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Effect of endothelin antagonists on the responses to prostanoid endothelium-derived contracting factor

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1 The effect of endothelin antagonists on endothelium-dependent contractions was studied in conditions of stimulated endothelium-derived contracting factor (EDCF) release and with exogenous activation of thromboxane A_2-endoperoxide receptors in the rat aorta.

2 The incubation of aortic rings with N^{ω}-nitro-L-arginine methyl ester (L-NAME) led to EDCF-mediated contraction upon stimulation with acetylcholine ($24 \pm 3\%$ of KCl contraction). When vessels were preincubated with bosentan, an endothelin$_A$- and endothelin$_B$-receptor antagonist, in addition to L-NAME, acetylcholine-induced contraction was reduced to $8 \pm 2\%$ ($P<0.01$) of KCl contractions. PD147953, a selective endothelin$_A$-receptor antagonist, reduced the contraction to $14 \pm 4\%$ ($P<0.05$) of KCl contractions.

3 Bosentan preincubation produced a significant parallel rightward shift of the contractions to U46619, a selective thromboxane A_2 receptor agonist. In contrast, PD147953 failed to exhibit any inhibitory effect on U46619 contractions.

4 These results suggest that endothelin antagonists inhibit EDCF-mediated contractions by blocking endothelin$_A$ receptors and that, in addition, bosentan antagonizes the direct stimulation of thromboxane A_2 receptors.

Keywords: Bosentan; PD147953; endothelin-1; endothelin antagonists; endothelium; EDCF; thromboxane A_2

Introduction

In addition to the vasodilator, nitric oxide, and the potent vasoconstrictor, endothelin-1 (ET), the vascular endothelium has been shown to release cyclo-oxygenase-dependent contracting factors in several vascular beds of different species (Lüscher *et al.*, 1992). Thromboxane A_2 (TxA_2) and the endoperoxide prostaglandin H_2 (PGH_2) are mediators that can act as endothelium-derived contracting factors (EDCF) depending on the vascular bed and the stimulus studied (Lüscher *et al.*, 1992). The rat aorta, when stimulated with acetylcholine, releases predominantly PGH_2 and not TxA_2 as the response can be blocked by a TxA_2 receptor antagonist, but not by a thromboxane synthase inhibitor (Auch-Schwelk *et al.*, 1990; Kato *et al.*, 1990). This vascular bed may therefore represent a good model to study prostanoid EDCF, especially when the concomitant formation of nitric oxide is prevented by N^{ω}-nitro-L-arginine methyl ester (L-NAME) (Küng & Lüscher, 1995).

PD147953 (also known as FR139317) is a well characterized tripeptide selective ET_A-receptor antagonist (Sogabe *et al.*, 1993). We have confirmed its selectivity in a recent study designed to determine the receptor subtypes involved in ET-induced contractions of resistance arteries (Takase *et al.*, 1995). Bosentan is an orally active competitive antagonist of both ET_A- and ET_B-receptors (Clozel *et al.*, 1994). Its specificity has been evaluated against several agonists, including TxA_2, and showed no inhibitory effect except for a slight displacement of neurokinin A binding (Clozel *et al.*, 1994). Bosentan is therefore considered to be a non-selective specific ET-receptor antagonist.

During chronic administration of bosentan, we observed an interaction with endothelium-dependent contractions (Moreau P., Takase H., Küng C.F. & Lüscher T.F., 1995, unpublished observation). This observation, together with the growing evidence that endothelium contractions are partly mediated by a prostanoid EDCF (Lin & Nasjletti, 1992; Taddei & Vanhoutte, 1993; Küng *et al.*, 1995), has prompted us to study the acute effect of a selective ET_A-receptor antagonist, PD147953, and of a non-selective $ET_{A/B}$-receptor antagonist, bosentan, on EDCF-mediated responses as well as on the direct stimulation of thromboxane receptors.

Methods

Twelve weeks old, male Wistar-Kyoto rats (IFFA CREDO, L'Arbresle, France) were anaesthetized with thiopentone, 50 mg kg^{-1}, i.p. The thoracic aorta was dissected free and cut into 4 mm long rings that were mounted horizontally between two stirrups to measure isometric tension in organ chambers filled with Krebs solution (composition in mM: NaCl 118.6, KCl 4.7, $CaCl_2$ 2.5, KH_2PO_4 1.2, $MgSO_4$ 1.2, $NaHCO_3$ 25.1, calcium disodium edetate 0.026, glucose 10.1) maintained at 37°C and bubbled with 95% O_2/5% CO_2. After a 30 min equilibrium period, the rings were progressively stretched to the optimal passive tension of 2.5 ± 0.2 g (Küng & Lüscher, 1995). Two successive contractions were then obtained with control solution containing 100 mM KCl.

