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Endothelial mediated inhibition of contraction and increase in cyclic GMP levels evoked by the α-adrenoceptor agonist B-HT 920 in rat isolated aorta

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1 In the presence of endothelium maximal contractions of rat aorta preparations evoked by B-HT 920 were about 10% of those evoked in the absence of endothelium.

2 6-Allyl-2-amino-5,6,7,8-tetrahydro-4H-thiazolo-(4,5-d)azepin dihydrochloride (B-HT 920, 0.1 μM to 0.1 mM) induced concentration-dependent contractions of rat aorta in the absence of endothelium. Maximal contractions were comparable in magnitude to those induced by noradrenaline.

3 In the presence of endothelium but not in its absence B-HT 920 (0.1 mM) stimulated an increase in tissue cyclic GMP levels of about 2 fold. Levels of cyclic AMP were unaffected. Removal of endothelium reduced basal tissue levels of cyclic GMP.

4 The guanylate cyclase inhibitor methylene blue (0.5 μM) potentiated B-HT 920-induced contractions in the presence of endothelium and inhibited increases in cyclic GMP.

5 In the presence of endothelium 5,8,11,14-eicosatetraynoic acid (ETYA; 0.1 mM), an inhibitor of both lipoxygenase and cyclo-oxygenase systems, inhibited the B-HT 920-induced increase in cyclic GMP but did not potentiate B-HT 920-induced contractions. ETYA also antagonized B-HT 920-induced contractions in the absence of endothelium.

6 It is concluded that endothelium continuously releases a product or products which influence the smooth muscle. Inhibition of B-HT 920-induced contractions in the presence of endothelium is associated with increased tissue levels of cyclic GMP.

Introduction

In the last 5 years the importance of the vascular endothelium as a mediator of many, but not all, relaxant responses has been well established (reviewed by Furchgott, 1983). The relaxation mediated by the endothelium is thought to be dependent on the integrity of the lipoxygenase system (Furchgott & Zawadzki, 1980; Furchgott, 1983) and is associated with increased tissue cyclic GMP (cyclic guanosine 3′,5′-monophosphate) levels (Holzmann, 1982; Diamond & Chu, 1983; Rapoport & Murad, 1983).

Recently it has been demonstrated that some but not all contractile effects are also potentiated in rat aorta and dog and pig coronary arteries by the removal of the endothelium (Allan *et al.*, 1983; Cocks & Angus, 1983; Godfraind & Miller, 1984). The greatest potentiating effect was seen in the case of clonidine, which induced a very small contraction of the rat aorta in the presence of endothelium, but a response comparable to that elicited by noradrenaline in the absence of endothelium (Eglème *et al.*, 1984a,b).

It, therefore, seemed of interest to find out if the modulation of the α-adrenergic responses in the rat aorta is due to the stimulated liberation of the same or a similar product to that released during relaxant responses and if there is a similar association with cyclic GMP.

The results show that contractile effects of the α_2-adrenoceptor selective agonist 6-allyl-2-amino-5,6,7,8-tetrahydro-4H-thiazolo-(4,5-d)azepin dihydrochloride (B-HT 920) are inhibited in the presence of endothelium. This inhibition is associated with a significant rise in tissue levels of cyclic GMP.

Methods

Contractile experiments

Female Wistar rats, 11 to 15 weeks old, were killed, the thoracic aorta exposed and cleaned of all loosely adherent tissue before being removed. Pairs of aortic rings 2 mm long were cut from close to the aortic arch. The endothelium was removed from one (or sometimes both) ring of each pair by rubbing with a wooden stick. The rings were then mounted under a tension of 2 g between hooks in a 10 ml organ bath containing a physiological solution (composition mM: NaCl 112, KCl 5, $NaHCO_3$ 25, KH_2PO_4 1, $MgSO_4$ 1.2, $CaCl_2$ 1.25 and glucose 11.5) maintained at 37°C and bubbled with a mixture of 95% O_2 and 5% CO_2. Isometric responses were recorded. After a 60 min equilibration period during which the solution was replaced periodically the tension was readjusted to 2 g. Cumulative concentration-effect curves to B-HT 920 were then elicited by approximately trebling the bath concentration with each addition of compound. When the maximal tension had been attained, the presence or absence of the endothelium was assessed by the addition of acetylcholine 1 μM (Furchgott & Zawadzki, 1980). Thereafter the tissues were washed every 15 min for 90 min before a contraction was elicited with noradrenaline 1 μM. When the maximal response was obtained (after 30 min) acetylcholine (1 μM) was again added to the bath. Occasionally noradrenaline-induced contractions preceded B-HT 920-induced responses. To study responses elicited by B-HT 920 in the presence of the guanylate cyclase inhibitor methylene blue or the lipoxygenase/cyclo-oxygenase inhibitor 5,8,11,14 - eicosatetraynoic acid (ETYA) tissues were preincubated with the respective antagonist (or vehicle alone in the case of controls) for 30 min.

