Involvement of TP and EP₃ Receptors in Vasoconstrictor Responses to Isoprostanes in Pulmonary Vasculature

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ABSTRACT
Although isoprostanes generally act on smooth muscle via TXA₂-selective prostanoid receptors (TPs), some suggest other prostanoid receptors or possibly even a novel isoprostane-selective receptor might be involved. We studied contractions to several isoprostanes in porcine pulmonary vasculature using organ bath techniques. 8-iso-prostaglandin E₂ (PGE₂) was the most potent and efficacious of the isoprostanes, with a log EC₅₀ of −7.0 ± 0.2 in the pulmonary artery and −6.8 ± 0.2 in the pulmonary vein. The responses to all the isoprostanes were essentially completely blocked by the TP receptor antagonist ICI 192605 (4(Z)-6-[2-(2,4,5-trifluorophenyl)-4-(2-hydroxyphenyl)]-3-dioxan-5-yl)hexenoic acid), and the equilibrium dissociation constants for ICI 192605 competing with U46619 or 8-iso-PGE₂ were both ≈2 nM, indicating that isoprostane-evoked responses involve primarily TP receptors. Only 8-iso-PGE₂ was able to evoke substantial contractions in the presence of ICI 192605 and only in the pulmonary vein. The EC₅₀ of these ICI 192605-insensitive responses was −6.1 ± 0.2. Using a variety of prostanoid antagonists, we found the pulmonary vein lacked excitatory PGF₂α-selective prostanoid receptor (FP) or PGD₂-selective prostanoid receptor (DP) but expressed excitatory EP₃ receptors. The ICI 192605-insensitive responses to 8-iso-PGE₂ were unaffected by the EP₁ antagonist SC-19220 [8-chloro-debenz[b,f] [1,4]oxazepine-10(11H)-carboxy-(2-acetyl)] hydrazine; 10⁻⁶ M), but were antagonized by the less selective DP/EP3/EP₁ antagonist AH6809 (6-isopropoxy-9-oxoanthene-2-carboxylic acid; 10⁻⁵ M) or by cyclopiazonic acid (10⁻⁶ M; depletes the internal Ca²⁺ store). Our data indicate that, whereas 8-iso-PGE₂ constricts pulmonary vasculature primarily through TP receptors, a substantial portion of this response is also directed through EP₃ receptors or possibly a novel isoprostane receptor.

Isoprostanes are metabolites of polyunsaturated fatty acids, such as arachidonic acid, and are produced by peroxidative attack of lipid membranes. They accumulate to substantial levels in a wide variety of clinical and experimental settings associated with oxidative stress, including systemic (Romero and Reckelhoff, 2000) and pulmonary (Jankov et al., 2000) hypertension, and during exposure to agents that are associated with hypertension, such as subpressor doses of angiotensin II (Haas et al., 1999; Reckelhoff et al., 2000), inflammatory mediators (Jourdan et al., 1997b, 1999), and growth factors (Natarajan et al., 1996). For this reason, they are used extensively as markers of oxidative stress in general and membrane lipid peroxidation in particular. However, they are much more than inert markers; there is a growing body of literature describing powerful biological effects of these autacoids on smooth muscle (Fukunaga et al., 1994a,b), platelets (Longmire et al., 1994; Yin et al., 1994), and endothelial cells (Yura et al., 1999). We have previously characterized the effects of several different isoprostanes on pulmonary vascular smooth muscle, finding them to exert vasoconstriction via activation of tyrosine and Rho kinases (Janssen et al., 2001).