To stimulate the release of EDCF, acetylcholine was added in a cumulative fashion from 10^{-9} to 10^{-4} M to aortic rings preincubated for 30 min with L-NAME (10^{-4} M) alone or in combination with 10^{-5} M bosentan or 10^{-5} M PD147953. Some aortic rings were simultaneously incubated with 10^{-7} M SQ30741, a TxA_2 receptor antagonist (Auch-Schwelk *et al.*, 1990), in addition to L-NAME, L-NAME plus bosentan or L-NAME plus PD147953. Concentration-response curves to the selective TxA_2-mimetic, U46619, were performed in control aortic rings as well as in rings preincubated for 30 min with either bosentan (10^{-5}M), PD147953(10^{-5} M) or SQ30741 (10^{-7} M). This protocol was performed in aortic rings with intact endothelium or after mechanical endothelium removal. The proper removal of the endothelium was confirmed by the lack of effect of acetylcholine (10^{-5} M) added to the organ chamber at the end of the U46619 concentration-response curve.

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Contractions in the aorta were expressed as a percentage of the response to the second KCl (100 mM)-induced contraction. Data are presented as mean ± s.e.mean. The maximal contraction and half maximal effective concentration (EC_{50}) were calculated for each concentration-response curve by non linear regression analysis. For comparison, the EC_{50} is presented as its negative logarithm: pD_2. Maximal contractions and pD_2 values were compared by analysis of variance with Bonferroni's correction for multiple comparisons (Wallenstein *et al.*, 1980). An unpaired Student's *t* test was used when appropriate (see legends). A type I error inferior to 5% ($P<0.05$) was considered significant.

Bosentan (Ro 47-0302 free sulphonamide, F. Hoffman-La Roche, Ltd., Basel, Switzerland) and SQ30741 (Squibb Institute for Medical Research, Princeton, NJ, U.S.A) were dissolved in absolute ethanol. PD147953 ((**R**)2-[(**R**)-2-[(**S**)-2-[[1-(hexahydro-1H-azepinyl)] carbonyl-]amino-4-methylpentanoyl]amino-3-[3-(1-methyl-1 *H*-inodyl)]pro-pionyl]amino-3-(2-pyridyl)propionic acid, formerly called FR139317) was obtained from Parke-Davis Pharmaceutical Research Division, Warner-Lambert Company, Ann Arbor, MI, U.S.A) and dissolved in 20% ethanol. Acetylcholine chloride, U46619 (9, 11-dideoxy-11a, 9a-epoxymethano-prostaglandin $F_{2\alpha}$) and L-NAME (all Sigma Chemical Co., Buchs, Switzerland) were dissolved in water and diluted with control solution. In order to eliminate any possible effect of the vehicle, all the experiments were performed with 0.1% ethanol in the organ chamber.

Results

In the presence of L-NAME, acetylcholine induced an endothelium-dependent contraction of 24 ± 3% (Figure 1a and b). The maximum contraction was significantly reduced by bosentan (8 ± 2%, $P<0.05$) and, to a lesser degree, by PD147953 (14 ± 4%, $P<0.05$). The addition of SQ30741 to the organ chamber decreased the acetylcholine-induced endothelium-dependent contraction to 2 or 3% of KCl contraction in all groups (Figure 1b). The sensitivity of the aorta to acetylcholine was not modified by any pretreatment (Figure 1a).

The direct stimulation of TxA_2 receptors by U46619 gave much stronger contractions than the stimulation of endogenous EDCF release (Figure 2). The amplitude of these contractions was not modified by endothelial removal, but the senstivity was enhanced (Table 1). Independently of the presence of the endothelium, bosentan significantly displaced the concentration-response curve of U46619 to the right (concentration shift at EC_{50}: 1.6 fold), while PD147953 had no effect (Figure 2, Table 1). The slope of the concentration-response curve with bosentan was similar to that of the control curve, suggesting that the antagonism may be competitive in nature (Table 1). The TxA_2 receptor antagonist, SQ30741, was more potent than bosentan in antagonizing U46619-induced contractions (concentration shift at EC_{50}: 8.5 fold, Figure 2, Table 1).