Determination of cyclic nucleotide levels

Rat thoracic aorta, prepared as described above were divided into 4 or 5 segments of about 5 mg each. When necessary the endothelium was removed mechanically. Each segment was then incubated in 2 ml of physiological solution at 37°C bubbled with a mixture of 95% O_2 and 5% CO_2 for 135 min during which time the physiological solution was changed periodically. B-HT 920 (0.1 mM) was then added and after 1 min, or in the case of kinetic studies after varying time intervals, the aortic segments were frozen using aluminium tongs precooled in liquid nitrogen. When necessary, the tissues were preincubated for 30 min with antagonists before the addition of B-HT 920. Control tissues, treated only with solvents were included in all treatment groups. Tissues were thawed in 400 μl of perchloric acid (1 N), then homogenized with a Potter glass/glass homogenizer followed by sonication (Ultrasons-Annemasse, type 75TS) for 15 s. The homogenate was centrifuged at 10,000 *g* for 5 min and the cyclic nucleotide content (cyclic AMP (adenosine cyclic 3',5'-monophosphate), cyclic GMP) of the supernatant assayed by a radioimmunological method (Cailla *et al.*, 1973; 1976). The DNA content of each tissue segment was assayed by a fluorometric method as adapted by Schoeffter & Stoclet (1982). Cyclic nucleotide levels were expressed as fmol μg^{-1} DNA.

Drugs

Noradrenaline bitartrate (Sigma) was dissolved in distilled water containing 7.9 mM Na_2SO_3 and 34 mM HCl as a stock solution of 10 mM. Acetylcholine chloride (Sigma) was prepared as a stock solution of 10 mM in NaH_2PO_4 (0.1 M). ETYA (Hoffmann la Roche) was dissolved in absolute ethanol. B-HT 920 (Docteur Karl Thomae) and methylene blue (R.A.L.) were dissolved in distilled water. Dilutions were prepared in physiological solution. Cyclic AMP and cyclic GMP specific antibodies were the generous gift of Drs Cailla and Delaage of the Centre d'Immunologie, Marseille–Luminy (France). Cyclic AMP, cyclic GMP and DNA (from Calf Thymus, Type I) were obtained from Sigma. Labelled antigens were prepared by iodination of the succinyl-cyclic nucleotide tyrosylmethylesters (Sigma). All drug concentrations are expressed in terms of the base.

Statistical analysis

The data are expressed as means ± s.e.mean. Tests of significance were made using Student's *t* test, or paired *t* test where possible, *P* values less than 0.05 being considered significant. The concentration of an agonist producing 50% of the maximal response for that agonist (EC_{50} value) was estimated from its concentration-effect curve.

Results

Contractile experiments

In preliminary experiments cumulative concentration-effect curves elicited by B-HT 920 in the absence of endothelium produced a maximal contraction of 1.22 ± 0.26 g and a maximal contraction elicited 90 min later by noradrenaline 1 μM amounted to 1.62 ± 0.30 g ($n = 3$). When the noradrenaline contraction preceded the concentration-effect curve elicited by B-HT 920 a maximal noradrenaline contraction of 1.89 ± 0.29 g was obtained. A following B-HT 920 concentration-effect curve eli-

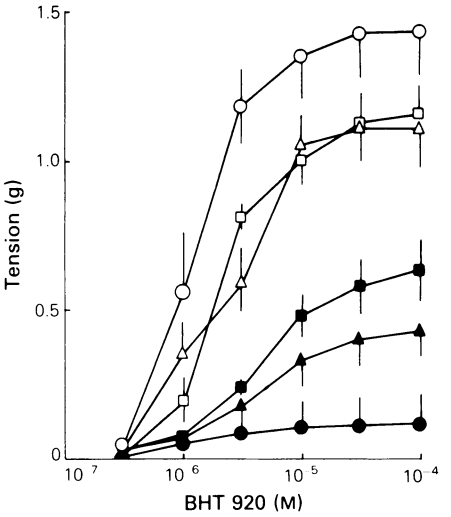

Figure 1 Concentration-effect curves of contractions elicited by B-HT 920 in rat aortic rings with (closed symbols) and without (open symbols) endothelium. Responses were elicited in the absence (○, ●) and in the presence of methylene blue 0.1 μM (△, ▲) or 0.5 μM (□, ■). Each curve is the mean of at least 5 observations. Vertical lines represent s.e.mean.