The excitatory effects of 8-iso-PGE₂ and 8-iso-PGF₂α are sensitive to a wide variety of agents, which are structurally distinct but all exhibit TP-receptor blocking activity, including ICI 192605 [4(Z)-6-[2-(2,4,5-cis)-2-(2-chlorophenyl)-4-(2-hydroxyphenyl)]-3-dioxan-5-yl]hexenoic acid (Jourdan et al., 1997a; Janssen et al., 2000, 2001), SQ 29548 (Banerjee et al., 1998), and 6-isopropoxy-9-oxoanthene-2-carboxylic acid (10⁻⁶ M) or by cyclopiazonic acid (10⁻⁶ M; depletes the internal Ca²⁺ store). Our data indicate that, whereas 8-iso-PGE₂ constricts pulmonary vasculature primarily through TP receptors, a substantial portion of this response is also directed through EP₃ receptors or possibly a novel isoprostane receptor.

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ABBREVIATIONS: ICI 192605, 4(Z)-6-[2-(2,4,5-cis)-2-(2-chlorophenyl)-4-(2-hydroxyphenyl)]-3-dioxan-5-yl]hexenoic acid; SC-19220, 8-chloro-debenz[b,f][1,4]oxazepine-10(11H)-carboxy-(2-acetyl)hydrazine; AH6809, 6-isopropoxy-9-oxoanthene-2-carboxylic acid; PG, prostaglandin; CPA, cyclopiazonic acid; SQ 29548, [1S-(1α,2β,3β,3a,4a)]-7-[[2-[phenylamino]carbonyl]hydrazinomethyl]-7-oxabicyclo[2.2.1]hept-2-yl-5-hepnoic acid; L 670596, (−)-6,8-difluoro-9-p-methyisylnonyl benzyl-1,2,3,4-tetrahydrocarbolin-1-yl-acetic acid; L 657925, 9,11-dimethyl-methano-1,12-methano-16-(3-iodo-4-hydroxy)-13-aza-15n,β-ω-tetranorthromboxane A₃; GR 32191, [1R-(1α-2β,3β,5α,6α,7α,9α,10α,11β,12β,13α,14β,15α,16α)-7-[1,1′-bibiphenyl]-4-yl)methoxy]-3-hydroxy-2-((1-piperidinyl)cyclopentyl)-4-4-heptanoic acid] hydrochloride; BMS 180291, [1S-(exo,exo)]-2-[3-(4-9-pentylamino)carbonyl]-2-oxazolyl]-7-oxabicyclo[2.2.1]hept-2-yl[methyl]-benzenepropanoic acid; BW245C, (4S)-(3-[GR,S]-3-cyclohexyl-3-hydroxypropyl)-2,5-dioxo-4-imidazolidine heptanoic acid.
1992; Fukunaga et al., 1993b; Mohler et al., 1996; Elmhurst et al., 1997; John and Valentin, 1997; Wagner et al., 1997; Sametz et al., 2000), L 657925 (Wagner et al., 1997), L 670596 (Elmhurst et al., 1997; Wagner et al., 1997), GR 32191 (Elmhurst et al., 1997; Oliveira et al., 2000), and BMS 180291 (Mohler et al., 1996). Thus, the bulk of the data would strongly suggest that TP receptors are involved.

However, certain findings have prompted some to suggest that isoprostanes act through some other receptor, perhaps even a novel isoprostane-selective receptor. For example, in aortic smooth muscle, 8-iso-PGF$_{2\alpha}$ displaces the binding of TP receptor-acting ligands with much less potency (two to three orders of magnitude less) than the homologids but stimulates IP$_3$ production and [H]thymidine incorporation with a higher potency than TP agonists (Fukunaga et al., 1992, 1993a, b). Binding experiments have indicated the presence of both low-affinity and high-affinity binding sites for 8-iso-PGF$_{2\alpha}$ (Fukunaga et al., 1993a, 1995, 1997; Yura et al., 1999), which could represent the TP receptor and a unique isoprostane receptor, respectively. Finally, astroglia, endothelial cells, and microvessel smooth muscle cells are all able to respond to the TP agonist U46619, whereas 8-iso-PGF$_{2\alpha}$ stimulates only the former two but not the smooth muscle cells (Hou et al., 2000); in platelets, 8-iso-PGF$_{2\alpha}$ is only a partial agonist on TP receptors (Morrow et al., 1992; Longmire et al., 1994; Yin et al., 1994). Collectively, these data point toward another receptor for the isoprostanes distinct from the TP receptor. There are limited data that isoprostanes can act on other prostanoid receptors. 12-iso-PGF$_{2\alpha}$ is a powerful agonist for FP receptors (Kunapuli et al., 1997), and there is evidence that some isoprostane responses may involve EP receptors (Elmhurst et al., 1997; Sametz et al., 2000; Unmack et al., 2001). In this study, we examined the actions of isoprostanes on pulmonary vascular smooth muscle using a variety of agonists and antagonists of prostanoid receptors.

