Augmentation of bovine airway smooth muscle responsiveness to carbachol, KCl, and histamine by the isoprostane 8-iso-PGE$_2$

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Catalli, Adriana, and Luke J. Janssen. Augmentation of bovine airway smooth muscle responsiveness to carbachol, KCl, and histamine by the isoprostane 8-iso-PGE$_2$. Am J Physiol Lung Cell Mol Physiol 287: L1035–L1041, 2004.First published July 16, 2004; doi:10.1152/ajplung.00138.2004.—Isoprostanes are generated during periods of oxidative stress, which characterize diseases such as asthma and cystic fibrosis. They also elicit functional responses and may therefore contribute to the pathology of these diseases. We set out to examine the effects of isoprostanes on airway responsiveness to cholinergic stimulation. Muscle bath techniques were employed using isolated bovine tracheal smooth muscle. 8-Isoprostaglandin E$_2$ (8-iso-PGE$_2$) increased tone directly on its own, although the magnitude of this response, even at the highest concentration tested, was only a fraction of that evoked by KCl or carbachol. More importantly, though, pretreatment of the tissues with 8-iso-PGE$_2$ (10 μM) markedly augmented responses to submaximal and even subthreshold concentrations of KCl, carbachol, or histamine, whereas maximal responses to these agents were unaffected by the isoprostane. The augmentative effect on cholinergic responsiveness was mimicked by PGE$_2$ (0.1 μM) and by the FP agonists PGF$_2$ (0.1 μM) and fluprostenol (0.1 μM), but not by the EP$_3$ agonist sulprostone (0.1 μM) or the TP agonist U-46619 (0.1 μM). Antagonists of EP$_1$ receptors (AH-6809 and SC-19920, 10 μM) and TP receptors (IC1-192605, 1 μM) had no effect on 8-iso-PGE$_2$-induced augmentation of cholinergic responsiveness. We conclude that 8-iso-PGE$_2$ induces nonspecific airway smooth muscle hyperresponsiveness through a non-TP non-EP prostanoid receptor.

Prostanoid receptors; contraction; asthma; oxidative stress; 8-isoprostaglandin E$_2$

Isoprostanes are a large group of free radical-generated, prostaglandin-like molecules that have proven useful as markers of oxidative stress. Only recently have researchers begun to recognize these molecules as having their own potent biological activity. Most research to date has focused solely on the excitatory actions of isoprostane 8-iso-PGF$_{2\alpha}$, which is responsible for such physiological responses as vasoconstriction, bronchoconstriction (22, 25, 27), and modulation of platelet aggregation (28, 38).

Isoprostanes are detected in normal human plasma, urine, bronchoalveolar lavage fluid (33–35), and exhaled breath condensate (31). More importantly, their concentrations are significantly elevated during periods of oxidative stress, which characterizes such diseases as asthma (2, 3, 31, 45), chronic obstructive pulmonary disorder (30, 37), interstitial lung disease (29), and cystic fibrosis (9, 11, 32) and as such may contribute to the pathology underlying these conditions.

There are a number of reports indicating that isoprostanes exert their biological effects through classic prostanoid receptors. This is not surprising considering the strong structural similarities between isoprostanes and prostaglandins; both groups of molecules possess a cyclopentane ring with two alkyl side chains but differ only in the orientation of those side chains (5, 43). Five classes of prostanoid receptors have been identified, DP, EP, FP, IP, and TP (some with subtypes and splice variants), which correspond to the arachidonic acid-derived endogenous ligands PGD$_2$, PGE$_2$, PGF$_2\alpha$, PGJ$_2$, and thromboxane A$_2$, respectively (6, 7, 16, 20). Isoprostane 8-iso-PGF$_{2\alpha}$ appears to act through the thromboxane-selective TP prostanoid receptor to induce vasoconstriction (13, 17) and bronchoconstriction (25). Some studies indicate that 8-iso-PGE$_2$ may act similarly on the TP receptor (12, 22–24, 42), whereas others indicate the involvement of FP and EP prostanoid receptors in mediating the excitatory activity of 8-iso-PGE$_2$ (40, 44). Inhibitory activity is also observed for the isoprostanes (8, 22), with 8-iso-PGE$_2$ seemingly acting at the inhibitory EP receptors to induce airway relaxation (8). Limited data are available with regard to the activities of the other isoprostane compounds, with studies to date showing both inhibitory and excitatory activities, depending on the tissue type, species, and isoprostane in question.