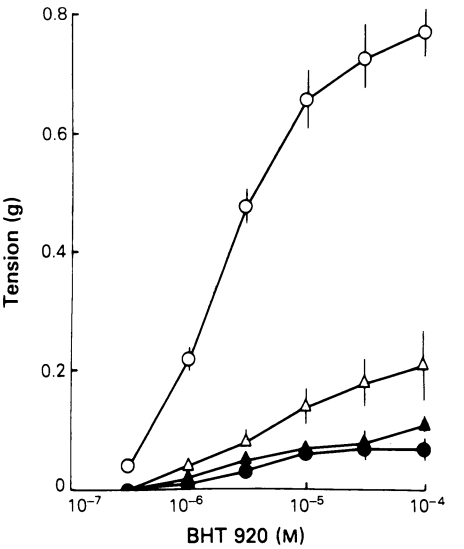

Figure 2 Concentration-effect curves of contractions elicited by B-HT 920 in rat aortic rings with (closed symbols) and without (open symbols) endothelium. Responses were elicited in the absence (○, ●) and in the presence of 5,8,11,14-eicosatetrayonoic acid (ETYA) 0.1 mM (△, ▲). Each curve is the mean of at least 5 observations. Vertical lines represent s.e.mean.

cited a significantly smaller maximal increase in tension of 0.35 ± 0.19 g ($n = 5$). Therefore, in all following experiments B-HT 920 concentration-effect curves were elicited without prior exposure to any other contractile agent.

B-HT 920 (0.1 μM to 0.1 mM) provoked concentration-dependent increases in tone of rat aorta preparations from which the endothelial cells had been removed. The EC_{50} value was 1.30 ± 0.25 μM and the maximum developed contraction amounted to 1.44 ± 0.16 g ($n = 6$). The absence of endothelial cells was verified by the addition of acetylcholine 1 μM to the stable maximal contractions and resulted in a slight (7.6 ± 1.7%) relaxation. In preparations of aorta with an intact endothelial layer, B-HT 920 evoked a small maximal contraction of 0.12 ± 0.11 g, or about 8% of that evoked in preparations from which the endothelium had been removed. The EC_{50} value was estimated to be about 1.1 μM (Figure 1). These small contractions were relaxed completely by the addition of acetylcholine 1 μM.

In separate experiments maximal contractions elicited to noradrenaline 1 μM were not significantly different in the absence or presence of endothelium and amounted to about 1.5 g. Acetylcholine 1 μM, in the presence of endothelium, relaxed the contractions to noradrenaline by about 50%, but in the absence of endothelium produced a relaxation of only about 2% ($n = 6$).

In the presence of methylene blue (0.5 μM) responses evoked by B-HT 920 (3 μM to 0.1 mM) in tissues with an intact endothelium were enhanced, maximal responses being increased by about 5 fold. The EC_{50} value (4.78 ± 0.87 μM, $n = 6$) was significantly ($P < 0.02$) increased from the EC_{50} values obtained in the absence of endothelium with or without methylene blue (EC_{50} values 2.14 ± 0.19 and 1.30 ± 0.25 μM respectively). Similar responses were obtained in the presence of 0.1 μM methylene blue. In preparations devoid of endothelium, neither concentration of methylene blue had a significant effect on B-HT 920 evoked contractions although responses tended to be reduced (Figure 1).

ETYA (0.1 mM) significantly inhibited contractions induced by B-HT 920 in preparations without endothelium and significantly increased the EC_{50} value from 2.3 ± 0.1 μM to 4.9 ± 1.5 μM (Figure 2, $n = 6$, $0.025 < P < 0.05$). In preparations with intact endothelium ETYA had no significant effect on B-HT 920-induced responses (Figure 2).

