



Effects of some isoprostanes on the human umbilical artery *in vitro*

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1 Cumulative concentration-effect curves for the selective prostanoid TP receptor agonist U46619 and six isoprostanes were constructed in the human isolated umbilical artery.

2 All compounds except 8-iso-PGF_{3α} produced concentration-dependent contractions. The contractile response to the isoprostanes increased with each cumulative addition up to a point, after which subsequent addition reduced the contraction below the previous level. This 'downturn' in the concentration-effect curve did not occur with U46619.

3 The potencies of the compounds tested were as follows ($pEC_{50} \pm$ s.e.mean): U46619, 6.7 ± 0.2 ; 8-iso-PGE₂, 6.5 ± 0.1 ; 8-iso-PGF_{2α}, 5.8 ± 0.2 ; 8-iso-PGE₁, 5.4 ± 0.1 ; 8-iso-PGF_{1α}, 5.0 ± 0.1 ; 8-iso-PGF_{2β} > 4.8; 8-iso-PGF_{3α} > > 4.8 ($n = 4-17$). Neither 8-iso-PGF_{2β} nor 8-iso-PGF_{3α} at 44 μM had a significant effect on cumulative concentration-effect curves to U46619.

4 The selective TP receptor antagonist GR32191 (0.1 μM) caused rightward shifts in the concentration-effect curves to all the active compounds. pA_2 values for GR32191 against U46619, 8-iso-PGE₂, 8-iso-PGF_{2α}, 8-iso-PGE₁ were 7.6 ± 0.2 , 9 ± 1 , 8.2 ± 0.3 and 7.7 ± 0.3 , respectively ($n = 4$).

5 Neither *Nω*-nitro-L-arginine methyl ester (100 μM) nor the selective DP receptor antagonist BW A868C (50 nM) affected the complex concentration-effect curve to 8-iso-PGE₂ ($n = 3$).

6 Stable contractions to U46619 (1–3 μM) were unaffected by anandamide at concentrations up to 60 μM.

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Abbreviations: BPSS, buffered physiological salt solution; HUA, human umbilical artery; L-NAME, *Nω*-nitro-L-arginine methyl ester; PG, prostaglandin; PSS, physiological salt solution

Introduction

The isoprostanes are stable arachidonic acid metabolites that appear in normal human plasma and urine at concentrations at least an order of magnitude greater than those of the prostaglandins (Morrow *et al.*, 1990). Isoprostanes differ structurally from prostaglandins by the *cis*-orientation at the cyclopentane ring junction compared to the *trans*-orientation in the classical prostanoids (see Figure 1). Although originally thought to be formed exclusively by a cyclo-oxygenase-independent, free-radical catalyzed mechanism (Morrow *et al.*, 1990), there is now mounting evidence to suggest that some isoprostanes can also be formed by a cyclo-oxygenase-dependent pathway (Jourdan *et al.*, 1999; Montuschi *et al.*, 1999). The isoprostane family is potentially very large. The F₂-isoprostane family (isomers of prostaglandin (PG) F_{2α}) alone is composed of four regioisomers each theoretically composed of eight racemic diastereomers (Morrow & Roberts, 1996). Not only F-isoprostanes, but also D- and E-ring isoprostanes are formed *in vivo* (Morrow *et al.*, 1994).

Concentrations of isoprostanes, particularly the most widely studied 8-iso-PGF_{2α}, are increased in a number of pathophysiological conditions including experimental diabetes (Wentzel *et al.*, 1999), Alzheimer's disease (Montine *et al.*, 1998), and anaphylaxis (Montuschi *et al.*, 1999). But whether they contribute to these pathologies, as opposed to being mere markers for them, is largely unknown. In this regard, the pharmacology of the isoprostanes is poorly

understood and knowledge is limited almost exclusively to 8-iso-PGF_{2α}.