In addition to their inhibitory and excitatory activities in airways, isoprostanes may also induce hypersensitivity to bronchoconstrictors in these tissues. A hallmark feature of asthma is hyperresponsiveness of the airway smooth muscle to physical, environmental, and chemical stimuli (e.g., cholinergic agonists, histamine, cysteinyl leukotrienes, and prostaglandins). This heightened responsiveness is associated with airway obstruction, as well as increased asthma severity and need for drug therapy (4, 36). Isoprostanes 8-iso-PGE$_2$ and 8-iso-PGF$_{2\alpha}$ enhance vasoconstrictor responses to norepinephrine and angiotensin II in perfused rabbit ear (39). 8-IsopGF$_{2\alpha}$ similarly augmented the aggregation response of platelets to a variety of stimuli including thrombin, arachidonic acid, and the thromboxane mimetic U-46619 (38). In addition, Held and Uhlig (21) demonstrated airway hyperreactivity to methacholine following pretreatment with 8-iso-PGF$_{2\alpha}$ in perfused mouse lung, a response mediated by the TP prostanoid receptor. However, this report gave no indication as to whether this effect was additive or synergistic. These data suggest that isoprostanes are capable of inducing hypersensitivity to vaso- and bronchoconstrictors and thus may contribute to the airway hyperresponsiveness observed in asthma. This is of particular...
importance in light of the fact that isoprostane production is markedly increased in such disease states.

The aim of this study was to enhance the current knowledge of isoprostanes by characterizing their effects in bovine airways and their ability to induce hyperresponsiveness to bronchoconstrictors. Studies of receptor involvement were also undertaken.

METHODS

Tissue preparation. All experimental procedures were approved by the McMaster University Animal Care Committee, the McMaster University Biosafety Committee, and the St. Joseph’s Healthcare Research Ethics Board and conform to the guidelines set out by the Canadian Council on Animal Care.

Bovine lungs were obtained from an abattoir and placed in ice-cold Krebs solution (see Solutions and chemicals). The epithelium was removed, and tracheal smooth muscle strips (~1–2 mm wide) were excised and used immediately or stored at 4°C for use up to 48 h. Tissues were suspended in 4-mL Krebs-filled baths and tied with silk thread such that one end of the tissue was anchored while the other was fastened to a Grass FT.03 force transducer. Changes in isometric force were digitized and recorded on-line using the DigitMed System Integrator program (MicroMed, Louisville, KY).

Protocol. Tissues were maintained in Krebs buffer at 37°C and bubbled with 95% O2/5% CO2 to maintain pH at 7.4. Tissues were equilibrated for 1 h, during which time a preload of ~1 g was applied and maintained to ensure maximal physiological response, and several washes were performed. After the equilibration period, tissues were challenged two to three times with 60 mM KCl (with intermittent washes), and the final KCl response was used to standardize all subsequent responses.

Concentration-response relationships. A single concentration of isoprostane was added 30 min before the generation of cumulative concentration-response curves for the contractile agonists carbachol, KCl, or histamine unless specified otherwise. The resulting isoprostane contractile response, if any, was not included in the measurement of contractile responses to carbachol, histamine, or KCl (i.e., the postisoprostane tone was set as baseline for those other agonists). For KCl contraction experiments, atropine and propranolol were added to eliminate the contributions of adrenergic and cholinergic nerve stimulation.

Cholinergic responsiveness: single concentration carbachol protocol. A single concentration (5 × 10−9 M) of carbachol was selected to produce a detectable but small carbachol response under control conditions. Tissues underwent three sequential treatments with 5 × 10−9 M carbachol (with intermittent washes), and the last carbachol response was used as the control response. After carbachol washout, the tissues were allowed to recover and then were exposed to either isoprostane or prostanoïd agonists for 30 min before being challenged again with 5 × 10−9 M carbachol. Finally, tissues were maximally stimulated with 10−5 M carbachol for use in standardizing the tissue. Where applicable, prostanoïd receptor antagonists were added 15 min before isoprostane treatment.