Discussion

The present experiments demonstrate that ET-receptor antagonists inhibit the contractions to an endogenous prostanoid EDCF. In some experimental conditions, part of the contractile effect of exogenous ET has been shown to be mediated by the release of EDCF, as a TxA_2-receptor antagonist could reduce the maximal contraction induced by ET (Lin & Nasjletti, 1992; Taddei & Vanhoutte, 1993). Furthermore, the ET-stimulated release of EDCF was attributed to the stimulation of ET_A-receptors (Taddei & Vanhoutte, 1993). In the present study, the effectiveness of ET-receptor antagonists in reducing endothelium-dependent contractions suggests that endogenous ET may also regulate EDCF release. Indeed, PD147953 re-

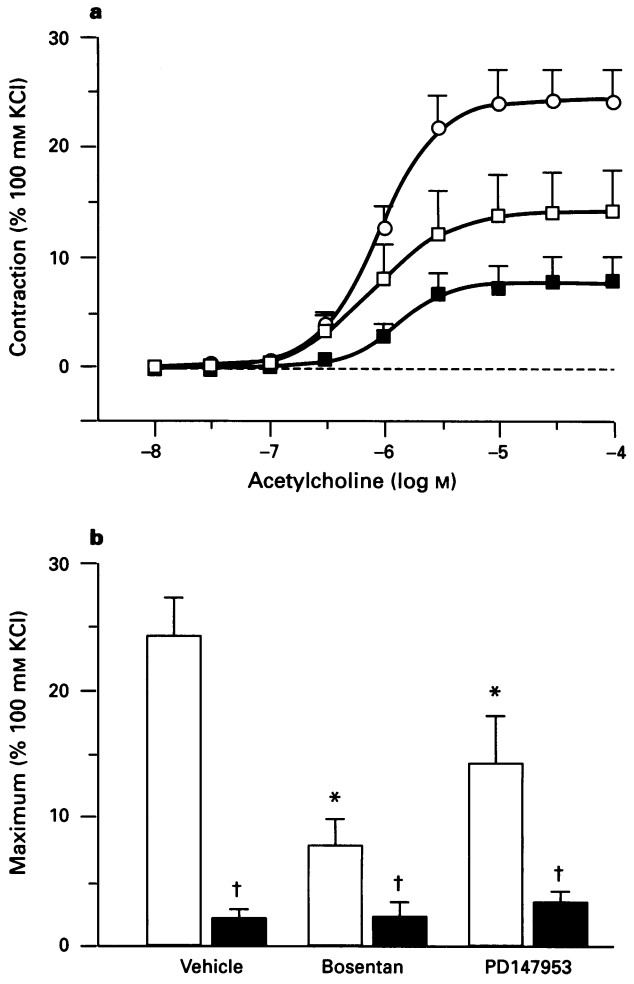

Figure 1 (a) Concentration-response curves to acetylcholine in aortic rings preincubated for 30 min with L-NAME (10^{-4} M). The vessels were also incubated with the vehicle ($n=8$, ○), bosentan ($n=8$, ■) or PD147953 ($n=8$, □). (**b**) Maximal contractions to acetylcholine in the absence (□) or presence (■) of SQ30741, a thromboxane A_2-receptor antagonist. All aortic rings were preincubated with L-NAME (10^{-4} M) and with either the vehicle, bosentan or PD147953. *$P<0.05$ as compared to the vehicle group (ANOVA + Bonferroni). †$P<0.05$ as compared to without SQ30741 (unpaired *t* test).

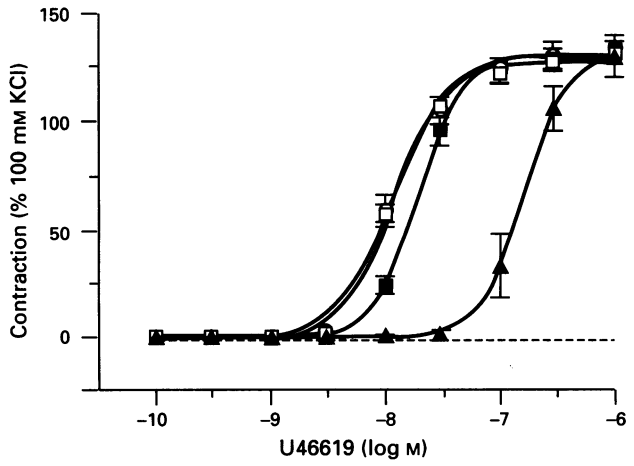

Figure 2 Concentration-response curves to the thromboxane A_2 receptor agonist, U46619, in endothelium-denuded aortic rings preincubated with vehicle ($n=8$, ○), bosentan ($n=8$, ■), PD147953 ($n=7$, □) or SQ30741 ($n=5$, ▲). Refer to Table 1 and results section for statistics.