8-iso-PGF_{2α} is a potent stimulant of vascular (Takahashi *et al.*, 1992; Kang *et al.*, 1993; Kromer & Tippins, 1996; Zhang *et al.*, 1996; John & Valentin, 1997), airway (Kang *et al.*, 1993; Kawikova *et al.*, 1996), intestinal (Elmhurst *et al.*, 1997) and uterine (Crankshaw, 1995) smooth muscles where its effects are sensitive to selective prostanoid TP receptor antagonists. Consequently, the effects of 8-iso-PGF_{2α} are generally, though not exclusively, thought to be mediated by action at TP receptors (Kromer & Tippins, 1999). 8-iso-PGF_{2α} is also a partial agonist at platelet TP receptors (Yin *et al.*, 1994). 8-iso-PGE₂ also has smooth muscle stimulant properties that are TP receptor antagonist-sensitive (Morrow *et al.*, 1994; Fukunaga *et al.*, 1993a) and this has led to speculation that spatial orientation of the side-chains is a more important determinant of isoprostane/receptor interactions than structural differences in the cyclopentane ring (Morrow & Roberts, 1996). Figure 1 shows the structure of 8-iso-PGE₂ and compares it to both PGE₂ and thromboxane A₂, the endogenous ligand for TP receptors.

We recently demonstrated that the human umbilical artery (HUA) operationally expresses only one excitatory prostanoid receptor subtype, namely TP (Boersma *et al.*, 1999). This preparation is therefore an ideal one in which to investigate operational aspects of the structure-activity relationships of isoprostanes with human TP receptors. In this study we have, therefore, compared the effects of a number of commercially available isoprostanes and the TP receptor agonist U46619 (Coleman *et al.*, 1994) on the contractility of the HUA *in vitro*. Some of these data have

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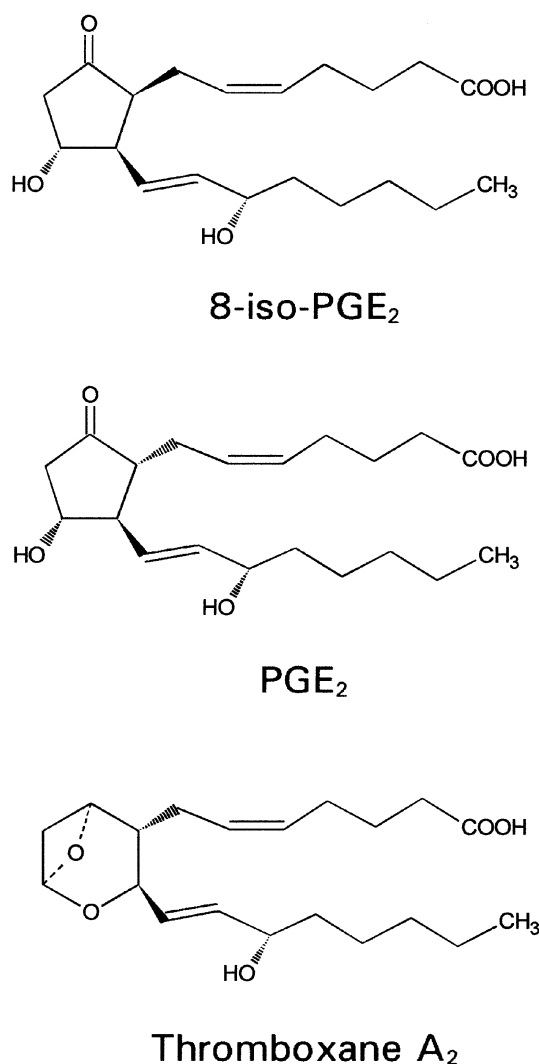


Figure 1 Chemical structures of 8-iso-PGE₂, PGE₂ and thromboxane A₂.

been communicated to the British Pharmacological Society (Oliveira & Crankshaw, 1998; Oliveira *et al.*, 1998).