**Materials and Methods**

**Preparation of Isolated Tissues and Single Cells.** Lobes of lung were obtained from pigs (20–90 kg) euthanized at a local abattoir and immediately put in ice-cold physiological solution for transport to the laboratory. After removing the overlying parenchyma and connective tissue, the pulmonary artery and vein were excised and cut into ring segments (~4- to 5-mm long (o.d. ~2–10 mm); no attempt was made to remove the endothelium.

**Muscle Bath Technique.** Ring segments were mounted into 3-mI 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 Plexiglas rod, which served as an anchor. Tissues were bathed in Krebs-Ringer 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 ~1 g (determined to allow maximal responses). Isometric changes in tension were amplified, digitized (two samples per second), and recorded on-line (DigIMed System Integrator; MicroMed, Louisville, KY) 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. Tissues were then washed, and the preload was readjusted just prior to onset of the actual study (i.e., at the end of the equilibrium period).

**Solutions and Chemicals.** Tissues were studied using Krebs-Ringer buffer containing 116 mM NaCl, 4.2 mM KCl, 2.5 mM CaCl$_2$, 1.6 mM NaH$_2$PO$_4$, 1.2 mM MgSO$_4$, 22 mM NaHCO$_3$, 11 mM d-glucose, 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 and SC-19220 [8-chloro-dibenzo[b,f][1,4]oxazepine-10(11H)-carboxy-(2-acetyl)hydrazide] were purchased from Cayman Chemical (Ann Arbor, MI), and ICI 192605 was a gift from Zeneca Pharmaceuticals plc (Alderley Park, UK); all other chemicals were obtained from Sigma-Aldrich (St. Louis, MO). Stock solutions (10 mM) were prepared in absolute ethanol (isoprostanes, U46619; prostanoids, AH6809) or dimethyl sulfoxide (ICI 192605, SC-19220, cyclopiazonic acid); the final bath concentration of dimethyl sulfoxide and ethanol did not exceed 0.1%, which we have found elsewhere to have little or no effect on mechanical activity.

**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. The equilibrium dissociation constant (K$_d$) for ICI 192605 was calculated using the equation: K$_d$ = [B]/[DR − 1], where [B] is the concentration of the antagonist and DR (dose ratio) is the ratio of EC$_{50}$ in the presence and absence of antagonist.

Responses were standardized relative to responses to either 60 mM KCl or to 10$^{-6}$ M U46619, as indicated and are reported as mean ± S.E.M; n refers to the number of animals. Statistical comparisons were made using analysis of variance (with Newman-Keuls post hoc test), as appropriate; P < 0.05 was considered statistically significant.

**Results**

**Excitatory Effects of Isoprostanes in Porcine Pulmonary Vein.** We first examined the ability of various isoprostanes to elevate tone in porcine pulmonary vasculature; isoprostanes tested included 8-iso-PGE$_1$, 8-iso-PGE$_2$, 8-iso-PGF$_{1\alpha}$, 8-iso-PGF$_{2\alpha}$, and 8-iso-PGF$_{2\beta}$.

8-iso-PGE$_2$ was the most potent and efficacious of the isoprostanes (Fig. 1). The log EC$_{50}$ values for 8-iso-PGE$_2$ in the artery and the vein were ~7.0 ± 0.2 and ~6.8 ± 0.2, respectively. Supramaximal concentrations of 8-iso-PGE$_2$ consistently reversed tone in the arterial segments but not the vein segments (Fig. 1).

