroblasts. When cAMP levels in the endothelial cells were elevated 3.7-fold by an inhibitor of cAMP phosphodiesterase, basal and stimulated release of NO was unchanged. Thus, cAMP levels did not influence NO formation in their endothelial cells[11], but cAMP may have been compartmentalized in these endothelial cells so that it failed to affect Ca^{2+} levels near NO-synthase or the enzyme itself. These cells may have lost cAMP control of NO release in culture.

In cultured bovine aortic endothelial cells, Iso or For induced biphasic elevations of $[Ca^{2+}]_i$ when added during the plateau phase of $[Ca^{2+}]_i$ increases induced by thrombin, bradykinin or ATP[9]. Neither Iso nor For affected the initial phase of $[Ca^{2+}]_i$ increase induced by these 3 stimuli. These authors

suggested that cAMP elevation augments Ca^{2+} mobilization in these endothelial cells[9]. Although NO release was not measured it seems likely that it would have been increased by cAMP elevation in this study. Conceivably in this study cAMP elevation occurred in a different compartment from that in the study in which a cAMP phosphodiesterase inhibitor was used. It is also possible that a release of Ca^{2+} from stores in endothelial cells is a necessary precondition for cAMP to enhance NO release.

Unresolved issues and possible interpretations Our results showed that MB reduced relaxations evoked by Iso and For in an endothelium-dependent manner. MB also antagonized endothelium-independent relaxations induced by SNP, Iso and For. This latter inhibition by MB may involve a common pathway, affecting smooth muscle function, perhaps involving superoxide anion formed by MB[12]. There is no evidence that Iso or For raise cGMP levels, so inhibition of guanylate cyclase and cGMP formation is not likely to be involved in the actions of MB in these cases.

β-Adrenoceptor stimulation (by Iso) of rat aorta rings caused endothelium-dependent relaxation of norepinephrine-induced contraction[10]. These relaxations were concentration-dependent and inhibition of NO synthase reduced the maximal relaxation. For also relaxed aortic rings precontracted by norepinephrine. Both Iso 1 μmol L^{-1} and For 0.1 μmol L^{-1} increased cAMP levels (4- and 25-fold, respectively) and cGMP levels (12-fold each). Inhibition of NO-synthase abolished the increases in cGMP but not those in cAMP[10]. Our findings are consistent with theirs. However, these authors reported that removal of the endothelium abolished relaxation to Iso 1 μmol L^{-1} in contrast to our observation that this procedure shifted relaxation responses so that higher concentrations of Iso or For were required for equivalent relaxations. Iso 1 μmol L^{-1} failed to raise cAMP in aortic muscle denuded of endothelium[10]. If cAMP was elevated by Iso and For in aortic muscle when endothelium was intact, a more plausible explanation of their results could arise from the studies showing that elevation of cGMP led to synergistic elevation of cAMP in platelets[13]. This occurred because cGMP inhibits a phosphodiesterase which hydrolyses cAMP. Removal of the endothelium would remove this potentiation. α_2-Adrenoceptors as well as β-adrenoceptors[14,15] may exist on endothelial cells and increase NO release. This may have confused the results obtained prior to endothelial removal. It is possible that injury to the vascular smooth muscle during the extensive removal of the endothelium in the earlier study[10] affected the ability of Iso to relax by elevating the cAMP in smooth muscle.

REFERENCES

1 Furchgott RF, Zawadzki JV. The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. Nature 1980; **288**: 373—6.

2 Moncada S, Palmer RMJ, Higgs EA. Nitric oxide: physiology, pathophysiology and pharmacology. Pharmacol Rev 1991; **43**: 109—42.

3 Rees DD, Palmer RMJ, Schulz R, Hodson HF, Moncada S. Characterization of three inhibitors of endothelial nitric oxide synthase *in vitro* and *in vivo*. Br J Pharmacol 1990; **101**: 746—52.

4 Martin W, Villani GM, Jothianandan D, Furchgott RF. Selective blockade of endothelium-dependent and glyceryl trinitrate-induced relaxation by haemoglobin and by methylene blue in the rabbit aorta. J Pharmacol Exp Ther 1985; **232**: 708—16.

5 Mulsch A, Bassenge E, Busse R. Nitric oxide synthesis in endothelial cytosol: Evidence for a calcium-dependent and a calcium-independent mechanism. Naunyn Schmiedebergs Arch Pharmacol 1989; **340**: 767—70.

6 Zheng XF, Guan YY, Kwan CY. Cyclopiazonic acid causes endothelium-dependent relaxation in rat aorta.

Acta Pharmacol Sin 1993; **14**: 21—6.

7 Ahmad S, Chretin P, Daniel EE, Shen SH. Characterization of beta adrenoceptors on cultured endothelial cells by radioligand binding. Life Sci 1990; **47**: 2365—70.

8 Freissmuth M, Hausleithner V, Nees S, Bock M, Schulz W. Cardiac ventricular β_2-adrenoceptors in guinea-pigs and rats are located on the coronary endothelium. Naunyn Schmiedebergs Arch Pharmacol 1986; **334**: 56—62.

9 Buchan KW, Martin W. Modulation of agonist-induced calcium mobilisation in bovine aortic endothelial cells by phorbol myristate acetate and cyclic AMP but not cyclic GMP. Br J Pharmacol 1991; **104**: 361—6.

10 Gray DW, Marshall I. Novel signal transduction pathway mediating endothelium-dependent β-adrenoceptor vasorelaxation in rat thoracic aorta. Br J Pharmacol 1992; **107**: 684—90.

11 Kuhn M, Otten A, Frolich JC, Forstermann U. Endothelial cyclic GMP and cyclic AMP do not regulate the release of endothelium-derived relaxing fator/nitric oxide from bovine aortic endothelial cells. J Pharmacol Exp Ther 1991; **256**: 677—82.

12 Ignarro LJ. Biological actions and properties of endothelium-derived nitric oxide formed and released from artery and vein. Circ Res 1989; **65**: 1—21.

13 Maurice DH, Haslam RJ. Molecular basis of synergetic inhibition of platelet function by nitrovasodilators and activators of adenylate cyclase: inhibition of cyclic AMP breakdown by cyclic GMP. Mol Pharmacol 1990; **37**: 671—81.

14 Bullock GR, Taylor SG, Weston AH. Influence of the vascular endothelium on agonist-induced contractions and relaxations in rat aorta. Br J Pharmacol 1986; **89**: 819—30.

15 Nyborg NCB. Actions of noradrenaline on isolated proximal and distal coronary arteries of rat: selective release of endothelium-derived relaxing factor in proximal arteries. Br J Pharmacol 1990; **100**: 552—6.