Methods

Tissue collection and preparation

Sections of umbilical cords within 20 cm of the placenta were obtained from full-term vaginal or Caesarean births and collected in cold buffered physiological salt solution (BPSS). Tissues were stored at 4°C and used within 24 h. Human umbilical arteries were dissected free of Wharton's Jelly and cut into transverse rings 3–5 mm in length. Endothelial cells were mechanically removed and removal of the cells was confirmed by histology.

Isometric contractions

Rings were suspended on stainless-steel hooks and mounted in individual 5 ml jacketed muscle baths containing oxygenated (95% O₂ and 5% CO₂) physiological salt solution (PSS) at 37°C. One hook was anchored in the bath while the other was attached with silk thread to a FT-03 force displacement transducer writing to a 7D 8-channel polygraph (Grass

Instruments). A resting tension of 30 mN was applied to each tissue ring. Individual rings were washed and allowed to equilibrate for 3 h under these conditions during which time spontaneous tone developed. Tissues were then challenged with 60 mM KCl. Once the maximum response to the KCl challenge was achieved, tissues were washed three times and allowed to equilibrate for 20 min to allow baseline to be reached again. The KCl challenge was performed a total of three times.

Agonist potency

Eighty minutes after the last KCl challenge had been washed out, concentration-effect experiments were performed by cumulative addition of agonists to produce approximately half log unit increases in the bath concentration per addition. When the response to the last agonist addition had reached a plateau, the PSS was washed from the bath and replaced with deionized water in order to induce a hypotonic shock. The contraction produced by the hypotonic shock was used to normalize all drug-induced responses (Boersma *et al.*, 1999).

Concentration-effect curves were constructed from the data obtained by fitting to a form of the logistic equation:

$$E = E_{min} + (E_{max} - E_{min}) / (1 + e^{-n_H (\log C + pEC_{50})}) \quad (1)$$

where E is the effect of the agonist, E_{min} is the effect in the absence of agonist, E_{max} is the maximum effect, C is the molar concentration of the agonist, n_H is the slope parameter, and pEC₅₀ is the negative log of the molar concentration of the agonist that produces a half-maximal response. Individual concentration-effect curves in which a reduced response was obtained at higher agonist concentrations were analysed by ignoring the points falling below and to the right of the peak response. In experiments where antagonists were used, they were added to the bath 60 min before the start of the concentration-effect experiment. Concentration-effect experiments were performed in antagonist-treated and matched control rings from the same donor. pA₂ values were calculated as described above. Single dose-ratio estimates of pA₂ values were then determined according to the equation:

$$pA_2 = \log((EC_{50A}/EC_{50C}) - 1) - \log[B] \quad (2)$$

where EC_{50A} is the agonist EC₅₀ in the presence of antagonist, EC_{50C} is the control agonist EC₅₀ and [B] is the molar concentration of antagonist.

Effects of anandamide on stable contractions

Stable contractions to U46619 were obtained by adding either 1 or 3 μM of the agonist and allowing 30 min for the response to equilibrate. Thereafter, anandamide was added cumulatively as described for agonist potency experiments.

Drugs and chemicals

Anandamide (arachidonlyethanolamide), U46619 (9,11-dideoxy-9α,11α-methanoepoxy prostaglandin F_{2α}), 8-iso-PGE₂, 8-iso-PGE₁, 8-iso-PGF_{3α}, 8-iso-PGF_{2α}, 8-iso-PGF_{2β} and 8-iso-PGF_{1α} were obtained from Cayman Chemical (Ann Arbor, MI, U.S.A.). Indomethacin, 5-HT and Nω-nitro-L-arginine methyl ester (L-NAME) were obtained from Sigma (Oakville, ON, Canada). The following compounds were received as gifts: BW A868C (3-benzyl-5-(6-carboxyhexyl)-1-(2-cyclohexyl-2-hydroxyethylamino)hydantoin) from Wellcome (Beckenham, U.K.); GR32191 ([1R-[1α(Z),2β,3β,5α]]-(+)-7-[5-[(1,1'-

biphenyl)-4-yl]methoxy]-3-hydroxy-2-(1-piperidinyl)cyclopentyl]-4-heptenoic acid) from Glaxo-Wellcome (Stevenage, U.K.); All other chemicals were from BDH (Toronto, ON, Canada). Indomethacin was prepared as described by Curry *et al.* (1982). All other drugs were made as solutions in ethanol. Immediately before experiments, appropriate serial dilutions of drugs were made in double distilled water from concentrated stock solutions.

