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invited review

Isoprostanes: an overview and putative roles in pulmonary pathophysiology

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Janssen, L. J. Isoprostanes: an overview and putative roles in pulmonary pathophysiology. *Am J Physiol Lung Cell Mol Physiol* 280: L1067–L1082, 2001.—Isoprostanes are produced during peroxidation of membrane lipids by free radicals and reactive oxygen species. Initially, they were recognized as being valuable markers of oxidative stress, and in the past 10 years, dozens of disease states and experimental conditions with diverse etiologies have been shown to be associated with marked increases in urinary, plasma, and tissue levels of isoprostanes. However, they are not just mere markers; they evoke important biological responses on virtually every cell type found within the lung, and these responses exhibit compound-, tissue-, and species-related variations. In fact, the isoprostanes may mediate many of the features of the disease states for which they are used as indicators. In this review, I describe the chemistry, metabolism, and pharmacology of isoprostanes, with a particular emphasis on pulmonary cell types, and the possible roles of isoprostanes in pulmonary pathophysiology.

oxidative stress; arachidonic acid; lipid peroxidation; inflammation; smooth muscle; endothelium; platelet

JUST OVER A DECADE AGO, Morrow et al. (106) first described the nonenzymatic production of a series of prostaglandin-like compounds during peroxidation of membrane phospholipids by free radicals and reactive oxygen species. Since that seminal discovery, many studies have pointed out the usefulness of these compounds, particularly 8-*iso*-$PGF_{2\alpha}$, as markers of oxidative stress under many different clinical and experimental conditions (reviewed in Refs. 59, 112, 144). Concurrently, others began to show that they also evoke important biological responses, first in vascular smooth muscle and platelets and then in many other cell types (see CELLULAR ACTIONS). From these two avenues of investigation, a large and rapidly growing literature has developed, beginning in 1992 with the first three papers by Morrow and colleagues (102, 103, 106) to use the term "isoprostane" in referring to these compounds and growing exponentially ever since (see Fig. 1). In response to this outpouring of data, there have been a number of reviews of the existing literature (112, 144); however, these have emphasized particularly the chemistry of the isoprostanes (e.g., their structure, genesis, nomenclature, and detection), with relatively less discussion about their biological actions. I (59) have previously reviewed some of the biological effects of isoprostanes on smooth muscle. However, since the writing of that review, the literature has doubled again (Fig. 1), and it is now known that isoprostanes have effects on virtually every cell type found in the lungs; moreover, there have been some important new advances in our understanding of the signaling pathways by which isoprostanes act. In this review, I collate these data and bring them to bear particularly on pulmonary physiology and pathophysiology.

PRODUCTS OF LIPID PEROXIDATION

For detailed descriptions of isoprostane chemistry (e.g., their generation and nomenclature), the reader is directed to those earlier reviews written by Morrow and Roberts (112) and Rokach et al. (144). Briefly, though, oxygen-centered radicals such as peroxide and

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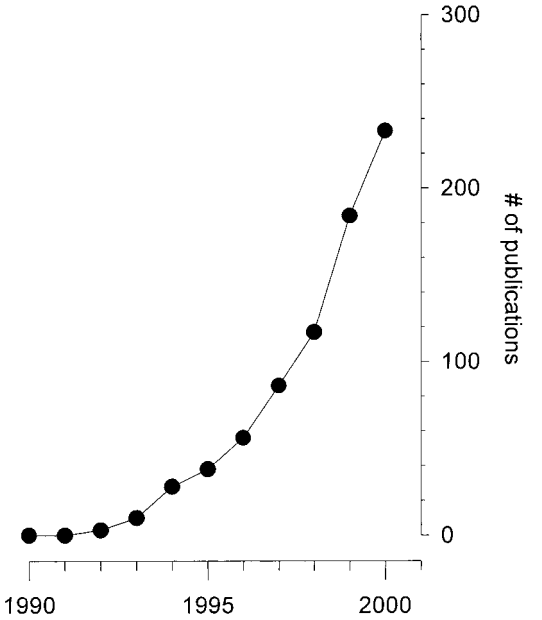

Fig. 1. A MedLine search for titles and abstracts containing the term "isoprostane" shows an exponential growth in publications over the past 10 years.

superoxide can react with the unsaturated bonds of arachidonic acid, leading to the formation of as many as four different bicycloendoperoxide intermediates differing in whether the peroxide group has been added to C-6/C-8, to C-9/C-11, or to C-12/C-14 (Fig. 2). Reduction of the intermediates leads to four different classes of isoprostane regioisomers, all composed of a cyclopentane ring with two alkyl chains *cis* to one another (Fig. 2). The prostanoids, on the other hand, have the two side chains *trans* to one another (Fig. 3).

In addition to its well-described role in the production of prostanoids, cyclooxygenase (COX) can also contribute to isoprostane production in vascular smooth muscle (68, 69), endothelium (167), platelets (74, 127, 132–134), monocytes (125, 129, 131), macrophages (149, 160), and mesangial cells (74). However, it is not entirely clear whether the isoprostanes are direct products of COX or arise secondarily to an "overflow" of COX-derived reactive oxygen species. Some recent data (167) suggest the latter may be the case; 8-*iso*-$PGF_{2\alpha}$ production in endothelial cells was shown to be COX dependent (in that it could be inhibited by indomethacin or aspirin) and also sensitive to catalase but not to superoxide dismutase, suggesting that hydrogen peroxide (H_2O_2) originating from COX was responsible.

Reactive nitrogen species may also participate in the production of isoprostanes. For example, nitric oxide (NO; on its own a weak oxidizing agent) reacts with superoxide to produce peroxynitrite ($ONOO^-$), a highly reactive and cytotoxic molecule (136). Exposure of myelin suspensions (162), low-density lipoproteins (101), or human plasma (101) to peroxynitrite leads to lipid peroxidation and generation of isoprostanes; this effect is not mimicked by NO or superoxide alone (162). BN-80933 (a new neuroprotective compound combining potent antioxidant and selective neuronal NO synthase inhibitory actions) significantly reduced 8-*iso*-$PGF_{2\alpha}$ levels in a rat model of cerebral ischemia-reperfusion injury (87); however, a follow-up study indicated that this effect of BN-80933 might be due more to its antioxidant properties than to its ability to inhibit NO synthesis (30). *N*-nitro-L-arginine methyl ester (a NO synthase inhibitor) greatly inhibited cytokine-induced production of 8-*iso*-$PGF_{2\alpha}$ from pulmonary artery smooth muscle (69); this effect did not involve modulation of COX activity by NO because cytokine-induced release of PGE_2 from these cells was unaffected.

Polyunsaturated fatty acids other than arachidonic acid are also vulnerable to reactive oxygen species and produce isoprostanes. For example, in addition to the four classes of F_2-isoprostanes that can arise from arachidonic acid (eicosatetraenoic acid), peroxidation of eicosapentaenoic acid is predicted to lead to the generation of six classes of F_3-isoprostanes (117, 144), α-linolenic and γ-linolenic acids to two classes of E_1- and F_1-isoprostanes, respectively (13, 123), and docosahexaenoic acid to eight classes of D_4-isoprostanes and eight classes of E_4-isoprostanes (140) (Fig. 2). Each of these classes comprise up to eight racemic isomers, leading to an astounding number of isoprostane molecules!