Solutions and chemicals. Krebs buffer consisted of 116 mM NaCl, 4.6 mM KCl, 2.5 mM CaCl2, 1.3 mM NaH2PO4, 1.2 mM MgSO4, 23 mM NaHCO3, 11 mM d-glucose, and 10−3 M indomethacin bubbled to maintain pH 7.4. Carbachol, KCl, and histamine were dissolved in doubly-distilled deionized water. The isoprostanes and prostanoïd agonists were dissolved in ethanol, with the exception of U-46619, which was dissolved in methyl acetate. ICI-192605, AH-6809, and SC-19220 were dissolved in DMSO. Stock solutions were diluted in Krebs buffer and discarded after 24 h. The isoprostanes and prostanoïd drugs were obtained from Cayman Chemical (Ann Arbor, MI). All other chemicals were obtained from Sigma Chemical (St. Louis, MO).

Data analysis. For experiments examining concentration-response relationships, contractile responses to isoprostanes, carbachol, KCl, and histamine were expressed as a percentage of the response to 60 mM KCl. For all other experiments, contractions induced by 5 × 10−9 M carbachol were expressed as the percentage of tone evoked by 10−5 M carbachol.

Values are expressed as means ± SE. One-way ANOVA and Student’s t-test were used to ascertain the statistical significance of the differences between the means; n is the number of animals used.

RESULTS

Isoprostane-induced augmentation of cholinergic response in bovine tracheal smooth muscle. Before examining their interactions with other bronchoconstrictor stimuli, we first evaluated the direct constrictor effects of the isoprostanes on tone in bovine airway smooth muscle. Cumulative contractile concentration-response relationships were generated for several different isoprostanes (Fig. 1). Of the isoprostanes tested, only 8-iso-PGE2 induced marked contraction, and this required micromolar concentrations; the mean response at the highest concentration tested (10−3 M) was 58 ± 19% of 60 mM KCl standard tone. In some tissues, 8-iso-PGE1 exhibited a small contractile response (~10% of KCl tone) at the highest concentration tested (10−5 M). All the other isoprostanes we tested were essentially without direct effect on tone in this airway preparation.

We next examined the influence of isoprostanes on cholinergic responsiveness. Cumulative carbachol concentration-response relationships were generated in the presence or absence of 10−5 M 8-iso-PGE2 (Fig. 2A). After a 30-min preincubation period with the isoprostane, carbachol concentrations <10−8 M, which typically evoked no response in control tissues, now evoked substantial contractions (Fig. 2B); for example, the mean response to 3 × 10−9 M carbachol was augmented from 0.5 ± 0.5% of KCl tone in control tissues to 52.1 ± 12.4% in 8-iso-PGE2-pretreated tissues. However, 8-iso-PGE2 did not significantly alter the responses to carbachol concentrations >3 × 10−8 M. As such, this effect of the isoprostane is not simply a leftward shift in the sigmoidal cholinergic concentra-

![Fig. 1. Isoprostane concentration-contraction relationships.](./image.png)
tion-response relationship but specifically an augmentation of only the lower portion of that sigmoid. This enhanced sensitivity to carbachol did not seem to be secondary to the change in baseline tone caused by the isoprostane, since a correlation analysis showed no significant relationship between these two parameters ($R = 0.123$, Fig. 2C): in fact, in several tissues, 8-iso-PGE$_2$ caused substantial augmentation without any change in tone whatsoever (Fig. 2C). A lower concentration ($10^{-6}$ M) of 8-iso-PGE$_2$ was still conducive to, though less effective at, augmenting carbachol responsiveness (Fig. 2D). This augmentation appears to be receptor mediated rather than through a nonspecific mechanism such as altered membrane fluidity or a vehicle effect, since other structurally similar isoprostanes, 8-iso-PGF$_{2\alpha}$ and 8-iso-PGE$_1$, were far less effective in enhancing carbachol responses (Fig. 3).

8-Iso-PGE$_2$ markedly augmented the response to KCl as well (atropine and propranolol were present to eliminate contributions of nerve stimulation by KCl) (Fig. 4A). For example, 20 mM KCl generally evoked no response in control tissues but evoked responses nearly 40% of maximum in the presence of the isoprostane (Fig. 4A). Likewise, histamine responsiveness was also enhanced by the isoprostane: concentrations $<10^{-8}$ M were noneffective in control tissues but evoked substantial contractions in isoprostane-pretreated tissues; maximal responsiveness to KCl or to histamine was unaffected by the isoprostane (Fig. 4B).