In both artery and vein, 8-iso-PGE$_1$ was much less potent than 8-iso-PGE$_2$, requiring approximately 10-fold higher concentrations to evoke a similar response as 8-iso-PGE$_2$ (Fig. 1), and the F-ring isoprostanes were generally even less effective, having little or no effect at micromolar concentrations and achieving only ~50% KCl response at 10$^{-5}$ M (Fig. 1).

**Involvement of Both TP and Non-TP Receptors in Mediating Isoprostane Contractions.** In many other smooth muscle preparations, the excitatory effects of isoprostanes are sensitive to antagonists of TP receptors (see Introduction). Therefore we examined the effect of the TP receptor antagonist ICI 192605 on isoprostane-evoked contractions in porcine pulmonary vein. Tissues were first preconstricted with the TP receptor agonist U46619 to standardize responses (10$^{-6}$ M) and treated with ICI 192605 (10$^{-6}$ M) for 20 min, after which the dose-response relationships for the different isoprostanes were reexamined (Fig. 2).

ICI 192605 completely inhibited U46619-evoked tone (data not shown). This agent also blocked completely the responses to all the isoprostanes in the pulmonary artery, as well as
those to 8-iso-PGF$_{1a}$ and 8-iso-PGF$_{2a}$ in the pulmonary vein. However, 8-iso-PGE$_2$ was still able to evoke a response of ≈25% that of the U46619 response (which is comparable with the response to 60 mM KCl) in the pulmonary vein pretreated with ICI 192605; likewise, 8-iso-PGF$_{2a}$ could still evoke a response of ≈8% that of the U46619 response (Fig. 2). It was not clear if these responses were maximal at 10$^{-5}$ M (the highest concentration tested) but, assuming that to be the case, the log EC$_{50}$ value for 8-iso-PGE$_2$ in the presence of ICI 192605 was $-6.1 \pm 0.2$.

**Derivation of Inhibitory Constant for ICI 192605.** To test the possibility that the isoprostane-evoked contractions in the presence of 10$^{-6}$ M ICI 192605 are due to incomplete block of the TP receptors, we ascertained the $K_B$ value for ICI 192605 in this preparation. Tissues were pretreated with vehicle or ICI 192605 (10$^{-9}$, 10$^{-8}$, or 10$^{-7}$ M) for 20 min and challenged with increasing concentrations of U46619 or 8-iso-PGE$_2$ in cumulative fashion (both 10$^{-9}$–10$^{-5}$ M; $n = 5$).

ICI 192605 caused a rightward shift in the dose-response relationships for U46619 (Fig. 3A); $K_B$ values derived in the presence of 10$^{-9}$, 10$^{-8}$, or 10$^{-7}$ M ICI 192605 were 1.4 × 10$^{-9}$ M, 2.9 × 10$^{-9}$ M, and 2.5 × 10$^{-9}$ M, respectively. Likewise, ICI 192605 displaced the 8-iso-PGE$_2$ dose-response relationship in similar fashion (Fig. 3B). $K_B$ values of 2.1 × 10$^{-9}$ and 1.9 × 10$^{-9}$ M were obtained in the presence of 10$^{-9}$ and 10$^{-8}$ M ICI 192605, respectively. In fact, 10$^{-7}$ M ICI 192605 inhibited 8-iso-PGE$_2$ responses to such an extent that a distinct $E_{\text{max}}$ was not obtained; however, assuming an $E_{\text{max}}$ of 115% KCl (comparable with that for the other data), $K_B$ was 5.9 × 10$^{-9}$ M. The potency of ICI 192605 against 8-iso-PGE$_2$ responses (log $K_B$ of $-2-5$ nM) argues strongly against the possibility that 8-iso-PGE$_2$ evokes constriction in the presence of 10$^{-6}$ M ICI 192605 by merely displacing the latter receptor blocker.