Although dozens (hundreds?) of different isoprostane molecules can be produced by such free radical-mediated peroxidation, the group of isoprostane molecules most commonly referred to in the literature are those in which peroxidation occurs at C-9/C-11 of arachidonic acid, producing the family of 8-isoprostanes. Moreover, the particular member of this family that is by far the most prominent in the literature is 8-*iso*-$PGF_{2\alpha}$ (Fig. 3). However, there is now evidence that D- and E-ring isoprostane species can also be produced in vivo and can accumulate to levels only slightly less than those of 8-*iso*-$PGF_{2\alpha}$ (i.e., 1–2 orders of magnitude higher than circulating levels of prostaglandins) (82, 105, 107, 108, 112, 113, 140). Also, like their PGD_2 and PGE_2 cousins, dehydration of D- and E-ring isoprostanes in vitro and in vivo has produced A_2- or J_2-isoprostanes (17, 18). The widely differing pharmacological actions of some of these isoprostanes are only just beginning to be understood (see CELLULAR ACTIONS).

The bicyclic endoperoxide intermediates that give rise to the isoprostanes can also rearrange to form other highly reactive molecules such as the isolevuglandins (12, 145) and isothromboxanes (104, 144), and isoleukotrienes have also been identified (48), but these are not discussed in this review.

MARKERS OF OXIDATIVE STRESS

For many reasons, isoprostanes are very well suited as biochemical markers of oxidative stress. First, they can be measured accurately down to picomolar levels (9, 152, 166) with analytic techniques such as HPLC (8), gas chromatography-mass spectrometry (1, 45, 108, 111, 133, 143, 144, 152), or radioimmunoassay (15, 166). The former two techniques are supremely

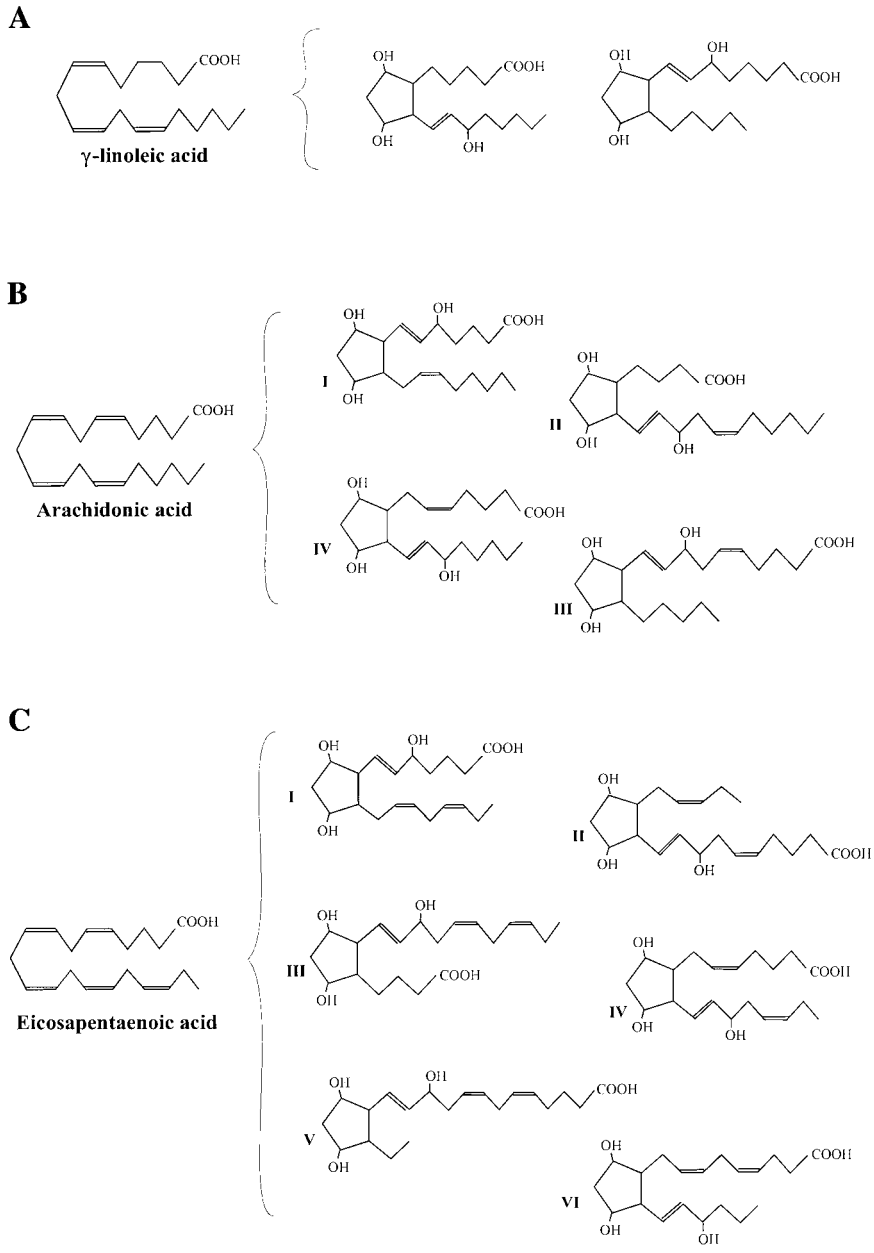

Fig. 2. Peroxidative attack of unsaturated fatty acids leads to a variety of isoprostanes: 2 classes of F_1-isoprostanes from γ-linoleic acid (*A*); 4 classes of F_2-isoprostanes from arachidonic acid (*B*), and 6 classes of F_3-isoprostanes from eicosapentaenoic acid (*C*). For each class, there are 8 diastereomers; stereochemistry is not indicated.

able to discriminate between the different types of isoprostanes but require extensive preparation of the material (e.g., phospholipid extraction, alkaline hydrolysis) and expensive instrumentation (111). Radioimmunoassays are somewhat easier to perform and are widely commercially available; however, many of these are not able (or have not yet been shown to be able) to distinguish between the prostanoids and the isoprostanes, let alone between the different types of isoprostanes. One group (15) has described a radioimmunoassay that uses a monoclonal antibody directed against 8-*iso*-$PGF_{2\alpha}$ conjugated to BSA; this assay exhibits a detection limit of 23 pmol/l, with very low cross-reactivity ratios of ~10:1 for 8-*iso*-$PGF_{2\beta}$, 100:1 for $PGF_{2\alpha}$, 8-*iso*-$PGF_{3\alpha}$, and 8-*iso*-15-keto-13,14-dihydro-$PGF_{2\alpha}$, and 10,000:1 for 8-*iso*-15-keto-$PGF_{2\alpha}$ and 15-keto-13,14-dihydro-$PGF_{2\alpha}$. It is even possible to use isoprostanes to assess oxidative stress or damage in specific target organs of interest (e.g., via biopsy); for example, in carbon tetrachloride-induced hepatoxicity, isoprostanes were markedly elevated in the liver, lung, and kidney but not in the brain or heart (103).

Second, they are stable in isolated samples of body fluids; their presence was first detected in fresh and stored samples of plasma and urine (3, 106) and then more recently in cerebrospinal (46), pericardial (86), and bronchoalveolar (43, 98) fluids. A particularly exciting recent development has been the demonstration that they can be detected in breath condensates (15, 100), providing an exceedingly noninvasive route for their measurement.

