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Vasoconstrictor responses, and underlying mechanisms, to isoprostanes in human and porcine bronchial arterial smooth muscle

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1 We investigated the effects of five different isoprostanes (8-*iso* PGE_1, 8-*iso* PGE_2, 8-*iso* $PGF_{1\alpha}$, 8-*iso* $PGF_{2\alpha}$ and 8-*iso* $PGF_{2\beta}$) on vasomotor tone in human and porcine bronchial arterial tissues.

2 In the human bronchial arteries, 8-*iso* PGE_2 and 8-*iso* $PGF_{2\alpha}$ evoked powerful constrictions (magnitudes several fold greater than the responses to high millimolar KCl) with negative log concentration causing 50% excitation (EC_{50}) values of 6.8 and 6.5, respectively; 8-*iso* PGE_1 was less potent (EC_{50} not calculated, since a clear peak contraction was not obtained), while the other isoprostanes were largely ineffective. In the porcine arteries, on the other hand, all three F-ring isoprostanes as well as 8-*iso* PGE_2 evoked constrictor responses, although the peak magnitudes were approximately 50% of the KCl-evoked response; 8-*iso* PGE_2 and 8-*iso* $PGF_{2\alpha}$ were the most potent, with negative log EC_{50} values of 6.5.

3 We next sought to characterize the signaling pathways underlying the vasoconstrictor responses to 8-*iso* PGE_2, since this was the most potent of the isoprostanes we tested. These responses were largely reversed by the thromboxane A_2-selective (TP) prostanoid receptor antagonist ICI 192605 (10^{-8} M; 4(Z)-6-[(2,4,5 *cis*)2-(2-chlorophenyl)-4-(2-hydroxy phenyl)1,3-dioxan-5-yl]hexenoic acid) as well as by the nonspecific tyrosine kinase inhibitor genistein (10^{-5} and 10^{-4} M), and were reversed approximately 50% by the Rho-kinase inhibitor Y27632 (10^{-5} M; (+)-(*R*)-*trans*-4-(1-aminoethyl)-*N*-(pyridyl) cyclohexanecarboxamide dihydrochloride).

4 We conclude, therefore, that 8-*iso* PGE_2 constricts bronchial vasculature through the activation of TP receptors, which in turn trigger tyrosine kinase and Rho-kinase activities, resulting in powerful vasoconstriction. These findings are highly relevant to lung transplantation and to exercise-induced asthma.

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Abbreviations: EC_{50}, concentration causing 50% excitation; E_{max}, maximal effect; IC_{50}, concentration causing 50% inhibition; ICI 192605, (4(Z)-6-[(2,4,5 *cis*)2-(2-chlorophenyl)-4-(2-hydroxy phenyl)1, 3-dioxan-5-yl]hexenoic acid; TP, thromboxane A_2-selective prostanoid receptor; Y27632, (+)-(*R*)-*trans*-4-(1-aminoethyl)-*N*-(pyridyl) cyclohexanecarboxamide dihydrochloride

Introduction

Isoprostanes are arachidonic acid metabolites produced by peroxidative attack of membrane lipids (Janssen, 2001). These accumulate to substantial levels in many clinical conditions characterized in part by accumulation of free radicals and reactive oxygen species, including asthma (Montuschi *et al*., 1999; Wood *et al*., 2000; Kostikas *et al*., 2002), hypertension (Romero and Reckelhoff, 1999; Yamada *et al*., 1999; Cracowski *et al*., 2001) and ischemia – reperfusion injury (Mathews *et al*., 1994; Reilly *et al*., 1997; Fischer *et al*., 2000). For this reason, they are frequently used as markers of oxidative stress; however, many are now finding that these molecules are not inert, but in fact evoke powerful biological responses in an increasing array of cell types (Janssen, 2001; Janssen *et al*., 2000; 2001; Catalli *et al*., 2002; Janssen & Tazzeo, 2002; Zhang *et al*., 2003). In many cases, these biological effects can account in part for the various features and manifestations of those clinical conditions. Thus, it may be possible that the isoprostanes are playing somewhat of a causal role in those disease states.