Solutions

The BPSS had the following composition (mM): N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid 5.0, NaCl 150, KCl 4.6, MgSO₄ 1.2, CaCl₂ 2.5, glucose 11.1, and indomethacin 0.01, pH 7.4. The PSS was composed as follows (mM): KCl 4.6, MgSO₄ 1.16, NaH₂PO₄ 1.16, CaCl₂ 2.5, NaCl 115.5, NaHCO₃ 21.9, glucose 11.1 and indomethacin 0.03.

Statistics

All data are expressed as means \pm s.e.mean. The concentration-effect parameters for U46619 in the absence and in the presence of isoprostanes were compared using a paired Student's *t*-test, values of $P < 0.05$ were considered significant.

Results

U46619 caused concentration-dependent contraction of HUA. Contractions were slow to develop and tonic in nature at low agonist concentrations, with relatively slow phasic contractions often superimposed on the tonic background at higher concentrations. All the isoprostanes tested, except 8-iso-PGF_{3 α} at concentrations up to 44 μ M, produced measurable contractions. Responses to the isoprostanes were similar in nature to those to U46619 except that the response to each cumulative addition of isoprostane

increased up to a point, after which a subsequent addition reduced the contraction below the previous level. In some cases the response to the most effective concentration of isoprostane was tonic and well maintained, in other cases it was transient. Illustrative traces of the responses to U46619 and 8-iso-PGE₂ are shown in Figure 2.

Responses to all active compounds were sensitive to the selective TP receptor antagonist GR32191 (Lumley *et al.*, 1989) at 0.1 μ M. GR32191 alone produced no measurable effect. Mean concentration-effect curves for the active compounds obtained from matched rings in the presence and absence of GR32191 are given in Figure 3. Concentration-effect parameters obtained from all control experiments with the compounds tested are given in Table 1. The pA_2 values for GR32191 against U46619, 8-iso-PGE₂, 8-iso-PGF_{2 α} and 8-iso-PGE₁ were 7.6 ± 0.2 , 9 ± 1 , 8.2 ± 0.3 and 7.7 ± 0.3 , respectively ($n = 4$). pA_2 values against 8-iso-PGF_{1 α} , 8-iso-PGF_{2 β} and 8-iso-PGF_{3 α} could not be calculated because of the low potency of the agonists.

We tested some isoprostanes for antagonist activity. Neither of the two least potent compounds, 8-iso-PGF_{2 β} (effective at 4 to 44 μ M with reduced maximum response, Figure 3), nor 8-iso-PGF_{3 α} (ineffective at concentrations up to 44 μ M) at 44 μ M had a significant effect on cumulative concentration-effect curves to U46619. However, 8-iso-PGE₂, at the same concentration, significantly reduced both the pEC_{50} and the maximum response to U46619. Data from these experiments are summarized in Table 2. By contrast, 8-iso-PGE₂ did not reduce the pEC_{50} for 5-HT. In these experiments pEC_{50} , n_H , and E_{max} values were 7.1 ± 0.5 , 3 ± 1 , and 68 ± 15 in control and 7.5 ± 0.4 , 12 ± 10 , and 67 ± 3 in isoprostane-treated rings, respectively ($n = 3$).