Third, and most importantly, their measured values do not exhibit diurnal variations (53, 166) but do vary markedly in clinical and experimental conditions char-

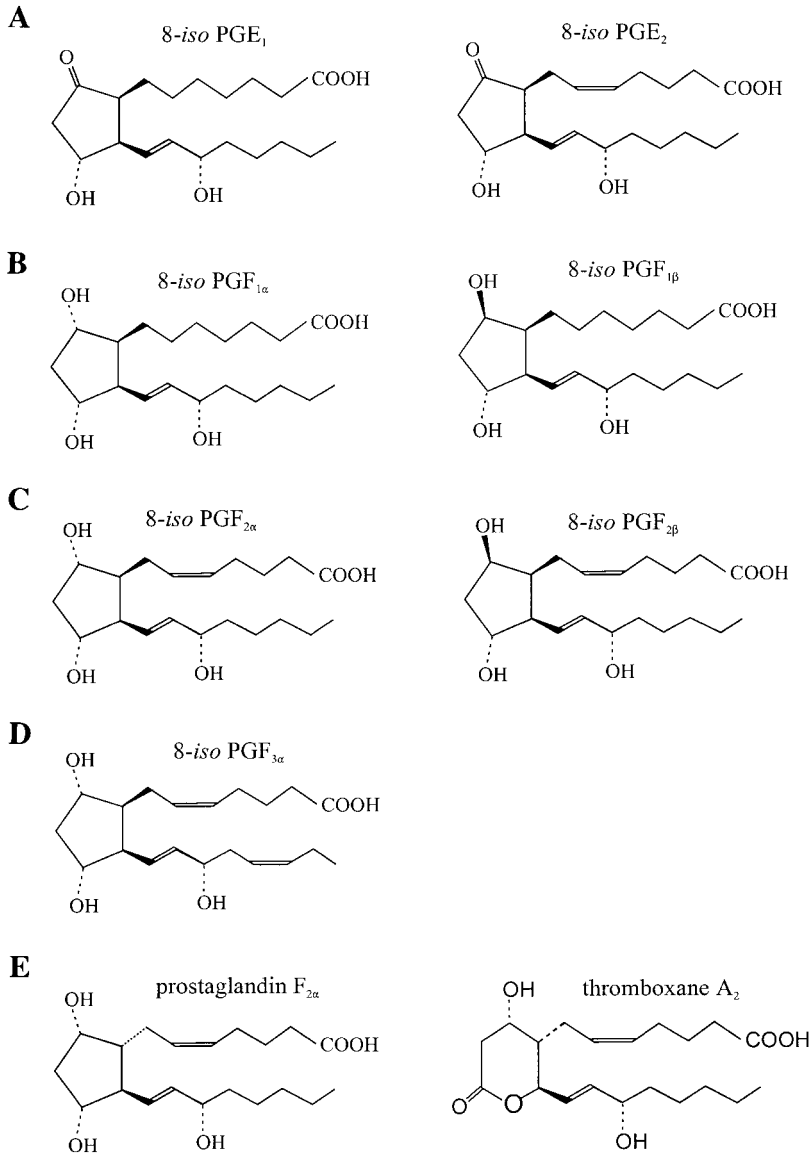

Fig. 3. Chemical structures of E-ring (*A*), F_1-ring (*B*), F_2-ring (*C*), and F_3-ring (*D*) isoprostanes and the prostanoids $PGF_{2\alpha}$ and thromboxane A_2 (*E*).

acterized by oxidative stress, and these values closely parallel disease severity.

In this way, a long list of respiratory-related pathologies has come to be associated with isoprostanes. 8-*iso*-$PGF_{2\alpha}$ is generated in substantial amounts in otherwise "normal" individuals exposed to cigarette smoke (19, 28, 105, 127, 141), allergen (32, 99), ozone (50), or hyperoxia (160, 161) and during ventilated ischemia (11) and is markedly elevated in patients with a wide variety of lung diseases such as asthma (32, 35, 97), chronic obstructive pulmonary disease (128), interstitial lung disease (98), cystic fibrosis (23, 100), acute lung injury including acute respiratory distress syndrome (15), and severe respiratory failure in infants (43).

Similarly, a number of cardiovascular conditions feature marked elevations in isoprostane levels, including renal (158), cerebral (87), and myocardial (29, 94, 142) ischemia-reperfusion injury; unstable angina (21); heart failure (86); coronary heart disease (90); acute ischemic stroke (163); hypercholesterolemia (26); atherosclerosis (131, 135); and preeclampsia (5, 156). Isoprostane levels are increased in pulmonary hypertension (20) and during exposure to agents that are associated with hypertension, such as subpressor doses of angiotensin II (47, 116, 139), inflammatory mediators (68, 69), and growth factors (116).

Finally, 8-*iso*-$PGF_{2\alpha}$ has been found to be a useful marker of oxidative damage and lipid peroxidation in disease states as diverse as Alzheimer's disease (96), multiple sclerosis (46), diabetes mellitus (27, 45), systemic lupus erythematosus (58), and several hepatic pathologies (10, 103, 110, 130) as well as in experimental settings ranging from humans in space flight (157) to sled dogs during endurance exercise training (55)!

STORAGE, RELEASE, AND METABOLISM

Although the synthesis of prostanoids by COX first requires that arachidonic acid be liberated from the

plasma membrane by phospholipases (22, 115), this is not necessarily true for the production of isoprostanes by free radicals; there is now good reason to believe that the latter can act on free fatty acids as well as on those still esterified to membrane phospholipids (102).

For example, isoprostanes have been identified within phospholipids extracted from surgical specimens of lung (11), atherosclerotic vasculature (119, 131, 135), lymphatics (118), brain (140), and preeclamptic decidua basalis (156). Levels of free and esterified isoprostanes in plasma from smokers remain significantly elevated above baseline for weeks after cessation of smoking, even though other markers of smoking such as urinary nicotine and cotinine become undetectable (105, 141). In carbon tetrachloride-induced hepatotoxicity (103), isoprostanes begin to accumulate almost immediately in phospholipids extracted from the liver (and to some extent in the lungs and kidney) and shortly thereafter in the circulation; the latter reach a peak at 4 h (77-fold) and remain markedly elevated above baseline at 24 (21-fold) and 48 (10-fold) h, indicating prolonged oxidative stress and/or on-going release of esterified isoprostanes. The presence of the isoprostanes within the membrane is expected to have profound effects on membrane fluidity as discussed in MEDIATORS OF PULMONARY PATHOPHYSIOLOGY? *Membrane properties*.

Isoprostanes esterified in the membrane may then be released at some later time by phospholipases (144) because they are suitable substrates for bee venom phospholipase A_2 (102, 108), and phospholipase A_2 inhibitors (mepacrine) partially reduce growth factor-induced isoprostane release in cultured cells (116).

Once isoprostanes are generated and released into the circulation, they appear to be quickly metabolized and eliminated. In one study done in the rabbit (8), it was shown that a bolus injection of 8-*iso*-$PGF_{2\alpha}$ was rapidly distributed in the circulation, with a half-life of distribution of ~1 min, and then was rapidly eliminated, with a half-life of ~4 min. Using HPLC, they went on to show that 8-*iso*-$PGF_{2\alpha}$ was degraded to several β-oxidized metabolites, culminating in formation of α-tetranor-15-keto-13,14-dihydro-8-*iso*-$PGF_{2\alpha}$. It was hypothesized that the metabolism of this isoprostane followed a sequential pathway similar to that ascertained for prostaglandins, involving *1*) oxidation of C-15 by 15-hydroxyprostaglandin dehydrogenase and *2*) reduction of the C-13,14 double bond by Δ^{13}-reductase followed by *3*) β-oxidation to α-tetranor-15-keto-13,14-dihydro-8-*iso*-$PGF_{2\alpha}$. The parent 8-*iso*-$PGF_{2\alpha}$ and its metabolites began to accumulate in the urine within 20 min; 80% of the original radioactive label was present in the urine by 4 h. These findings parallel closely those made in an earlier study (143) using a single human volunteer and a rhesus monkey; 75% of the original radioactivity was found in the urine 4.5 h after infusion of the parent compound, with the primary metabolite being 2,3-dinor-5,6-dihydro-$PGF_{2\alpha}$. A more recent study done by another group (19) showed that the metabolites also include 2,3-dinor-$PGF_{2\alpha}$ in humans and 2,3,4,5-tetranor-5,6-dihydro-$PGF_{2\alpha}$ in rats.