**Involvement of prostanoid receptors.** Isoprostanes generally appear to exert their excitatory and inhibitory effects through classic prostanoid receptors (8, 24). We therefore investigated whether excitatory prostanoid receptors (EP$_1$, EP$_3$, FP, and TP) mediate the effect of 8-iso-PGE$_2$ on cholinergic responses by examining whether the enhancement was suppressed by selec-

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Fig. 2. 8-Iso-PGE$_2$ enhances carbachol (CCh) responses in bovine tracheal smooth muscle (TSM). A: 2 representative traces of CCh concentration-contraction relationships generated in the presence (bottom) or absence (top) of $10^{-5}$ M 8-iso-PGE$_2$. These were obtained on the same day from trachea of the same animal. The isoprostane was applied to tissues 30 min before the generation of the CCh concentration-response relationships. Dashed lines $a$ and $b$ represent the CCh baselines in control and 8-iso-PGE$_2$-treated tissues, respectively. B: effects of 8-iso-PGE$_2$ on CCh responsiveness were examined by the protocols shown in A. 8-Iso-PGE$_2$ enhanced the response to subthreshold concentrations of CCh ($n = 4-6$). C: correlation analysis of the relationship between the change in baseline due to 8-iso-PGE$_2$ ($10^{-5}$ M) and its effect on the response to $5 \times 10^{-9}$ M carbachol. D: concentration dependence of the 8-iso-PGE$_2$-induced effect on cholinergic responsiveness ($n = 5-6$). * and †, significant difference from control, with $P < 0.05$ and $P < 0.1$, respectively.

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Fig. 3. Other isoprostanes are much less effective in altering cholinergic responsiveness. Bovine TSM were pretreated with the indicated isoprostane for 30 min before generation of CCh concentration-response relationships (as shown in Fig. 2A). 8-iso-PGE$_{2\alpha}$ and 8-iso-PGE$_2$ were less effective at enhancing CCh responses compared with 8-iso-PGE$_2$ (isoprostanes, $n = 5-7$; control, $n = 11$). *Significant difference from control, with $P < 0.05$. 

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*Please note that the diagrams and figures mentioned in the text are not included in this text representation.*
tive prostanoid receptor antagonists, using the experimental protocol represented in Fig. 5

A.

B.

Fig. 4. 8-ISO-PGE2 also enhances responses to KCl and histamine. KCl (n = 4, A) and histamine (n = 6, B) concentration-response relationships were generated in the presence (●) or absence (○) of 8-ISO-PGE2. * and †, significant difference from control, with P < 0.05 and P < 0.1, respectively.

Fig. 5. 8-ISO-PGE2 may act via prostanoid receptors. A: representative tracing showing the experimental protocol used to assess responsiveness to submaximal cholinergic stimulation (5 × 10⁻⁹ M CCh). B: mean responses to CCh (5 × 10⁻⁹ M) before (black bars) and after (gray bars) incubation with 8-ISO-PGE2 in the presence (n = 5–6) or absence (n = 11) of prostanoid antagonist ICI-192605 (TP selective, 10⁻⁶ M), AH-6809 (EP₁/DP selective, 10⁻⁵ M), or SC-19220 (EP₁ selective, 10⁻⁵ M) for 15 min before addition of 8-ISO-PGE2. All experiments were concluded by the addition of 10⁻⁵ M carbachol for use in standardizing the data. The data are

for 30 min before being challenged again with 5 × 10⁻⁹ M carbachol. In some cases, tissues were pretreated with the prostanoid receptor antagonists ICI-192605 (TP selective, 10⁻⁶ M), AH-6809 (EP₁/DP selective, 10⁻⁵ M), or SC-19220 (EP₁ selective, 10⁻⁵ M) for 15 min before addition of 8-ISO-PGE2. All experiments were concluded by the addition of 10⁻⁵ M carbachol for use in standardizing the data. The data are
summarized in Fig. 5B. 8-Iso-PGE₂ markedly enhanced airway smooth muscle responsiveness to $5 \times 10^{-9}$ M carbachol, and this enhancement was not attenuated by ICI-192605 nor AH-6809. Although SC-19220 appeared to reduce the augmentative effect, the difference was not statistically significant ($P = 0.16$).