When cumulative concentration-effect experiments to 8-iso-PGE₂ were performed in matched rings in either the presence or the absence of 100 μ M L-NAME, the resulting concentration-effect curves were superimposable. Similar results were obtained when the selective DP receptor antagonist, BW A868C (Giles *et al.*, 1989), at 50 nM was

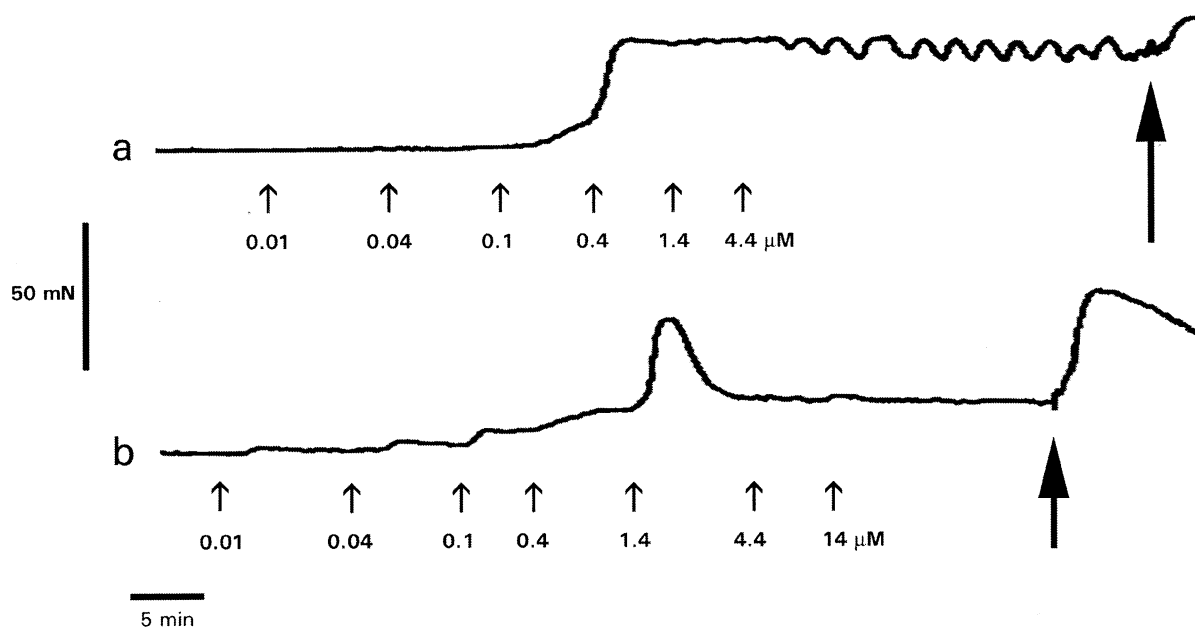


Figure 2 Sample traces showing the effect of cumulative addition of (a) U46619 and (b) 8-iso-PGE₂ on tension development by rings of human umbilical artery. Agonists were added to the baths at the points indicated by the small arrows to give the cumulative concentrations shown. The large arrows indicate the points at which the bath contents were replaced with deionized water in order to induce a hypotonic shock. The peak response to hypotonic shock was used to normalize the drug-induced responses.

substituted for L-NAME. Data from these experiments are shown in Figure 4.

Stable contractions of HUA to 1 or 3 μM U46619 were not affected in any way by cumulative addition of the endogenous cannabimimetic eicosanoid, anandamide (Felder & Glass, 1998), up to 60 μM .