Other more chemically reactive isoeicosanoids such as the A_2-isoprostanes (18) and isolevuglandins (12) covalently adduct to proteins and are therefore protected from rapid clearance via the kidney.

CELLULAR ACTIONS

Although there is an abundant literature pertaining to isoprostanes as markers of oxidative stress, we are only just now beginning to appreciate the wide variety of actions that the isoprostanes exert on the function of many different cell types. Thus isoprostanes should be considered not just "markers" but perhaps also as "mediators" of disease (see MEDIATORS OF PULMONARY PATHOPHYSIOLOGY?). However, the current literature describing the effects of isoprostanes is woefully inadequate at this time.

First, almost all the studies to date have focused on 8-*iso*-$PGF_{2\alpha}$ despite the growing awareness that hundreds of isoprostane species can potentially exist, and evidence for the in vivo generation of some of these is now in hand (see PRODUCTS OF LIPID PEROXIDATION). Thus this fixation on 8-*iso*-$PGF_{2\alpha}$ is distracting and detrimental; consider the results if the bulk of our understanding of prostanoid pharmacology came from studies of prostacyclin alone [or thromboxane (Tx) A_2 alone or some other single eicosanoid] and the effects of the other prostanoids were extrapolated from them. Comparisons of the effects of 8-*iso*-$PGF_{2\alpha}$ and 8-*iso*-PGE_2 in a given tissue often reveal important differences; 8-*iso*-PGE_2 evokes an electrophysiological response in epithelial cells (33), relaxation in canine airway smooth muscle (62), biochemical responses in calcifying vascular cells (124), and altered sensory neuronal function (34), whereas 8-*iso*-$PGF_{2\alpha}$ is ineffective in all these respects. Only a handful of studies (62, 63, 79, 121, 146) have been done with isoprostanes other than 8-*iso*-$PGF_{2\alpha}$ and 8-*iso*-PGE_2; these, too, describe important differences between the isoprostanes. For example, in platelets pretreated with SQ-29548 to eliminate confounding effects via TxA_2 (TP) receptors, eight different isoprostanes were tested and only 8-*iso*-PGE_2 markedly increased cAMP accumulation (83). These marked differences in pharmacology between nearly identical chemical species attest to a highly discriminative mechanism, such as the involvement of specific membrane receptors, rather than to nonspecific effects, such as altered membrane fluidity (see *Classic prostanoid receptors or unique isoprostane receptors?* and *Membrane properties*).

Second, most of the existing pharmacological data were obtained in rodents, primarily the rat. Relatively few studies have been done with human tissues (25, 62, 71, 118, 121, 154), and there is strong evidence for species-related differences in the responses to isoprostanes: *1*) in contrast to the airways in other species, those in the rabbit (54) and dog (62) do not constrict in response to 8-*iso*-PGE_2 or 8-*iso*-$PGF_{2\alpha}$; *2*) rat pulmonary arteries exhibit a relaxant response to 8-*iso*-

$PGF_{2\alpha}$ (67), in contrast to the vasoconstriction seen in other species; and *3*) 8-*iso*-PGE_2 constricts porcine and bovine but not ovine coronary arteries (76).

Third, the majority of the literature pertains to the effects of isoprostane on vascular smooth muscle and platelets; their effects on other cell types are only just now beginning to be understood.

Clearly, then, there is currently a very limited understanding of the physiological effects of these autacoids!

Classic prostanoid receptors or unique isoprostane receptors? PGs were the first group of arachidonic acid metabolites to be identified and characterized in detail. There is now an extensive literature for these compounds that is not reviewed here; for this, the reader is referred elsewhere (22, 115). Briefly, however, there are five major prostanoid receptor types, termed D-prostanoid (DP), E-prostanoid (EP), F-prostanoid (FP), I-prostanoid (IP), and TP receptors that are each approximately an order of magnitude more selective for either PGD_2, PGE_2, $PGF_{2\alpha}$, PGI_2, or TxA_2, respectively (22, 115). The EP receptors can be further subclassified into four subtypes and the TP receptors into two subtypes (22, 115).

The selectivity of these receptors arises from interactions with the ketone or hydroxyl groups on the central cyclopentane ring as well as on the hydrocarbon chains. Although a different orientation (*cis* versus *trans*) of otherwise identical hydrocarbon chains on the cyclopentane ring might, on the surface, appear to be only a minor difference, it leads to either a roughly flat planar, densely packed molecule (the isoprostanes) or a wedge-shaped, arrowheaded molecule (prostanoids) (Fig. 3). Clearly, this would profoundly affect the way in which these molecules interact with membrane receptors. Thus it should not be assumed that 8-*iso*-$PGF_{2\alpha}$ necessarily acts on FP receptors and/or that 8-*iso*-PGE_2 acts on one of the EP receptors. In fact, many different groups have found the excitatory effects of both isoprostanes to be sensitive to a wide variety of agents that are structurally distinct but which all exhibit TP receptor-blocking activity, including ICI-192605 (62, 63, 67, 71), SQ-29548 (4, 33, 38, 66, 70, 76–78, 88, 95, 147, 164), L-657925 (164), L-670596 (33, 81, 164), GR-32191 (33, 121), BAY U 3405 (120), LCB-2853 (91), BMS-180291 (95, 174), and BM-13505 (76). Thus the bulk of the data would strongly suggest that TP receptors are involved.

However, mounting evidence suggests that there might be a novel isoprostane-selective receptor. To better understand the signaling mechanisms underlying the TP receptor-mediated responses that Banerjee et al. (4) and Takahashi et al. (158) had observed in vascular smooth muscle, Morrow, Roberts, and colleagues went on to examine the effects of 8-*iso*-$PGF_{2\alpha}$ on platelets, which also express TP receptors. To their surprise, Longmire et al. (85), Morrow et al. (109), and Yin et al. (170) found that it is only a weak agonist for TP receptors and, in fact, acts primarily as an antagonist of the TP receptors on the platelet. After considering other possible explanations, they cautiously proposed the existence of a novel isoprostane receptor that happens to also show sensitivity to TP receptor antagonists. Concurrent studies by Fukunaga and colleagues (37, 38) using aortic smooth muscle showed that 8-*iso*-$PGF_{2\alpha}$ displaces the binding of TP receptor-acting ligands with much less potency (2–3 orders of magnitude less) than the homoligands themselves but stimulated inositol 1,4,5-trisphosphate [Ins(1,4,5)P_3] production and [^{3}H]thymidine incorporation with a higher potency than the TP agonists, leading them to also conclude that its effects were therefore probably transduced through a distinct receptor with close homology to the TP receptor. Later, they (40) described binding data and pharmacological responses to U-46619 in vascular smooth muscle that were not mimicked by 8-*iso*-$PGF_{2\alpha}$ and provided evidence for low-affinity and high-affinity binding sites for 8-*iso*-$PGF_{2\alpha}$, which they interpreted to represent the TP receptor and a unique isoprostane receptor, respectively. High- and low-affinity binding sites for 8-*iso*-$PGF_{2\alpha}$ have also been identified in endothelial cells (39, 171). A recent study (57) of brain microvessel smooth muscle cells described U-46619 responses that were not mimicked by 8-*iso*-$PGF_{2\alpha}$ even though both acted on TP receptors in astroglia and endothelial cells from the same preparation. These accumulating data support the proposal that isoprostanes interact with a novel isoprostane-selective receptor on vascular smooth muscle while also acting nonselectively as an antagonist on TP receptors on the vascular smooth muscle and platelets. However, this view is not held by all (147, 174).

12-*iso*-$PGF_{2\alpha}$ (but not 8-*iso*-$PGF_{2\alpha}$) is a powerful agonist for FP receptors (79), and there is evidence that some isoprostane responses may involve EP receptors (33, 147).