We next examined the ability of various selective prostanoid receptor agonists to mimic the effects of isoprostanes using a similar protocol as described above. Briefly, following the three consecutive carbachol ($5 \times 10^{-9}$ M) administrations, tissues were exposed to a prostanoid agonist for 30 min, after which they were retested with $5 \times 10^{-9}$ M carbachol (Fig. 5C). Neither a TP (U-46619) nor an EP₃ (suprostenol) receptor agonist augmented carbachol response when added at concentrations that are selective for their respective receptors ($10^{-7}$ M). However, cholinergic responsiveness was significantly enhanced by pretreatment with the EP receptor agonist PGE₂, the DP receptor agonist PGD₂, and the FP receptor agonists PGF₂α, and fluprostenol (all applied at concentrations that are selective for their respective receptors: $10^{-7}$ M).

Collectively, these data suggest that augmentation of cholinergic responsiveness by 8-iso-PGE₂ is not mediated by TP receptors but may instead involve one or more other prostanoid receptors or possibly a novel isoprostane-selective receptor.

**DISCUSSION**

Several previous studies have described various direct actions of isoprostanes on airway tissues, including bronchoconstriction (22, 25), bronchodilation (8, 22), regulation of gene transcription (10), and altered neurotransmission (41). We have previously shown that isoprostanes are primarily excitatory in human airway smooth muscle tone but inhibitory toward that in canine and porcine tissue (8, 22). The results of this study indicate that bovine airways are generally resistant to their direct excitatory effects: only one isoprostane (8-iso-PGE₂) evoked contractions $>10\%$ of the response to KCl, and even this required supramicromolar concentrations. These stark differences in responsiveness to isoprostanes between species are most likely due to differences in expression of prostanoid receptor populations on the membrane.

Isoprostanes can be detected in plasma, urine, bronchoalveolar lavage fluid, and exhaled breath condensates of normal individuals, and production of these molecules is greatly enhanced in oxidative stress-related diseases (3, 30, 32). This finding, coupled with the growing knowledge that they are also highly biologically active compounds, suggests they may actually contribute to the underlying pathology of these conditions. Indeed, the results of this study demonstrate that isoprostanes are capable of augmenting airway smooth muscle sensitivity and responsiveness to various agonists, a hallmark feature of asthma. In particular, 8-iso-PGE₂ augmented responses to submaximal concentrations of the bronchoconstrictors carbachol, histamine, and KCl in bovine trachea. For example, carbachol concentrations that were otherwise ineffective ($3 \times 10^{-10}-3 \times 10^{-9}$ M) induced significant contractions in the presence of 8-iso-PGE₂, and the response to the threshold concentration ($10^{-8}$ M) was more than doubled. We further showed that this augmentation was independent of any direct contractile effect of the isoprostane itself. Interestingly, there was no significant difference in the maximal constrictor responses: thus the leftward shift in the concentration-response relationships for the agonists in isoprostane-treated tissue was not parallel. We would point out that the lower portion of the concentration-response relationships are nonetheless highly physiologically (and pathologically) relevant: the upper end of these relationships are never achieved in real life without dire consequences.