Prostanoid formation is completely enzyme dependent, requiring arachidonic acid to be first liberated from the membrane by phospholipases, and then metabolized further by other enzymes including cyclooxygenase. Isoprostane formation, however, is quite different: in this case, the arachidonic acid is converted to an isoprostane directly following peroxidative attack of its unsaturated double bonds, and, more importantly, this can occur while arachidonic acid is still esterified within the membrane, as well as after it has been liberated (Janssen, 2001). Thus, isoprostanes can accumulate both within the fluid compartments surrounding the tissues and within the membranes of the tissues, to be liberated at some later time by phospholipases. Ischemia – reperfusion injury, then, is accompanied by an immediate rise in

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isoprostane levels, which can persist long after perfusion has been restored. This may be highly relevant during organ transplantation, which represents a form of ischemia – reperfusion injury.

We have previously reported the effects of isoprostanes on airway (Janssen *et al*., 2000; Catalli *et al*., 2002), pulmonary vascular (Janssen *et al*., 2001; Janssen & Tazzeo, 2002) and coronary vascular (Zhang *et al*., 2003) smooth muscles. In general, we found the isoprostanes to act on thromboxane A_2-selective prostanoid receptors (TPs), leading to the stimulation of tyrosine kinase and Rho-kinase activities, and resulting in powerful constriction of the muscle tissues. In the present study, we sought to investigate the effects of several different isoprostanes on human bronchial arterial tissues. Parallel studies were also performed using porcine bronchial artery.

Methods

Preparation of isolated tissues

Bronchial arteries were obtained from six surgical patients, each of whom gave their consent for use of their tissues; prior approval had also been obtained from the Research Ethics Board of our institution. Tracheae were obtained from pigs (20 – 90 kg) euthanized at a local abattoir, from which the overlying vasculature was excised. These vessels (0.5 – 1 mm outer diameter) were immediately put in ice-cold physiological solution and transported to the laboratory; these were cleaned of overlying connective and adipose tissues, then carefully cut into ring segments $\approx$4 mm long. No attempt was made to remove the endothelium.

Muscle bath technique

Ring segments were mounted into 3 ml muscle baths using stainless-steel hooks inserted into the lumen. One hook was fastened to a Grass FT.03 force transducer using silk thread (Ethicon 4-0); the other was attached to a plexiglass rod, which served as an anchor. Tissues were bathed in Krebs – Ringer's buffer (see below for composition) containing indomethacin (10 μM), bubbled with 95% O_2/5% CO_2, and maintained at 37°C; tissues were passively stretched to impose a preload tension of 0.5 – 0.6 g. Isometric changes in tension were amplified, digitized (two samples per second) and recorded on-line (DigiMed System Integrator, MicroMed, Louisville, KY, U.S.A.) for plotting on the computer. Tissues were equilibrated for 1 h before commencing the experiments, during which time the tissues were challenged with 60 mM KCl at least once to assess the functional state of each tissue; the mean magnitudes of the responses to KCl in human and porcine segments were 0.34 ± 0.16 g ($n = 6$) and 0.54 ± 0.10 g ($n = 10$), respectively.

Solutions and chemicals

Tissues were studied using Krebs – Ringer's buffer containing (in mM) NaCl, 116; KCl, 4.2; $CaCl_2$, 2.5; NaH_2PO_4, 1.6; $MgSO_4$, 1.2; $NaHCO_3$, 22; D-glucose, 11; and bubbled to maintain pH at 7.4. Indomethacin (10 μM) was also added to the latter to prevent generation of cyclooxygenase metabolites of arachidonic acid.