Vascular smooth muscle. The biological actions of 8-*iso*-$PGF_{2\alpha}$ were first investigated in the renal vasculature in which they elicit profound vasoconstriction (106). Since then, 8-*iso*-$PGF_{2\alpha}$ and 8-*iso*-PGE_2 have been found to have excitatory effects on virtually every vascular smooth muscle in which they have been tested, as might be predicted by the widespread distribution of TP receptors on the vasculature including the aorta (77, 164, 174) and carotid (95), coronary (76, 78, 94, 153), cerebral (56), pial (56), pulmonary (4, 66, 70, 164), renal (38, 106, 158), portal (88), umbilical (121), and retinal (81, 91) arteries.

Recently, Janssen et al. (63) examined the responses to seven different isoprostanes, two of the E-ring series (8-*iso*-PGE_1 and 8-*iso*-PGE_2) and several different F-ring compounds (8-*iso*-$PGF_{1\alpha}$, 8-*iso*-$PGF_{1\beta}$, 8-*iso*-$PGF_{2\alpha}$, 8-*iso*-$PGF_{2\beta}$, and 8-*iso*-$PGF_{3\alpha}$), in small-diameter pulmonary arteries and veins; this was done with ~10th-order (diameter of 0.5–3.0 mm) human vasculature and 4th- to 6th-order (diameter 2–6 mm) canine vasculature. 8-*iso*-PGE_2 was by far the most potent and effective of these isoprostanes in human pulmonary arteries and was roughly equivalent to 8-*iso*-$PGF_{2\alpha}$ in human and canine pulmonary veins, evoking responses two to three times larger than those to po-

tassium chloride. The other isoprostanes were either weakly effective (8-*iso*-PGE_1, 8-*iso*-$PGF_{1\alpha}$, and 8-*iso*-$PGF_{2\beta}$) or noneffective (8-*iso*-$PGF_{1\beta}$ and 8-*iso*-$PGF_{3\alpha}$), attesting to the high degree of specificity of the mechanism transducing these responses. The canine pulmonary artery was essentially unresponsive to any of the isoprostanes. Consistent with the literature, these contractions were sensitive to the TP receptor antagonist ICI-192605; they were also sensitive to the EP receptor antagonist (AH-6809), but we believe this represents a nonspecific effect of AH-6809 on TP receptors because these tissues do not exhibit any excitatory response to the EP-selective agonist PGE_2 (63).

Three major signaling pathways have been found to be involved in mediating isoprostane contractions in vascular smooth muscle. On one hand, some studies of rat vasculature describe 8-*iso*-$PGF_{2\alpha}$-triggered activation of phospholipase C and elevation of cytosolic levels of Ins(1,4,5)P_3 (37, 38) as well as of 8-*iso*-$PGF_{2\alpha}$-evoked contractions, which are sensitive to the protein kinase C inhibitor calphostin C and to blockers of voltage-dependent Ca^{2+} channels (164). Others, however, show that the isoprostane does not alter intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) (40, 57, 63) but instead greatly enhances mitogen-activated protein (MAP) kinase activity when applied at nanomolar concentrations in rat aorta (40) and in porcine carotid artery (95). Unfortunately, the assays used to show this effect (phosphorylation of a peptide substrate) could not definitively identify the MAP kinase nor its downstream target(s).

My laboratory (63) also investigated the mechanisms underlying isoprostane-mediated excitation-contraction coupling in pulmonary vasculature using patch-clamp electrophysiological, fura 2 fluorometric, and standard muscle bath-based pharmacological techniques and also found that the isoprostanes caused little or no change in $[Ca^{2+}]_i$ in single cells (although other agonists such as caffeine or phenylephrine did) nor were the isoprostane contractions affected by blocking the voltage-dependent Ca^{2+} channels (with nifedipine) or depleting the internal Ca^{2+} store (with cyclopiazonic acid). These findings led us to pursue the possibility that the contractions involve a change in the Ca^{2+} sensitivity of the contractile apparatus (rather than mobilization of Ca^{2+} per se). In smooth muscle, this phenomenon seems to generally involve activation of Rho and Rho kinase, which go on to phosphorylate and thereby suppress myosin light chain phosphatase (155); other pathways under investigation include protein kinase C, extracellular signal-regulated kinase (ERK) 1/ERK2, and p21-activated kinase (PAK) (24, 36, 155). We found (63) that the isoprostane contractions were insensitive to two different blockers of protein kinase C with different mechanisms of action (chelerythrine and calphostin C, which interact with protein kinase C at its regulatory and catalytic sites, respectively). They were also insensitive to inhibitors of ERK1/ERK2 (PD-98059) or p38-activated kinase (SB-203580) but were essentially abolished by two structurally different blockers of Rho kinase (Y-27632 and HA-1077). Thus isoprostanes act in the pulmonary vasculature via Rho and Rho kinase, which presumably suppress myosin light chain phosphatase activity, resulting in a net accumulation of phosphorylated myosin light chains (Fig. 4). Moreover, the responses are blocked by the tyrosine kinase inhibitor genistein (but not by its less active analog daidzen), suggesting that tyrosine kinases also play a key role, possibly in triggering the sequence of events outlined above; my laboratory is currently investigating the possible targets that are tyrosine phosphorylated during stimulation with isoprostanes.

Finally, in contrast to the two direct mechanisms summarized above, data from porcine retinal (81) and periventricular brain (57) microvessels suggest that isoprostanes may activate vascular smooth muscle indirectly via the endothelium: *1*) 8-*iso*-$PGF_{2\alpha}$ elevates $[Ca^{2+}]_i$ in the retinal vascular endothelium but not within the smooth muscle per se and stimulates release of endothelin (81) and/or TxA_2 (57), *2*) vasoconstriction is blocked by inhibitors of the synthesis of those autacoids (i.e., the endothelin-converting enzyme inhibitor phosphoramidon or the TxA_2 synthase inhibitor CGS-12970, respectively) or by inhibitors of their receptors [i.e., the endothelin (ET) A receptor blocker BQ-123 or the TP receptor blocker L-670596, respectively], and *3*) blockade of receptor-operated Ca^{2+} influx inhibited both the change in $[Ca^{2+}]_i$ in the endothelium and contraction in the smooth muscle.

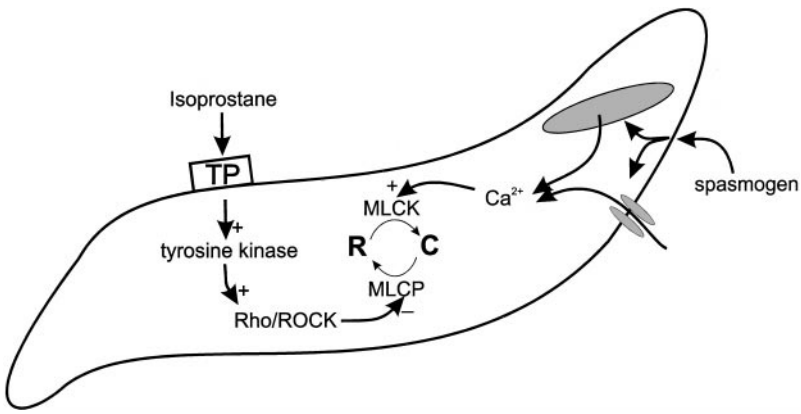

Fig. 4. Proposed isoprostane signaling pathway. Many spasmogens act by mobilizing Ca^{2+}, leading to stimulation of myosin light chain kinase (MLCK). Isoprostanes, on the other hand, appear to act through tyrosine kinase, Rho, and Rho kinase (ROCK), leading to decreased activity of myosin light chain phosphatase (MLCP). In both cases, the net response is increased levels of phosphorylated myosin light chain and contraction. TP, thromboxane A_2 receptor; R, relaxation; C, contraction.