Determination of cyclic nucleotide levels

When compared directly in preparations from the same aorta, the basal level of cyclic GMP was significantly lower in the absence than in the presence of endothelium (9.0 ± 1.0 fmol μg^{-1} DNA and 22.5 ± 5.0 fmol μg^{-1} DNA respectively, $0.025 < P <$

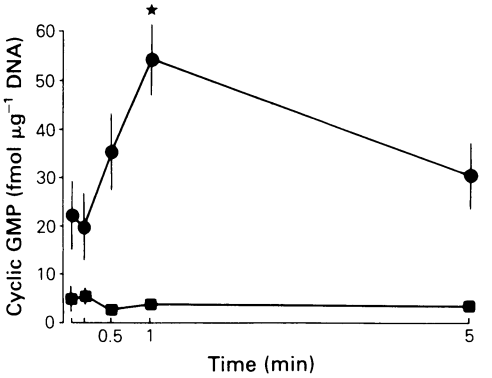

Figure 3 Effect of B-HT 920 0.1 mM on the tissue level of cyclic GMP in the presence (●) and in the absence (■) of endothelium as a function of time. Each point is the mean of 5 observations determined in duplicate. Vertical lines represent s.e.mean. *Significantly different from basal values.

0.05, $n = 4$) while cyclic AMP levels were not significantly different (70.0 ± 16.0 fmol μg^{-1} DNA and 55.3 ± 4.0 fmol μg^{-1} DNA respectively, $P > 0.4$). B-HT 920, 0.1 mM, a concentration which induced a maximal contraction of arteries, increased cyclic GMP levels in arterial samples with endothelium in a time-dependent manner (Figure 3). A maximal significant increase of about 2 fold in the level of cyclic GMP was apparent about 1 min after the addition of B-HT 920 (Figure 3, Table 1) while the cyclic AMP level was unchanged. This elevated level of cyclic GMP was maintained for 1 to 2 min (data not shown) and then declined. In aortic samples without endothelium neither cyclic GMP levels (Figure 3) nor cyclic AMP levels were significantly altered by exposure to B-HT 920 0.1 mM for 5 min.

In tissues with endothelium neither methylene blue 0.5 μM nor ETYA 0.1 μM had a significant effect on basal levels of cyclic AMP (data not shown) or cyclic GMP, although the latter tended to be reduced. B-HT 920, 0.1 mM, had no significant effect on either cyclic AMP or cyclic GMP levels in the presence of either methylene blue or ETYA (Table 1).

Table 1 Tissue levels of cyclic GMP (fmol μg^{-1} DNA) measured in the absence (control) and 1 min after the addition of B-HT 920 (0.1 mM) in the rat aorta complete with endothelium

Pretreatment	*Control*	*B-HT 920*
None	25.5 ± 2.4	47.7 ± 4.0*
Methylene blue	15.6 ± 4.5	25.4 ± 10.2
ETYA	15.2 ± 3.0	27.6 ± 5.7

*Significantly different from control ($P < 0.001$). Each value is the mean of at least 4 observations.

Discussion

These results demonstrate that in the rat aorta contractile responses elicited by the α_2-adrenoceptor selective agonist B-HT 920 (Kobinger & Pichler, 1980) were comparable with those elicited by noradrenaline if the endothelium had been removed, but in its presence B-HT 920 evoked only a slight contraction. Thus it might be assumed that B-HT 920 stimulates the endothelial cells to produce a product or products which modify the response of the smooth muscle cells to the direct effect of the agonist. A similar endothelial mediated inhibition of contractile responses evoked by clonidine and angiotensin I and II in rat aorta and by noradrenaline and 5-hydroxytryptamine (5-HT) in dog and pig coronary arteries has also been described (Allan *et al.*, 1983; Cocks & Angus, 1983; Eglème *et al.*, 1984a,b). In the case of the coronary artery, responses elicited by the thromboxane A_2 mimetic U46619 were unaffected by removal of the endothelium. Noradrenaline, phenylephrine, 5-HT and prostaglandin $F_{2\alpha}$ concentration-effect curves in rat aorta were shifted to the left about 3 to 6 fold by removal of the endothelium, but maximal responses were little affected (Eglème *et al.*, 1984a,b; Godfraind & Miller, 1984).