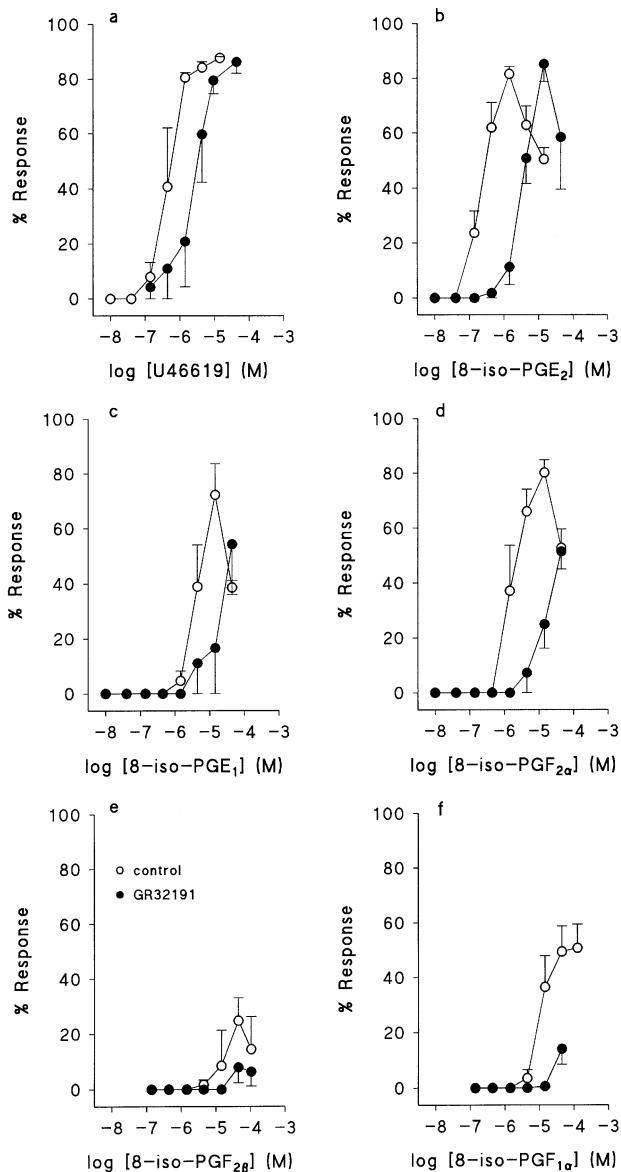


Figure 3 Effects of U46619 and some isoprostanes on tension development by rings of human umbilical artery. Mean concentration-effect curves obtained in the absence and presence of GR32191, 0.1 μM . (a) U46619, $n=4$; (b) 8-iso-PGE₂, $n=17$; (c) 8-iso-PGE₁, $n=4$; (d) 8-iso-PGF_{2 α} , $n=4$; (e) 8-iso-PGF_{2 β} , $n=4$; (f) 8-iso-PGF_{1 α} , $n=4$. Values are means \pm s.e.mean.

Discussion

Under the conditions of our experiments, the HUA operationally expresses only one subtype of excitatory prostanoid receptor, TP (Boersma *et al.*, 1999). All the active isoprostanes we tested were sensitive to the selective TP receptor antagonist GR32191, and it is therefore most likely that the effects we saw were mediated by human TP receptors. The slopes of the concentration effect-curves were very steep (Figure 3) compatible with our previous observations on HUA under these conditions (Boersma *et al.*, 1999). The relatively large variability in slope parameters (Tables 1 and 2) may be attributed in part to inaccuracies introduced by the necessity to ignore the downturn phase in the analysis of excitatory concentration-effect curves. However, considerable variability in the slope parameter was also observed for agonists that did not produce a downturn (Boersma *et al.*, 1999) suggesting significant inter-preparation variation. The reason for this variation is unclear to us.

The potency order shown in Table 1 suggests several conclusions regarding the structural requirements for isoprostane/receptor activation. Firstly, the superior potency of 8-iso-PGE₂ over 8-iso-PGF_{2 α} and of 8-iso-PGE₁ over 8-iso-PGF_{1 α} demonstrates that E-ring compounds are more potent than F-ring compounds. Secondly, the superior potency of 8-iso-PGE₂ over 8-iso-PGE₁ and of 8-iso-PGF_{2 α} over 8-iso-PGF_{1 α} demonstrates that doubly unsaturated compounds are more potent than singly unsaturated compounds. Thirdly, the much greater potency of 8-iso-PGF_{2 α} over 8-iso-PGF_{2 β} shows the importance of the α -configuration. 8-iso-PGF_{1 α} appears to be a partial agonist since it elicited a lower maximum response than U46619 (Figure 2, Table 1) and did not demonstrate any inhibitory effects. The extremely low potencies of 8-iso-PGF_{2 β} and 8-iso-PGF_{3 α} are likely to be attributable to a loss of affinity, since we were unable to demonstrate any antagonist activity for these compounds as opposed to that shown by 8-iso-PGE₂ (Table 2).