There are no published data yet on the effects of isoprostane on ion conductances in any smooth muscle tissue. However, in ongoing studies with patch-clamp electrophysiological techniques, my laboratory has found that 8-*iso*-PGE_2 suppresses K^+ currents in airway and pulmonary arterial smooth muscle cells (D. Zhang, unpublished observations).

Airway smooth muscle. Several groups have described TP antagonist-sensitive excitatory responses to 8-*iso*-PGE_2 and 8-*iso*-$PGF_{2\alpha}$ in airway smooth muscle of the rat (70), guinea pig (71), human (62, 71), and pig (Janssen, unpublished observations). Interestingly, rabbit (54) and canine (62) trachealis do not exhibit a contractile response, whereas canine bronchi show only moderate contractions (62), consistent with the distribution of TP receptors in the airways of different species (22, 115). Once again, Janssen et al. (62) found that 8-*iso*-PGE_2 was generally 10–100 times more potent than 8-*iso*-$PGF_{2\alpha}$, the isoprostane most commonly studied elsewhere. Furthermore, they were amazed at the specificity of these responses. For example, two isoprostanes that are nearly identical in structure to 8-*iso*-$PGF_{2\alpha}$, 8-*iso*-$PGF_{2\beta}$ (differs solely in the orientation of a single hydroxyl group on the cyclopentane ring) and 8-*iso*-$PGF_{3\alpha}$ (differs only in possessing one extra double bond; Fig. 3), lacked any spasmogenic activity; in fact, 8-*iso*-$PGF_{3\alpha}$ evoked dose-dependent relaxations (Fig. 5). In ongoing studies of the mechanisms underlying these bronchoconstrictor responses in human airway smooth muscle, my laboratory has identified many aspects in common with the signaling pathway underlying vasoconstriction; that is, the responses are sensitive to the TP receptor antagonist ICI-192605, the tyrosine kinase inhibitor genistein, and the Rho kinase inhibitor Y-27632 (Janssen, unpublished observations).

The E-ring isoprostanes 8-*iso*-PGE_1 and 8-*iso*-PGE_2 are powerful bronchodilators in canine airway smooth muscle, mimicking the effects of PGE_2 itself (62). The latter finding suggests that these responses are mediated through EP receptors. Experiments are ongoing, aimed at elucidating the signaling pathways involved (Janssen, unpublished observations).

Platelets. 8-*iso*-PGE_2 and 8-*iso*-$PGF_{2\alpha}$ stimulate shape change and aggregation in platelets (75, 83, 85, 109, 134) and reduce the antiadhesive effects of NO (93). In fact, several groups hypothesized that elevated levels of isoprostanes contribute to the increased activation of platelets (as indicated by plasma levels of TxA_2 metabolites) seen in hypercholesterolemia (26), acute ischemic stroke (163), diabetes mellitus (27), and

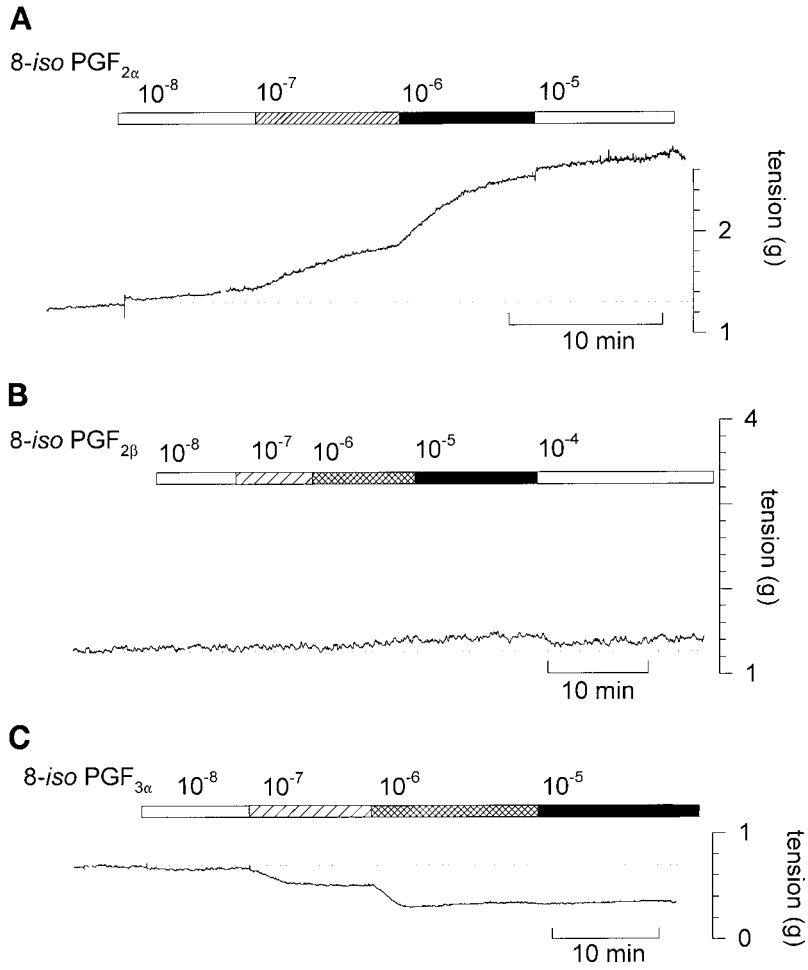

Fig. 5. Mechanical responses in human airway smooth muscle to 3 different isoprostanes; although these isoprostanes differ only slightly in structure (see Fig. 3), they evoke substantially different responses in human airways. Nos. over bars, molar concentration. [Reproduced with permission from Janssen et al. (62).]

preeclampsia (156). Interestingly, 8-*iso*-$PGF_{3\alpha}$ interferes with ADP-induced platelet aggregation (83).

As outlined above, these effects are exerted through TP receptors that couple to the G proteins G_q and G_{11} and cause generation of Ins(1,4,5)P_3 and elevation of $[Ca^{2+}]_i$ (72, 73, 134). 8-*iso*-PGE_1 also stimulates adenylate cyclase activity (83), whereas several other isoprostanes including 8-*iso*-PGE_2 and 8-*iso*-$PGF_{2\alpha}$ do not (83).

Epithelium. There have been no studies yet on the effect of isoprostanes on airway epithelium. However, in gastrointestinal epithelium (33), 8-*iso*-PGE_2 and/or 8-*iso*-$PGF_{2\alpha}$ alter membrane ion channel activity (as indicated by increased short-circuit current), apparently via EP receptors (because their effects are not blocked by TP receptor antagonists but are reduced by desensitization of EP receptors).

Endothelium. Diverse responses to isoprostanes have been described in cultured endothelial cells. In the endothelium of microvessels, 8-*iso*-$PGF_{2\alpha}$ stimulates DNA synthesis and proliferation, ET-1 mRNA and protein expression, TxA_2 synthesis, Ins(1,4,5)P_3 formation, and elevation of $[Ca^{2+}]_i$ (39, 57, 81), the latter effect being, in part, sensitive to a blocker of receptor-operated Ca^{2+} influx (SKF-96365) but not to one of voltage-dependent Ca^{2+} influx (nifedipine). Another study (172) with human umbilical vein endothelial cells showed that 8-*iso*-PGE_2 and/or 8-*iso*-$PGF_{2\alpha}$ did not increase expression of intercellular adhesion molecule-1 or E-selectin but did induce some change in the endothelial cells, which, in turn, was sufficient to enhance neutrophil adhesion (see *Inflammatory cells*). At the whole animal level, isoprostanes increase plasma exudation in guinea pig airways (assessed via Evans blue dye extravasation) (108) and edema formation in rabbit lung (4).