Table 1 Comparison of the concentration-response curves to U46619 with different preincubations with or without the endothelium

	Vehicle	*Bosentan*	*PD147953*	*SQ30741*
With endothelium	(7)	(8)	(8)	(6)
Max (%)	140±6	138±4	133±3	133±3
pD_2	7.57±0.04	7.37±0.06*	7.65±0.07	6.64±0.06*
Slope	37.4±3.0	33.5±1.1	37.6±1.6	38.4±1.2
Without endothelium	(8)	(8)	(7)	(5)
Max (%)	130±6	130±4	128±4	132±7
pD_2	7.95±0.05†	7.73±0.03*†	7.94±0.03†	6.86±0.10*
Slope	40.5±3.6	43.8±3.9†	38.2±2.0	42.6±4.1

The maximum contraction (Max %), half maximum effective concentration (negative log molar, pD_2) and slope of the concentration-response curves were obtained by non-linear regression analysis. The number in parentheses represents the number of experiments. *$P<0.05$ as compared to the vehicle group (ANOVA + Bonferroni). †$P<0.05$ as compared to with endothelium (unpaired t test).

duced the acetylcholine response without affecting the concentration-response curve to U46619. The reduction of endothelium-dependent contractions by bosentan and PD147953 may therefore constitute an indirect mechanism secondary to their inhibition of ET_A-receptors.

In addition to its ET-receptor antagonistic properties, bosentan seems to antagonize directly the stimulation of TxA_2 receptors. Although it is less potent than SQ30741 in inhibiting U46619-induced contractions, the antagonism could be of importance when contractions are of smaller magnitude, such as with acetylcholine-induced release of the endogenous agonist. The similar slope of the U46619 concentration-response curves in the absence or presence of bosentan suggests that the inhibition is competitive in nature, although this represents only a crude evaluation. Our observations differ from a previous report suggesting that bosentan did not affect TxA_2 binding properties in radiolabelled ligand binding experiments (Clozel *et al.*, 1994). This discrepancy could be explained by the suggestion that U46619 may stimulate two different subtypes of TxA_2 receptors (Ge *et al.*, 1995).

The relevance of the present observations for the *in vivo* use of ET-receptor antagonists depends on their difference of potency in antagonizing ET- or EDCF-mediated contractions, and on the effective concentration needed *in vivo*. In a previous study, we have reported that bosentan and PD147953 were equipotent in antagonizing ET-1-induced contractions (Takase *et al.*, 1995). At 10^{-5} M, both antagonists shifted ET-1 concentration-response curves by a factor of 100 (2 log shift). In the present study, PD147953 did not seem to interact directly with TxA_2 receptors, while bosentan shifted the U46619 concentration-response curve by a factor of 1.6 (at 10^{-5} M). It is therefore clear that bosentan and PD147953 are more potent ET-receptor antagonists than TxA_2-receptor antagonists. However, the effective plasma concentration of bosentan reached *in vivo* in a recent study involving heart failure patients (Kiowski *et al.*, 1995) is very similar to the concentration that we used *in vitro*. Indeed, intravenous doses of 100 and 200 mg of bosentan yielded maximal plasma concentrations of 1.7×10^{-5} M and 5.62×10^{-5} M, respectively. Thus, the inhibition of EDCF-mediated contractions by ET-receptor antagonists that we observed *in vitro* may also be present with clinically effective doses of these antagonists.

In conclusion, our experiments suggest that bosentan inhibits the direct stimulation of TxA_2 receptors, even in the absence of the endothelium. This effect, together with the indirect inhibition of EDCF responses by the blockade of ET_A-receptors, may inhibit as much as 70% of acetylcholine-induced endothelium-independent contraction in the rat aorta. As EDCF-mediated responses are likely to contribute to impaired endothelium-dependent relaxation in disease states such as in essential hypertension (Taddei *et al.*, 1993), this effect of ET-receptor antagonists may be clinically relevant. Hence, the contribution of this ancillary property of ET-receptor antagonists, and particularly of bosentan, to inhibit the vascular effect of prostanoid EDCF could have important implications for cardiovascular pharmacotherapy.

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