GR32191 has a pK_b of 8.1 against U46619 in HUA (Boersma *et al.*, 1999) and a pA_2 of 8.2 against U46619 in human pulmonary artery (Lumley *et al.*, 1989), evidence

Table 1 Concentration-effect parameters for various agonists on the HUA *in vitro*

Compound	pEC_{50}	n_H	E_{max}	n
U46619	6.7 ± 0.2	5 ± 1	82 ± 3	10
8-iso-PGE ₂	6.5 ± 0.1	4 ± 2	79 ± 3	17
8-iso-PGF _{2α}	5.8 ± 0.2	5 ± 1	80 ± 5	4
8-iso-PGE ₁	5.4 ± 0.1	6 ± 1	83 ± 6	4
8-iso-PGF _{1α}	5.0 ± 0.1	7 ± 2	66 ± 7	4
8-iso-PGF _{2β}	> 4.4	—	—	4
8-iso-PGF _{3α}	$> > 4.4$	—	—	4

Concentration-effect curve parameters were determined in rings of HUA as described in Methods. Data are means \pm s.e.mean.

Table 2 Effect of isoprostanes at 44 μM on the response of the HUA *in vitro* to U46619

Compound	pEC_{50}	Control		Treated			n
		n_H	E_{max}	pEC_{50}	n_H	E_{max}	
8-iso-PGE ₂	6.8 ± 0.3	4 ± 1	88 ± 3	$4.9 \pm 0.04^*$	6 ± 2	$53 \pm 8^*$	3
8-iso-PGF _{2β}	6.9 ± 0.3	5 ± 1	74 ± 6	6.5 ± 0.5	3 ± 1	82 ± 2	3
8-iso-PGF _{3α}	7.0 ± 0.2	4 ± 1	75 ± 8	6.9 ± 0.1	4 ± 2	79 ± 7	3

Concentration-effect curve parameters for U46619 were determined in the absence (Control) and presence (Treated) of isoprostane using paired rings of HUA as described in Methods. Data are means \pm s.e.mean. *Significantly different from control value, $P < 0.05$.

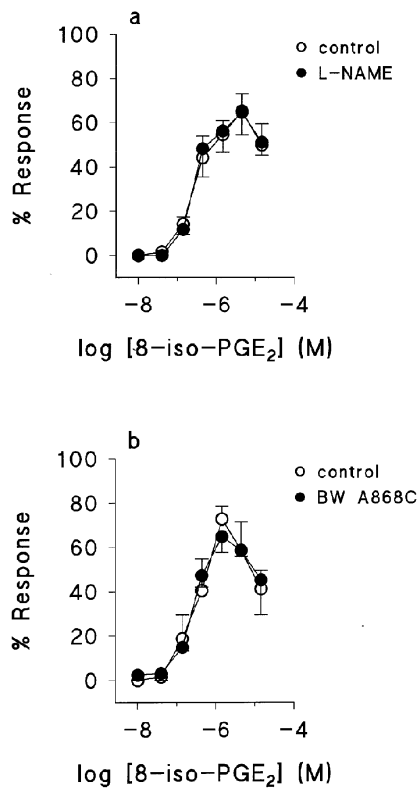


Figure 4 The effect of L-NAME and BW A868C on the response of human umbilical artery to 8-iso-PGE₂. Mean concentration-effect curves obtained in the absence and presence of (a) 100 μ M L-NAME, (b) 50 nM BWA 868C. In both cases $n=3$. Values are means \pm s.e.mean.

supports action at TP receptors in both cases. The pA_2 values that we obtained for GR32191 against isoprostanes in the present study are compatible with action at TP receptors. The large standard error on the pA_2 estimate against 8-iso-PGE₂ results from one tissue that was almost completely blocked by GR32191. Isoprostanes have been suggested to act at distinct isoprostane receptors (Fukunaga *et al.*, 1993b) but the present results do not support the existence of distinct isoprostane receptors in HUA. However, isoprostanes do have actions that are not TP receptor antagonist-sensitive (Elmhurst *et al.*, 1997; Jourdan *et al.*, 1997), the structure-activity requirements for these actions remain unknown.