This inhibitory effect of endothelium on contractile responses of vascular smooth muscle, which is evidently agonist-dependent, may also be dependent on species and tissue since contractions of rat tail artery and of dog femoral artery induced by noradrenaline and of rabbit aorta induced by phenylephrine and noradrenaline were unaffected by removal of the endothelium (Busse *et al.*, 1983; Furchgott, 1983). These observations indicate a variability of endothelial cell sensitivity to agonists or of the vascular smooth muscle to products liberated by the endothelium. The seemingly uniform sensitivity of vascular smooth muscle to endothelial cell mediated acetylcholine induced relaxation of contraction (Furchgott, 1983) might favour the former interpretation.

Removal of the endothelium in rat aorta was associated with a significant decrease in the basal tissue level of cyclic GMP by about 2.5 fold with no change in cyclic AMP levels. This change in cyclic GMP levels indicates a constant basal level of influence of the endothelium on the smooth muscle, assuming that the contribution of the endothelium to the total tissue cyclic GMP content is negligible. These basal levels of cyclic nucleotides in the absence of en-

dothelium were unaffected by a concentration of B-HT 920 (0.1 mM) which produced a maximal contraction.

The inhibition of B-HT 920-mediated contractions in the presence of endothelium was associated with a small but significant increase in the tissue content of cyclic GMP. Methylene blue, an inhibitor of the activation of guanylate cyclase (Katsuki *et al.*, 1977), tended to reduce basal levels of cyclic GMP in the presence of endothelium and inhibited the increase in cyclic GMP levels induced by B-HT 920 to a level about equal to that seen in control tissues in the absence of methylene blue. These reduced levels of cyclic GMP in the presence of B-HT 920 were associated with an enhanced contraction (Figure 1). Methylene blue also increased B-HT 920 EC_{50} values in the presence and absence of endothelium, which may indicate that it has other effects unconnected with inhibition of guanylate cyclase. The 8-bromo derivative of cyclic GMP has been shown to relax noradrenaline-induced contractions of rat and rabbit aorta (Schultz *et al.*, 1979) and an increased tissue content of cyclic GMP has also been correlated with endothelial dependent relaxant responses of rabbit and rat aorta and beef coronary arteries (Holzmann, 1982; Diamond & Chu, 1983; Rapoport & Murad, 1983). However, the maximal increase in cyclic GMP levels associated with acetylcholine mediated relaxations of pre-contracted rat and rabbit aorta and beef coronary artery (about 10 to 30 fold) was far in excess of that induced by B-HT 920. If cyclic GMP is directly involved in the inhibition of contraction/relaxation of contractile responses, then this marked difference in cyclic GMP levels may imply that either different mechanisms are involved or that different pools of cyclic GMP are being activated by acetylcholine and B-HT 920.

It has been proposed that the mediator of endothelium-dependent relaxant responses is a product of the lipoxygenase metabolism of arachidonic acid (Furchgott, 1983). A similar type of product may be produced by stimulation of the endothelial cells by B-HT 920 and other α-adrenoceptor agonists. The cyclo-oxygenase inhibitor indomethacin (3 μM) did not significantly affect noradrenaline evoked responses in the presence or absence of endothelium (unpublished observations), but ETYA, an inhibitor of both lipoxygenase and cyclo-oxygenase enzyme systems tended to reduce basal tissue levels of cyclic GMP in the presence of endothelium and did inhibit the B-HT 920-induced increase in tissue cyclic GMP levels, such that they did not exceed the basal level of cyclic GMP seen in the absence of ETYA. However, ETYA did not potentiate B-HT 920-induced contractions in the presence of endothelium, probably because of its marked direct depressant effect on B-HT 920-induced contractions (Figure 2). This depressant effect of ETYA is not seen in rabbit aorta but is evident in some other arteries (Furchgott, 1983).

It is interesting that in rat aorta the most striking inhibitory effect of the endothelium is apparent when stimulation is induced by agonists usually described as α_2-adrenoceptor selective. In this tissue these agonists are much more dependent on extracellular Ca^{2+} to induce contractions than are noradrenaline or phenylephrine whose concentration-effect curves are simply shifted to the left when the endothelium is removed (Godfraind *et al.*, 1982; Eglème *et al.*, 1984a,b). This might mean that cyclic GMP is involved in calcium gating in this tissue.

It can be concluded that the endothelium of the rat aorta releases continuously a product (or products) which influences the smooth muscle and plays an important modulating role in the contractile responses induced by α-adrenoceptor agonists. This effect is most pronounced in the case of α_2-adrenoceptor selective compounds which might indicate the presence on the endothelium of a receptor resembling the α_2 subtype. This modulating effect is associated with an increased tissue level of cyclic GMP.

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