Isoprostanes were purchased from Cayman Chemicals (Ann Arbor, MI, U.S.A.) and ICI 192,605 (4(Z)-6-[(2,4,5 *cis*)2-(2-chlorophenyl)-4-(2-hydroxy phenyl)1,3-dioxan-5-yl]hexenoic acid) was a gift from Zeneca (Alderley Park, U.K.); all other chemicals were obtained from Sigma Chemical Company. Stock solutions (10 mM) were prepared in absolute ethanol (isoprostanes; Y27632) or DMSO (ICI 192605); the final bath concentration of DMSO and EtOH did not exceed 0.1%, which we have found elsewhere to have little or no effect on mechanical activity (Janssen *et al*., 2000; 2001).

Data analysis

The maximal contraction (E_{max}) produced with the highest concentration and the half-maximum effective concentration (EC_{50}) for the isoprostanes were interpolated from the individual concentration – effect curves. Responses are reported as means $\pm$ s.e.m.; n refers to the number of animals. Statistical comparisons were made using Student's t-tests; $P < 0.05$ was considered statistically significant.

Results

Examination of isoprostane responses

We first investigated the concentration – response relationships of these tissues to 8-*iso* PGE_1, 8-*iso* PGE_2, 8-*iso* $PGF_{1\alpha}$, 8-*iso* $PGF_{2\alpha}$ and 8-*iso* $PGF_{2\beta}$.

In the human bronchial artery, 8-*iso* PGE_2 and 8-*iso* $PGF_{2\alpha}$ evoked powerful vasoconstrictor responses, with peak magnitudes more than 300% larger than the responses to stimulation with 60 mM KCl. 8-*iso* PGE_2 was the more potent of the two, with log EC_{50} values of -6.8 ± 0.2 and -6.5 ± 0.1, respectively. 8-*iso* $PGF_{2\alpha}$ was somewhat less potent: an EC_{50} value for this molecule was not determined, as the responses did not appear to be maximal at the highest concentration tested. Interestingly, 8-*iso* $PGF_{1\alpha}$ was much less effective (achieving a peak magnitude at the highest concentration tested of roughly half that of the other isoprostanes mentioned above) and less potent (EC_{50} not determined), and 8-*iso* $PGF_{2\beta}$ was essentially ineffective, even though these compounds have a chemical structure nearly identical to that of 8-*iso* $PGF_{2\alpha}$.

The porcine bronchial artery responded somewhat differently to the isoprostanes. That is, contractile responses were evoked by all three of the F-ring compounds as well as by 8-*iso* PGE_2, and the peak magnitudes were smaller (approximately 50% of that to KCl) than those seen in human tissues (Figures 1 and 2; right panel), even though the mean magnitudes of the KCl responses *per se* did not differ markedly between the two tissue preparations (see Methods). Log EC_{50} values were determined for 8-*iso* PGE_2 and 8-*iso* $PGF_{1\alpha}$ (both -6.6 ± 0.1), but not for 8-*iso* $PGF_{2\alpha}$ or 8-*iso* $PGF_{2\beta}$.

Characterization of underlying signaling pathways

The contributions of various signaling pathways to these isoprostane-evoked responses were examined pharmacologically in the human tissues. 8-*iso* PGE_2 was used, as this was the most potent of the isoprostanes we assayed above. Tissues were first preconstricted with a maximally effective