There are several possible explanations for the downturn in the concentration-effect curves to the isoprostanes at high agonist concentrations (Figure 2). In rat isolated pulmonary artery 8-iso-PGF_{2 α} causes constriction by a TP receptor-mediated pathway, and dilatation by a non-TP receptor mechanism that involves the release of NO (Jourdan *et al.*, 1997). If such a mechanism were operative in HUA, a complex concentration-effect curve to isoprostanes would likely result. However, NO is unlikely to contribute to the effects we observed since we had mechanically removed the endothelium, the primary source of NO in blood vessels (Pollock *et al.*, 1991). This notion is supported by the failure of the NO synthase inhibitor, L-NAME, to alter the concentration-effect curve to 8-iso-PGE₂ in any way (Figure 3a).

Alternatively, isoprostanes might simultaneously activate mutually opposing prostanoid receptor populations. The role of DP receptors in opposing the constrictor effects of 8-iso-PGE₂ was ruled out by the failure of BW A868C to alter the concentration-effect curve to 8-iso-PGE₂ in any way (Figure 3b). Less direct evidence also argues against the involvement of EP₂, EP₄ or IP receptors. The inability of PGE₂ or cicaprost to reverse a U46619-induced contraction (Boersma *et al.*, 1999) suggest that none of these receptors is operationally expressed at a sufficient level to mediate the downturn in response to the isoprostanes. The arachidonic acid metabolite, anandamide relaxes vascular smooth muscle by a number of putative mechanisms, including stimulation of cannabinoid receptors (Mombouli *et al.*, 1999). The failure of anandamide to reverse U46619-induced contractions provides indirect evidence that isoprostanes do not relax the HUA at high concentrations through release of anandamide or mimicry of its actions.

A third possible explanation for the downturn in the concentration-effect curves to isoprostanes is receptor desensitization at high agonist concentrations. Two observations are consistent with this hypothesis. Firstly, the downturn was shifted by GR32191 (Figure 2), suggesting it was a TP receptor-mediated event. This argument is dependent upon the well-recognized selectivity of GR32191 (Lumley *et al.*, 1989; Coleman *et al.*, 1994), however, if the downturn is mediated by a heretofore-uncharacterized receptor, insensitivity to GR32191 cannot be assumed. Secondly, the partial agonist 8-iso-PGF_{1 α} did not produce a downturn. If receptor desensitization were the explanation, we would have expected U46619 to produce a downturn also, which it did not (Figure 2). The discrepancy is unlikely to result from isoprostanes and U46619 acting at different receptors since 8-iso-PGE₂ antagonized U46619, but not 5-HT. Differential effects of the agonists could be mediated by the same receptor if there is agonist-specific dual coupling of TP receptors to both excitatory and inhibitory pathways, however, we have no current evidence to support such a mechanism.

In conclusion, we have demonstrated that a number of isoprostanes cause constriction of the HUA, most likely by activation of TP receptors. Structural determinants of the potency of these compounds in this system reside in both the cyclopentane ring and the side chains. Free plasma concentrations of isoprostanes during oxidative stress associated with smoking (Morrow *et al.*, 1995) or cystic fibrosis (Collins *et al.*, 1999) are between 0.2 and 0.9 nM. Concentrations at the site of production are likely to be considerably higher and capable of TP receptor activation. Our data suggest that the isoprostanes should be considered to be important pharmacological agents, not mere markers for oxidative stress.

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