Inflammatory cells. 8-*iso*-PGE_2 and 8-*iso*-$PGF_{2\alpha}$ were both found to enhance human polymorphonuclear granulocyte activity and adhesion to endothelial cells but did so via some indirect mechanism because neither isoprostane increased expression of CD11b or P-selectin nor were the levels of interleukin (IL)-6 or IL-8 altered, but CD11b expression was increased when naive neutrophils were exposed to isoprostane-pretreated endothelial cells or to supernatants from pretreated endothelial cells (172). Interestingly, this indirect activation was not inhibited by antagonists of TP or ET receptors (SQ-29548 and bosentan, respectively).

Neuronal cells. 8-*iso*-$PGF_{2\alpha}$ augments sensory neuronal function in that it increases the release of transmitters from isolated sensory neurons, enhances the firing of C-nociceptors in situ and of cultured sensory neurons in vitro, and significantly reduces the threshold levels of mechanical and thermal stimuli required to trigger hind paw withdrawal (34). E-ring isoprostanes inhibit norepinephrine release in rat hypothalamus (31). Finally, 8-*iso*-$PGF_{2\alpha}$ triggers Ca^{2+} influx in astrocytes via N-type (i.e., ω-conotoxin-sensitive) Ca^{2+} channels (57).

Nonpulmonary cell types. Isoprostanes also constrict myometrium (25), muscularis mucosa of the canine proximal colon (33), gastric fundus, (147), ileum (147), and lymphatic vessels (118, 154), stimulate alkaline phosphatase activity and differentiation in calcifying vascular cells but inhibit these in preosteoblasts (124), and induce hypertrophy in cardiac ventricular myocytes. The latter effect is accompanied by inositol phosphate formation and is dependent on activation of c-Jun amino-terminal kinase (JNK1), c-Jun, phosphatidylinositol 3-kinase, and p70 S6 kinase (80).

Summary and physiological relevance. It is clear, then, that isoprostanes are not just markers but that they can exert a wide range of biological effects, particularly on pulmonary cell types, depending on the species, tissue, and isomer being tested. Collectively, these findings emphasize the need for further investigations in this area and the danger in generalizing from studies done with 8-*iso*-$PGF_{2\alpha}$ in rodent tissues.

One might question whether isoprostanes can accumulate to levels sufficient to trigger functional responses such as those listed above. Numerous independent measurements of isoprostane levels yield values in the nanomolar range in total plasma or urine of normal or healthy individuals (26, 27, 35, 106, 127, 163), and these levels are markedly elevated (as much as 200-fold; 112) during oxidative stress (see MARKERS OF OXIDATIVE STRESS). Although these systemic concentrations might be subthreshold for some of the responses listed above, they are sufficient to markedly augment the responses evoked by other agonists (i.e., to induce nonspecific hyperresponsiveness; see *Airway hyperresponsiveness*) and to stimulate MAP kinase activity (40, 95). Also, this measured value pertains to total plasma or urine after the isoprostanes have been diluted throughout the systemic circulation and metabolized by the kidney and liver; it is entirely reasonable that isoprostane levels are substantially higher at the site of oxidative stress (e.g., within the lung per se or in the immediate vicinity of the cells releasing the reactive oxygen species).

MEDIATORS OF PULMONARY PATHOPHYSIOLOGY?

The recent awareness that isoprostanes are produced in abundance during oxidative stress and that they possess a diverse repertoire of biological actions may provide a whole new perspective on our understanding of pulmonary pathophysiology.

Membrane properties. Much attention has been focused recently on the effects of free radicals in the lungs (7, 61, 89, 137); it may be that these are, in part, the result of actions exerted by isoprostanes on the membrane. For example, arachidonic acid is a long hydrocarbon chain that contributes to some extent to the fluidity and hydrophobic nature of the membrane; conversion of this molecule into a hairpinlike structure by forming a cyclopentane ring at its center as well as by introducing several hydroxyl groups on that cyclopentane ring while it is still esterified within the membrane would markedly alter membrane fluidity, integrity, and hydrophobicity (102, 112), all of which are common features of lipid peroxidation.

Mechanical responses to free radicals. Similarly, the mechanical responses evoked by reactive oxygen species may, in fact, be mediated by isoprostanes produced by those free radicals. Canine airway smooth muscle exhibits a relaxant response to H_2O_2 (41, 61), whereas human airways show a bronchoconstrictor response (Fig. 6) (137). The substantial body of literature summarized above predicts that such exposure to H_2O_2 would lead to generation of a variety of isoprostanes, although the precise composition of this mixture of isoprostanes (the relative proportions of different isoprostane species) might vary from tissue to tissue. Although it might therefore seem hard to predict the response of the tissues to such complex mixtures, it is worth pointing out that in the dog, most isoprostanes evoke substantial relaxations, but none exhibit any appreciable excitatory activity (62), so the net effect that one might predict from a mixture of isoprostanes is bronchodilation, which is, in fact, the observed response to H_2O_2 (Fig. 6) (41, 61). In human airway smooth muscle, on the other hand, several isoprostanes are powerful constricting agents, and only one (8-*iso*-$PGF_{3\alpha}$) is moderately inhibitory at very high concentrations (62), which would account for the observed bronchoconstrictor response to H_2O_2 (Fig. 6) (137). Likewise in pulmonary vascular smooth muscle, both H_2O_2 and isoprostanes evoke contractions with similar pharmacological sensitivities (14, 60, 62, 65).

Airway hyperresponsiveness. Isoprostanes may contribute to nonspecific airway hyperresponsiveness because subthreshold concentrations of 8-*iso*-PGE_2 and 8-*iso*-$PGF_{2\alpha}$ have been shown in vascular smooth muscle (146) and platelets (134) to amplify the responses to norepinephrine, angiotensin II, and the Tx mimetic U-46619. Recently, it has been shown that lipopolysaccharide-induced murine airway hyperresponsiveness is COX independent and yet sensitive to a blocker of TP receptors or to the free radical scavenger *N*-acetylcysteine (51, 52); these observations are all consistent with the generation and pharmacological actions of isoprostanes.

Lung transplantation. Cerebral, myocardial, and renal ischemia-reperfusion injury are all associated with generation of isoprostanes (see MARKERS OF OXIDATIVE