**Involvement of Other Prostanoid Receptors in Mediating Non-TP Contractions.** It may be that 8-iso-PGE$_2$ is able to evoke constriction in the presence of ICI 192605 through an action on some prostanoid receptor other than TP receptors. We therefore characterized the sensitivity of this preparation to various prostanoid receptor agonists to determine which other excitatory prostanoid receptors might be present. Agonists included prostaglandin D$_2$, prostaglandin...
E₂, prostaglandin F₂α, BW245C (DP-selective), sulprostone (EP₃-selective), and fluprostenol (FP-selective). Tissues were pretreated with ICI 192605 (10⁻⁶ M) to rule out confounding effects of these prostanoids on TP receptors.

Prostaglandins E₂ and F₂α were both able to markedly elevate tone, although the former autacoid was considerably more potent than the latter (Fig. 4). PGE₂ responses increased in magnitude over the concentration range 10⁻⁸ to 10⁻⁶ M, with a log EC₅₀ value of 7.1 ± 0.2, comparable with the published pD₂ value for PGE₂ acting at an EP receptor; at 10⁻⁵ M, however, PGE₂ responses decreased in magnitude (i.e., PGE₂ seemed to cause relaxation). PGF₂α responses, on the other hand, increased in magnitude over the concentration range 10⁻⁷ to 10⁻⁵ M; since a plateau was not attained, we could not calculate an EC₅₀ value (but it is clearly greater than 1 μM). Sulprostone was even more potent (EC₅₀ value of 7.2 ± 0.2) and effective than both these prostaglandins, eliciting contractions nearly twice as large as those evoked by PGE₂ or PGF₂α.

Contractions were not evoked by fluprostenol, PGD₂, or BW245C (Fig. 4). In fact, the DP-selective agonists both evoked relaxations, with BW245C being particularly effective in this respect. Thus, the porcine pulmonary vein seems to be endowed with excitatory EP₃ and TP receptors, as well as inhibitory DP receptors, but not with FP receptors.

**Do Non-TP Contractions Involve EP Receptors?** It may be, then, that 8-iso-PGE₂ is also acting through excitatory EP receptors, in addition to the TP receptors characterized above. EP₁ receptors couple to Gq and phospholipase C and mediate excitation via IP₃-induced Ca²⁺ release, whereas EP₃ receptors couple to Gi and thereby inhibit adenylate cyclase (Coleman et al., 1994; Narumiya et al., 1999). We therefore examined the effect of the DP/EP₁/EP₂ receptor antagonist AH6809 and the EP₁-selective antagonist SC-19220, as well as the effect of depleting the internal Ca²⁺ pool using cyclopiazonic acid. Tissues were pretreated with ICI 192605 (10⁻⁶ M) to rule out confounding effects of these agonists on TP receptors (n = 4–7).

SC-19220 had no statistically significant effect on the concentration-response relationships for 8-iso-PGE₂ or PGE₂ (Fig. 5). The less selective blocker AH6809, however, had no effect on PGE₂ responses but significantly antagonized those evoked by 8-iso-PGE₂ (Fig. 6). Isoprostane responses were abolished by cyclopiazonic acid (Fig. 6).
Discussion