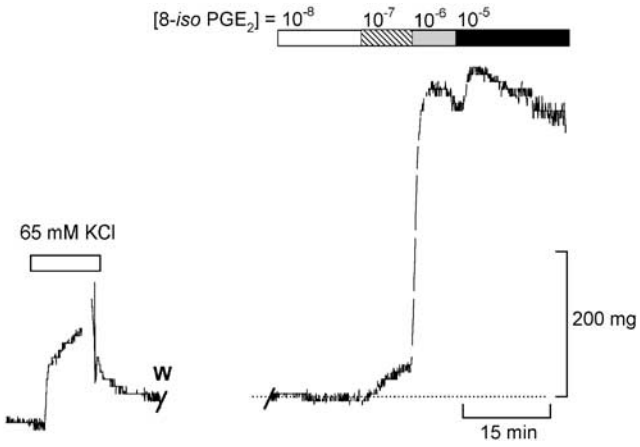
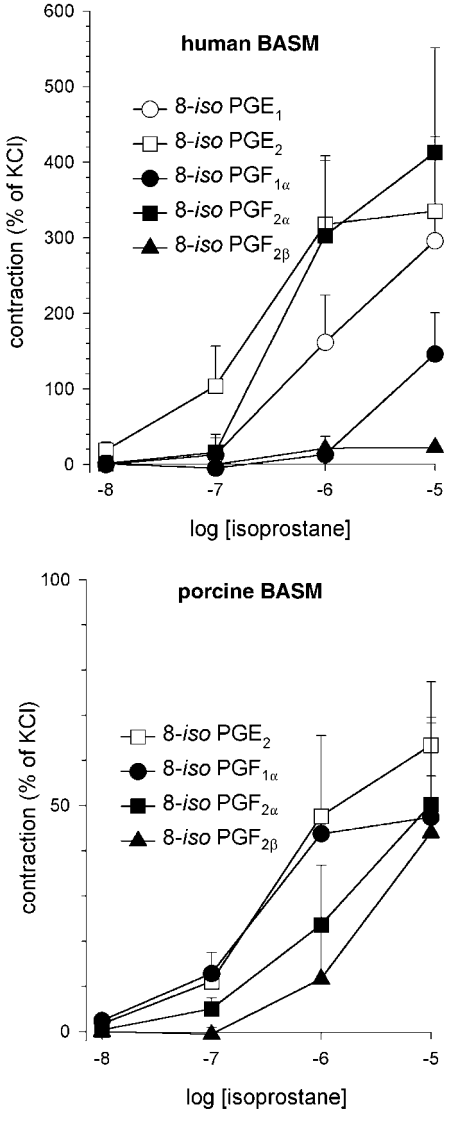


Figure 1 Representative tracing showing the vasoconstrictor response in a human bronchial arterial segment to 65 mM KCl, followed 30 min later (after several washes: **W**) by the responses to 8-*iso* PGE_2, added in 10-fold increasing concentrations in cumulative fashion, as indicated.



concentration of this agent (10^{-5} M), then challenged with a number of pharmacological antagonists.

ICI 192605 is a highly selective TP receptor blocker with pA_2 of approximately 8 (Brewster *et al*., 1988; Brown *et al*., 1990). In the present study, 8-*iso* PGE_2-evoked contractions were markedly and significantly reduced by 10^{-8} M ICI 192605, and abolished when the concentration of this blocker was increased 10-fold (Figures 3 and 4), indicating that they are likely directed through TP receptors.

Likewise, the nonspecific tyrosine kinase inhibitor genistein significantly reduced the responses when applied at 10^{-5} M concentrations, and eliminated them at 10^{-4} M (Figures 3 and 4).

Finally, we assessed the involvement of the monomeric G-protein Rho and its downstream effector molecule Rho-kinase in these responses using the highly selective Rho-kinase inhibitor Y27632 (10^{-6} and 10^{-5} M); this agent had little effect at 10^{-6} M, but reduced 8-*iso* PGE_2-evoked tone more than 60% at 10-fold higher concentrations.