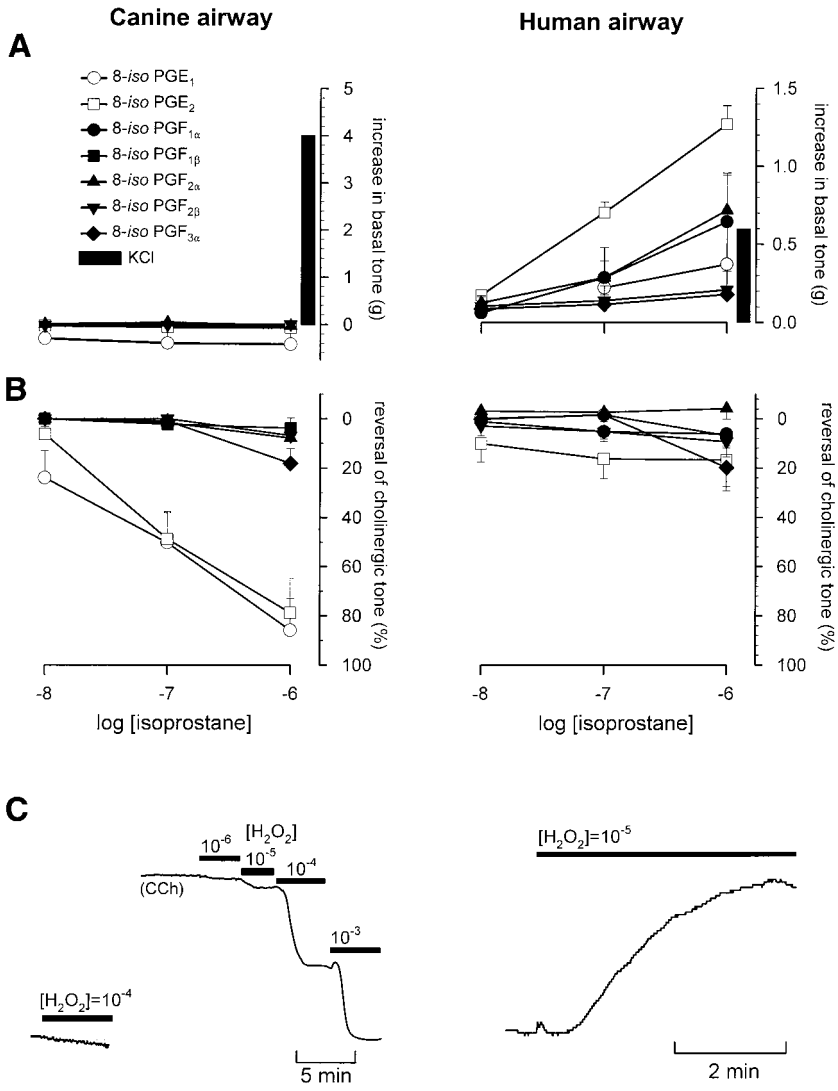

Fig. 6. Similarity in the effects of isoprostanes and H_2O_2 on airway smooth muscle. In canine airway smooth muscle tissues, none of the isoprostanes evoked a constrictor response (*A*), whereas the 2 E-ring isoprostanes tested evoked a complete reversal of cholinergic tone (*B*); H_2O_2 also evoked relaxation but not constriction in this tissue (*C*). In human airway smooth muscle tissues, however, many isoprostanes (particularly 8-iso-PGE_2) are powerful bronchoconstrictor agents at submicromolar concentrations (*A*), but none are effective as bronchodilators (*B*); likewise, H_2O_2 evokes constriction (*C*) but not relaxation (data not shown). CCh, carbachol. [Adapted from Janssen and colleagues (61, 62).]

STRESS), a substantial proportion of which may remain esterified within membrane phospholipids and be released over a prolonged period of time (see STORAGE, RELEASE, AND METABOLISM). These facts may be highly pertinent to lung transplantation; the donor lungs may, in fact, represent a major source of isoprostanes for days or weeks after the operation, affecting many parameters of lung function. Related to this, restoration of bronchial blood flow is recognized to be vital to the success of lung transplantation (126), and this may be adversely affected by the ongoing release of isoprostanes from the oxidatively stressed lungs.

Acute lung injury and pulmonary hypertension. Acute lung injury and pulmonary hypertension are associated with increased metabolism of arachidonic acid (6, 44, 64, 92, 122, 138, 165, 173) and are sensitive to inhibitors of TP receptors (16, 42, 114, 122, 151, 159, 165, 173), Tx synthase (16, 64, 114), COX (16, 64, 122, 151), and phospholipase A_2 (84). Thus some speculate that TxA_2 plays a central role in mediating these pathological changes, although others conclude differently (44). However, recent data (2, 44, 150, 168, 169) link superoxide and peroxide to these changes, which raises another possibility: isoprostanes generated by COX or nonenzymatically by free radicals and acting through TP receptors may mediate these changes. 8-*iso*-$PGF_{2\alpha}$ is released from several sources under conditions associated with acute lung injury and hypertension: from deendothelialized pulmonary artery smooth muscle on stimulation with growth factors (platelet-derived growth factor, transforming growth factor-β), proinflammatory cytokines (tumor necrosis factor-α, interferon-γ, and IL-1β), or superoxide (68, 69, 116); from pulmonary arterial endothelium stimulated with H_2O_2 (49); and from renal mesangial cells stimulated with IL-1 (74). 8-*iso*-$PGF_{2\alpha}$ generated in this way would potentially disrupt endothelial barrier function (49) and trigger pulmonary and systemic vasoconstriction (see *Vascular smooth muscle*).

Summary. There is growing interest in isoprostanes as putatively important mediators in lung pathophysiology. First, they are produced in abundance by reactive oxygen species released from inflammatory cells in the airways or in a COX-dependent fashion by virtually every cell type present in the lungs (smooth muscle, epithelium, endothelium, platelets, and inflammatory cells; see PRODUCTS OF LIPID PEROXIDATION). Second, they exert important biological actions on airway and pulmonary vascular smooth muscles, epithelium or endothelium, sensory nerves, and lymphatics (see CELLULAR ACTIONS); these actions can account, in part, for the nonspecific smooth muscle hyperresponsiveness, bronchoconstriction, hypertension, edema, and hypertrophy that characterize many lung-related diseases. Third, the unique nature of the generation and pharmacology of isoprostanes can account for the sometimes paradoxical effects of steroids, free radical scavengers, COX inhibitors, and TP receptor blockers in experimental and clinical settings.

FUTURE DIRECTIONS?

The isoprostane field is still in its infancy, and many important questions remain unanswered. Clearly, one important avenue of investigation pertains to the therapeutics designed to prevent the actions of isoprostanes in the lung.

On one hand, it should be possible to interfere with their production by using free radical scavengers. Numerous studies have documented an inverse relationship between the levels of isoprostanes and free radical scavengers such as vitamins A (23), C (130, 141), and E (21, 23, 26, 27, 130, 135, 148); β-carotene (23, 130); selenium (35); superoxide dismutase and catalase (11), or a superoxide dismutase mimetic (4-hydroxy-2,2,6,6-tetramethyl piperidynoxyl) (149). In some cases, oral supplementation with these scavengers was accompanied by amelioration, proportional to the reduction in isoprostane levels, of the pathophysiology being studied (11, 26, 130, 149). COX inhibitors and steroids, both used with success in the treatment of many pulmonary disorders, have also been shown to suppress isoprostane production by inflammatory cells stimulated with proinflammatory cytokines and growth factors (125, 129, 131, 149, 160).

Rather than interfering with isoprostane production, an alternative approach would be to interfere with isoprostane signaling at the receptor level; TP receptor blockers have already been shown to be useful in this respect (see *Classic prostanoid receptors or unique isoprostane receptors?*). Further work is needed to clarify whether a novel isoprostane-specific receptor is, in fact, involved, and if so, what agents can be used to block those selectively. It is worth noting that although isoprostanes mediate such a variety of effects within the lungs, many of the currently available therapies for pulmonary diseases, including leukotriene antagonists, antihistamines, anticholinergics, α-adrenergic blockers, and β-agonists, are ineffective against the isoprostanes. Alternatively, a better understanding of the second messenger signaling pathways underlying isoprostane responses might also allow for the development of novel treatments for pulmonary diseases characterized, in part, by oxidative stress.

More investigation is needed to further elucidate the cellular actions of isoprostanes in the lungs. There have been no studies of their effects on airway epithelium or innervation and virtually none pertaining to their effects on different inflammatory cell types and the endothelium. Although some of their effects on smooth muscle have been described, little or nothing is known about their effects on gene expression, cytoskeletal rearrangement, hypertrophy, or apoptosis in smooth muscle. Also, their interactions with other spasmogens (resulting in nonspecific hyperresponsiveness) needs to be studied in more detail. The biological effects of many isoprostanes and their metabolites have not yet been examined, even though there is now evidence that isoprostanes with nearly identical structures can have very different pharmacological effects (see CELLULAR ACTIONS). The signaling pathways in-

volved are still poorly understood, and almost nothing has been published about their effects on kinases, ion channels, and Ca^{2+} handling.

Assuming that the exponential growth in the number of publications pertaining to isoprostanes continues (Fig. 1), the next few years are certain to hold answers to many of these questions.

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