For over a decade, isoprostanes have been recognized as being useful as markers of oxidative stress in clinical and experimental settings. Now it is also known that these autacoids exert powerful biological effects. Some have described the vasoconstrictor effects of 8-iso-PGF$_2\alpha$ on pulmonary vasculature (Hill et al., 1997; John and Valentin, 1997) and on other vascular beds (Mohler et al., 1996; Wagner et al., 1997; Oliveira et al., 2000). However, very few have compared the effects of a wide range of isoprostanes, as we have done in this study. We found several E- and F-ring isoprostanes to increase tone in pulmonary vasculature to varying degrees. In particular, the most potent and efficacious of these was 8-iso-PGE$_2$, much more so than 8-iso-PGF$_2\alpha$, the isoprostane upon which most previous studies of isoprostane effects have focused solely. In fact, others have described vasodilatory responses to 8-iso-PGF$_2\alpha$ in the rat pulmonary artery (Jourdan et al., 1997a). We were again struck by the very high degree of specificity in these actions of the isoprostanes; 8-iso-PGE$_2$ differs only very slightly from 8-iso-PGF$_2\alpha$ (a ketone versus a hydroxyl group, respectively, on the central cyclopentane ring) or from 8-iso-PGE$_1$ (two versus one unsaturated bonds, respectively) but differs tremendously with respect to biological activity. This argues strongly for a receptor-mediated mechanism, rather than some nonspecific mechanism such as altered membrane fluidity.

In general, isoprostanes seem to mediate their effects on vascular smooth muscle via TP receptors. Consistent with this, we found that nearly all the excitatory responses of the isoprostanes in the pulmonary vasculature were prevented by pre-exposure to the TP receptor blocker ICI 192605. Moreover, we obtained similar values of $K_B$ for ICI 192605 acting against U46619 (10$^{-5}$ M) and against 8-iso-PGE$_2$ (10$^{-5}$ M), and both of these values compare favorably with published literature values for this antagonist (Narumiya et al., 1999). These data indicate that, in the porcine pulmonary vein, ICI 192605, U46619, and 8-iso-PGE$_2$ compete at a common receptor.
Although the actions of the other isoprostanes were essentially completely prevented by TP receptor blockade, this was not true of 8-iso-PGE₂ in the porcine pulmonary vein; only this isoprostane was able to evoke substantial contraction in the maintained presence of ICI 192605 (at concentrations three orders of magnitude above the \( K_d \) value we obtained for this blocker in this preparation). Once again, this high degree of compound-related specificity speaks toward a receptor-mediated mechanism. Others have provided limited evidence that isoprostanes can activate other prostanoid receptors, including EP (Elmhurst et al., 1997; Sametz et al., 2000; Ungrin et al., 2001) and FP (Kunapuli et al., 1997) receptors.

8-iso-PGE₂ has been shown elsewhere to be only a partial agonist at TP receptors in the coronary artery (Kromer and Tippins, 1996) and in platelets (Morrow et al., 1992; Longmire et al., 1994; Yin et al., 1994). If this were true also of the pulmonary vein, this might complicate interpretation of the effects of ICI 192605 on 8-iso-PGE₂-evoked responses. We found, however, that in tissues studied concurrently with either the TP agonist U46619 or with 8-iso-PGE₂, the former evoked a peak response of \( \approx 120\% \) KCl, whereas the response to the latter was already \( \approx 110\% \) KCl before it reached a peak (we were unable to use sufficiently high concentrations to reach a peak). This observation is not consistent with partial agonism. Furthermore, partial agonism would not explain the different sensitivity of these responses to CPA; the responses that persist in the presence of ICI 192605-sensitive responses are unaffected (Janssen et al., 2001) and in platelets (Morrow et al., 1992; Longmire et al., 1994). If this were the case, this would not explain the different sensitivity of these responses to CPA, which effectively depletes the internal Ca²⁺ pool. In a previous study of the effects of isoprostanes in human and canine pulmonary vasculature (Janssen et al., 2001), CPA was largely ineffective against the TP receptor-mediated effects of 8-iso-PGE₂.

In conclusion, we find that 8-iso-PGE₂ evokes vasoconstriction in the pulmonary vasculature via an action on TP receptors. In the pulmonary vein, 8-iso-PGE₂ can also act upon another type of excitatory receptor, likely EP₁ receptors or possibly a unique isoprostane receptor. This may represent an important mechanism during hypertension, which is characterized in part by production of large amounts of isoprostanes (Jankov et al., 2000; Romero and Reckelhoff, 2000).

**References**


John GW and Valentín JP (1997) Analysis of the pulmonary hypertensive effects of


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