Discussion

In this study, we characterized the vasoconstrictor responses of human and porcine bronchial arteries to five different isoprostanes, finding them to be highly reactive to the E-ring isoprostanes as well as to 8-*iso* $PGF_{2\alpha}$, but nearly nonresponsive to the other F-ring isoprostanes, even though the latter are nearly identical in chemical structure. For example, 8-*iso* $PGF_{2\alpha}$ and 8-*iso* $PGF_{2\beta}$ differ solely in the orientation of a single hydroxyl group on the central cyclopentane ring of these molecules, while 8-*iso* $PGF_{1\alpha}$ and 8-*iso* $PGF_{2\alpha}$ differ only with respect to having either one or two unsaturated double bonds in their lipid side chains, respectively. This high degree of stereochemical dependence in the responses speaks strongly toward a receptor-mediated signaling mechanism, rather than a nonspecific change such as a change in membrane lipid fluidity or redox effects. Consistent with this, we found the responses to be highly sensitive to the TP-receptor blocker ICI 192605, strongly indicating the involvement of these prostanoid receptors. Many others examining isoprostane-evoked constrictor responses have come to similar conclusions based on pharmacological sensitivity to a variety of TP-selective antagonists. However, others posit the hypothesis that isoprostanes act through a novel class of isoprostane-selective receptors that exhibit pharmacology remarkably similar to that of prostanoid receptors, largely due to the marked structural homology between prostanoids and isoprostanes (Janssen, 2001). It will not be possible to discriminate between these two possibilities until newer and more discriminating pharmacological tools are identified.

Interestingly, the receptors that respond to the isoprostanes in the porcine bronchial vasculature appear to be less discriminating, responding nearly identically to 8-*iso* PGE_2 and 8-*iso* $PGF_{1\alpha}$, whereas the other two F-ring isoprostanes were only slightly less potent. The porcine tissues also differed

Figure 2 Mean vasoconstrictor responses evoked by 8-*iso* PGE_1, 8-*iso* PGE_2, 8-*iso* $PGF_{1\alpha}$, 8-*iso* $PGF_{2\alpha}$ and 8-*iso* $PGF_{2\beta}$ in human (top panel) and porcine (bottom panel) bronchial artery ring segments. Responses were expressed as a percent of the response to 65 mM KCl, $n = 5-8$.

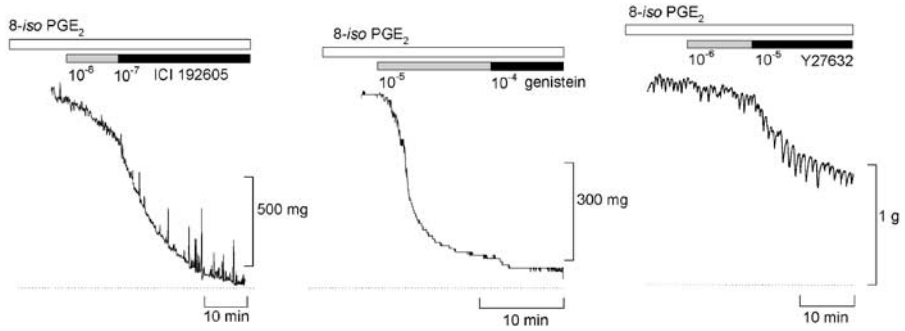


Figure 3 Representative tracings showing reversal of 8-*iso* PGE_2-evoked tone in human bronchial arterial ring segments by ICI 192605 (left), genistein (middle) or Y27632 (right) at concentrations indicated. Dotted lines indicate resting tone before addition of 8-*iso* PGE_2 (10^{-5} M).

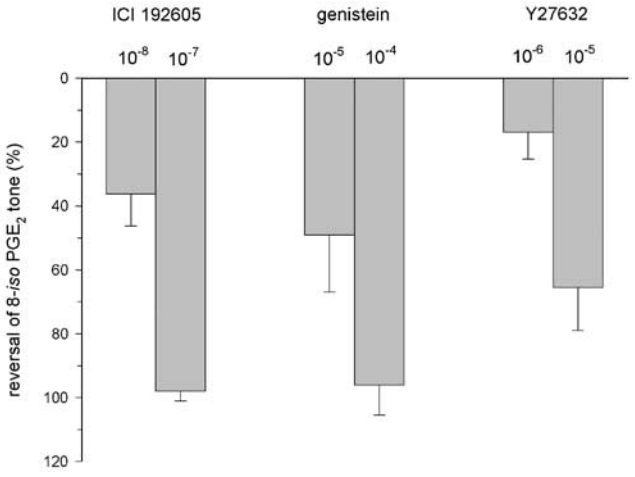


Figure 4 Mean reversals of 8-*iso* PGE_2-evoked tension in response to addition of Y27632 (10^{-6} and 10^{-5} M), genistein (10^{-5} and 10^{-4} M) or ICI 192605 (10^{-8} and 10^{-7} M), $n=4$.