Two other structurally related isoprostanes, 8-iso-PGF₂α and 8-iso-PGE₁, had much less of an effect on cholinergic responsiveness than did 8-iso-PGE₂, suggesting a receptor-mediated effect was involved rather than a nonspecific effect (such as altered membrane fluidity). Many studies indicate that isoprostanes exert their excitatory and inhibitory effects via the classic prostanoid receptors. This is not surprising considering the structural similarities between prostanoid and isoprostane compounds. Three of these prostanoid receptors are generally excitatory in smooth muscle: these being the FP, TP, and EP (EP₁ and EP₃ subtypes) classes of prostanoid receptors (6). 8-ISO-PGF₂α activity at the TP receptor has been documented (13, 17, 25); similarly, 8-ISO-PGE₂ activity at TP, FP, and EP receptors has also been observed (12, 22–24, 42). We attempted to ascertain the contribution of these receptors in the 8-ISO-PGE₂-mediated augmentation of cholinergic responsiveness. Few selective antagonists are available for the prostanoid receptors. Of these, the TP, EP₁/DP, and EP₃ prostanoid receptor antagonists ICI-192605, AH-6809, and SC-19220, respectively, failed to significantly attenuate 8-ISO-PGE₂ response. Using receptor-selective concentrations of various prostanoid receptor agonists, we found the FP (PGF₂α and fluprostenol)- and EP-selective agonists (PGE₁) to mimic the activity of 8-ISO-PGE₂, although with lesser efficacy. The TP agonist U-46619 and EP₁ agonist sulprostone, on the other hand, had little effect on cholinergic response. Together, these data suggest that 8-ISO-PGE₂ does not act through EP₃ or TP receptors (since its effects were not blocked by selective antagonists nor mimicked by selective agonists) but may do so through FP receptors; confirmation of this hypothesis will require the development of receptor-selective antagonists for FP receptors and/or the use of receptor knockout animals. Although PGD₂ also caused enhancement of cholinergic responsiveness, activation of the G₁, protein-coupled DP prostanoid receptor, which is typically inhibitory, induces cAMP production (6) and thus would not be expected to enhance cholinergic contractions. PGD₂ can also act through a novel DP₂/CRTTH₂ receptor, which has been identified in immune cells and which couples through G₁ to mediate chemoattraction (20). The paradoxical effect of PGD₂ may also represent a nonspecific action through one of the other prostanoid receptors: even at submicromolar concentrations, PGD₂ can bind with high affinity to the FP prostanoid receptor (1, 6, 19, 26). On the other hand, it could be argued that a nonprostanoid receptor, such as a novel isoprostane receptor, may mediate 8-ISO-PGE₂ activity, as has been suggested by others (14, 15).

The signaling pathways that are downstream of the receptor(s) mediating augmentation of bronchoconstrictor responsiveness are at present unclear; a knowledge of the specific receptor(s) involved would facilitate studies into this question. A previous study has documented a similar effect of histaminergic stimulation on cholinergic responses in canine tracheal smooth muscle (18): in that study, the effect seemed to involve a change in Ca²⁺ handling and/or in Ca²⁺ sensitivity. Likely...
candidates include 1) enhancement of the phosphoinositide pathway and release of Ca\(^{2+}\) from the sarcoplasmic reticulum, 2) increased Ca\(^{2+}\) influx via plasma membrane channels, and 3) modulation of the RhoA/Rho-kinase Ca\(^{2+}\) sensitization pathway. Augmentation of any or all of these would account for the nonspecific nature of the hyperresponsiveness seen in asthma, since many bronchoconstrictors act in part through them.

Non-specific airway hyperreactivity to a variety of stimuli such as cholinergic agonists, histamine, leukotrienes, and pros-taglandins is a characteristic feature of asthma that correlates with severity of disease symptoms and the need for drug therapy (4, 36). In particular, the airways can show an increase in sensitivity to these spasmogens (and others) of two to three orders of magnitude; however, it is not possible to say anything about the degree of any leftward shift in the concentration-response relationship, since such studies are only able to assess responsiveness at the low end of the concentration-response relationships without killing the individual. The results of this study illustrate that isoprostanes are capable of markedly increasing smooth muscle sensitivity and responsiveness to contractile agents: for example, the responses to subthreshold concentrations of CCh (10^{-10}–3 \times 10^{-5} M) were enhanced greater than 10-fold, and even that to 10^{-8} M carbachol was more than doubled in magnitude (see Fig. 2B). Because production of isoprostanes is markedly enhanced in asthma and other airway disorders, they may be contributing to the pathology of these disease states. Thus further study of the mechanisms underlying isoprostane activity and the receptors involved may provide a better understanding of the pathology of oxidative stress-related diseases and lead to new therapeutic strategies.

In conclusion, we found that 8-iso-PGE\(_2\) exerts modest direct excitatory activity in bovine airway smooth muscle but, more importantly, also markedly augments the responses to submaximal concentrations of the bronchoconstrictors carbachol, histamine, and KCl. This effect is receptor mediated, although we were unable to ascertain the nature of the receptor(s) involved, other than to exclude certain classic prostanoid receptors that are generally found to be involved in isoprostane-mediated effects.

GRANTS

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