Functional Characterization of the Ocular Prostaglandin F_{2α} (PGF_{2α}) Receptor

ACTIVATION BY THE ISOPROSTANE, 12-iso-PGF_{2α}*

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Prostaglandin F_{2α} (PGF_{2α}) is a product of cyclooxygenase-catalyzed metabolism of arachidonic acid. Recently, PGF_{2α} analogs have been hypothesized to reduce intraocular pressure via relaxation of the ciliary muscle. To investigate the molecular basis of PGF_{2α} receptor (FP) activation in the eye, we cloned the FP from a human ciliary body (hcb) cDNA library. The open reading frame of the hcb-FP cDNA was identical to the uterine FP cDNA. The hcb-FP appeared to be predominantly membrane-localized, as visualized by an FP-specific peptide antibody, and coupled to inositol phosphate formation when stably expressed in HEK 293 cells. Interestingly, the hcb-FP could also be activated by the F_{2} isoprostane, 12-iso-PGF_{2α}, in addition to its cognate ligand, PGF_{2α}. 12-iso-PGF_{2α} was less potent (EC_{50} = 5 μM) than PGF_{2α} (EC_{50} = 10 nM) in generating inositol phosphates via the hcb-FP in HEK 293 cells. Both ligands also stimulated mitogenesis in NIH 3T3 cells. Although 12-iso-PGF_{2α} caused a dose-dependent activation of the FP, it failed to activate the recombinant human prostacyclin receptor and caused only minimal activation of the thromboxane receptor isofoms stably expressed in HEK 293 cells. Four additional F_{2} isoprostanes, 8-iso-PGF_{2α}, IPF_{2α}, IPF_{2α}III, and 9β,11β-PGF_{2α} caused trivial, or no, activation of the FP. Consistent with these observations, only PGF_{2α} and 12-iso-PGF_{2α} caused rapid homologous desensitization of FP and also exhibited cross-desensitization, with PGF_{2α} resulting in a maximum of ~60% desensitization. The human FP may thus be activated specifically, by the free radical-catalyzed F_{2} isoprostane, 12-iso-PGF_{2α}, in addition to the cyclooxygenase product, PGF_{2α}. Incidental receptor activation by isoprostanes may complement the actions of PGF_{2α} in clinical syndromes where oxidant stress and augmented prostaglandin biosynthesis coincide.

Prostaglandins are arachidonic acid metabolites that may play a major role as mediators of cellular function. PGF_{2α} has diverse physiological actions ranging from being a potent luteolytic agent (1, 2) to causing smooth muscle contraction in the uterus (3, 4), vasculature (5), and gastrointestinal (6) and respiratory tracts (7, 8). PGF_{2α} induces DNA synthesis and cell proliferation in 3T3 fibroblasts (9, 10). Neuronal astrocytes respond to PGF_{2α}, which may mediate pain transmission (11). Recently, PGF_{2α} has also been shown to cause hypertrophy of cardiac myocytes and induction of myofibrillar genes, independent of muscle contraction. These observations suggest a role for the eicosanoid during development, in compensatory hypertrophy and/or in recovery of the heart from injury (12).

Recently, PGF_{2α} analogs have been shown to reduce intraocular pressure (IOP), in patients with glaucoma (13, 14). Although the precise mechanisms involved remain unclear, the effects of PGF_{2α} analogs on IOP may be attributed, at least in part, to their actions on the ciliary muscle. PGF_{2α} reduces IOP by increasing the uveoscleral outflow of aqueous humor (15, 16), possibly by reducing the resistance between the ciliary muscle bundles, via an effect on the extracellular matrix (17).

A single PGF_{2α} receptor (FP) has been cloned from myometrial tissue (18–22). Given that there is evidence consistent with splice variation of the FP (23), as has been described for other prostanooid receptors (24, 25), we wished to address the possibility that a distinct isoform might mediate the actions of PGF_{2α} in the ciliary muscle. Clarification of the nature of the human ciliary FP and development of an antibody that specifically recognized the receptor protein would facilitate investigation of the effects of PGF_{2α} and its analogs on IOP.