from the human tissues with respect to the efficacy of these compounds: all four isoprostanes exerted maximal contractions which were 50 – 70% of the response to high millimolar KCl, while the responses in human tissues were as much as 400% of the response to KCl.

TP receptor-mediated responses generally do not involve electromechanical coupling mechanisms (i.e. membrane depolarization with consequent voltage-dependent Ca^{2+} influx). Instead, TP receptors generally activate signaling pathways that enhance the Ca^{2+} sensitivity of the contractile apparatus (Coleman *et al*., 1994; Narumiya *et al*., 1999). These signaling pathways include the monomeric G-protein Rho and its effector molecule Rho-kinase, which goes on to phosphorylate the downstream target molecule myosin light-chain phosphatase (Somlyo & Somlyo, 2000). In the present study, we found 8-*iso* PGE_2-evoked responses to be markedly reduced by the Rho-kinase inhibitor Y27632. Although the concentration required to achieve this effect (10^{-5} M) is considerably higher than the published concentration causing 50% inhibition (IC_{50}) for this drug (800 nM; Davies *et al*., 2000), it should be pointed out that such IC_{50} values are obtained in *in vitro* kinase assays, which are not hindered by the membrane permeability issues that plague studies carried out using intact tissues. A recent study that compared the specificity of several commonly used protein kinase inhibitors found that Y27632, even at a concentration of 10^{-5} M, exerts little or no inhibitory effect (less than 10% inhibition) on 20 different kinases, including protein kinases A and C, SAP kinases, and several mitogen-activated kinases (Davies *et al*., 2000). We feel, then, that our data strongly indicate the involvement of Rho-kinase in 8-*iso* PGE_2-evoked responses. The fact that Y27632 did not completely abolish the responses may indicate that some other signaling pathway is also involved in mediating these responses, such as TP-receptor-mediated release of internally sequestered Ca^{2+}: we did not test this possibility in the present study.

Interestingly, we also found the nonspecific tyrosine kinase inhibitor genistein to be completely effective in reversing the 8-*iso* PGE_2-evoked responses. Unfortunately, our data do not allow us to speculate on which of the many tyrosine-kinase activities might be important in this respect.

Isoprostanes accumulate in many different clinical conditions characterized in part by oxidative stress (Janssen, 2001), including ischemia – reperfusion injuries. Lung transplantation represents a form of an ischemia – reperfusion injury. The success of this clinical intervention depends in large part upon restoration of adequate circulation within the transplanted lung. However, the newly transplanted lungs can represent a major source of isoprostanes introduced into the circulation of the recipient host, and our data suggest that these isoprostanes can seriously compromise reperfusion. It may be useful, then, to employ free radical scavengers during harvesting of the donor lungs to prevent the generation of isoprostanes and/or to use TP receptor blockers to prevent their vasoconstrictor effects after transplantation. Our findings may also be relevant to exercise-induced asthma, since many postulate an important role for the bronchial circulation in this clinical condition, and others have shown isoprostanes to be elevated in asthma (Montuschi *et al*., 1999; Wood *et al*., 2000) and following exercise (Kirschvink *et al*., 1999; Hinchcliff *et al*., 2000).

In conclusion, we found human and porcine bronchial arterial vasculature to exhibit a profound vasoconstrictor response to several isoprostanes, particularly 8-*iso* PGE_2 and 8-*iso* $PGF_{2\alpha}$. The responses to 8-*iso* PGE_2 appear to be mediated through TP receptors and stimulation of tyrosine kinase and Rho/Rho-kinase activities.

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