PGF_{2α} is formed from arachidonic acid via metabolic transformation sequentially catalyzed by phospholipases, cyclooxygenases, and a specific PGF synthase (26). However, it is now appreciated that a series of PGF_{2α} isomers, the F_{2} isoprostanes, may also be formed in vivo via a free radical-dependent pathway (27–29). It has been speculated that these F_{2} isoprostanes may function as incidental ligands at eicosanoid receptors, and, possibly, activate related receptors of their own (30). To date, attention has focused particularly on 8-iso-PGF_{2α}. This compound is a potent vasoconstrictor. It is also a mitogen and may activate human platelets (31–33). Curiously, despite its F prostaglandin configuration, 8-iso-PGF_{2α} has been shown to activate thromboxane receptors (TPs), and its biological effects are blocked by TP antagonists (31–33).

We now report the cloning of an FP receptor from the human ciliary body (hcb) cDNA library and its localization on the cell membrane. The gene product is identical to that cloned from pair(s); BSA, bovine serum albumin; FP, prostaglandin F_{2α}; hcb, human ciliary body; InsP, inositol phosphates; IOP, intraocular pressure; IP, prostacyclin receptor; DMEM, Dulbecco’s modified Eagle’s medium; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; TP, thromboxane A_{2} receptor.
human uterus (18). Additional to activation by PGF<sub>2α</sub>, F<sub>2</sub> isoprostanes may ligate the FP. Consistent with the observation that 8-iso-PGF<sub>2α</sub> is virtually ineffective in competing for binding of a PGF<sub>2α</sub> analog to the ovine FP (22), the hcb-FP is minimally activated by this isoprostane. However, a structurally related F<sub>2</sub> isoprostane, 12-iso-PGF<sub>2α</sub>, results in a significant, dose-dependent activation of both recombinant and native FPs. Moreover, 12-iso-PGF<sub>2α</sub> exhibits receptor specificity as a ligand; it fails to activate the prostacyclin receptor (IP) and only minimally activates TP isofoms. Furthermore, consistent with these observations, 12-iso-PGF<sub>2α</sub> also desensitizes FP-mediated responses. Oxidant stress is thought to be a feature of heart failure and ocular diseases (34–36). In such clinical conditions, incidental activation of distinct eicosanoid receptors by isoprostanes may modulate the course of disease or the response to therapy.

EXPERIMENTAL PROCEDURES

Materials—Wild type human embryonic kidney (HEK 293) cells and NIH 3T3 cells were from the American Type Culture Collection (Rockville, MD). (α<sup>32</sup>P)dCTP, (α<sup>32</sup>S)dATP, [γ<sup>32</sup>P]ATP, myo-[2-3H]inositol, [methyl-14C]thymidine, [32P]GTPγS, Rapid-hyb buffer, Redi-prim RNA labeling kit and iodoset were purchased from Amersham Life Sciences. Human multiple tissue Northern blots were purchased from CLONTECH (Palo Alto, CA). Tissue culture reagents were purchased from Life Technologies, Inc. Dotap, restriction enzymes, and other molecular biology reagents were purchased from Boehringer Mannheim. Ampli-Taq DNA polymerase and dNTPs were purchased from Perkin-Elmer. The anion exchange resin AG 1-XS (formate form, 200–400-mesh) was purchased from BioRad. U46619, SQ29548, and PGF<sub>2α</sub> were purchased from Cayman Chemicals (Ann Arbor, MI). Horseradish peroxidase-conjugated anti-rabbit IgG and fluorescein isothiocyanate-labeled anti-rabbit IgG were purchased from Jackson Immunologicals (West Grove, PA). 18 S ribosomal RNA was from BD Gentra (Minneapolis, PA).

Cell Culture—HEK 293 cells were routinely maintained in DMEM with 10% fetal bovine serum, 1% glutamine, and 0.5% penicillin/streptomycin. Stable transformants were maintained in HEK medium with 1.3 mg/ml G418. HEK 293 cells were kept in humidified 5% CO<sub>2</sub>, 95% air at 37 °C. NIH 3T3 cells were from the American Type Culture Collection (Rockville, MD). NIH 3T3 cells were maintained in DMEM with 10% fetal bovine serum, 1% glutamine, and 0.5% penicillin/streptomycin in humidified 10% CO<sub>2</sub>, 90% air at 37 °C.

Cloning of the Human Ocular FP Receptor cDNA—Phage DNA was prepared from the hcb-cDNA library (kindly donated by Dr. Miguel CocaPrados, Yale University, New Haven, CT) and subjected to PCR. PCR was performed on 200 ng of human ciliary body cDNA with 100 pmol of the FP-specific primer 5′-TGCAAGGACCTGGTGTTTCT-TGCGACGATG-3′ and a degenerate antisense primer 5′-CCAAGCRTCRCAATTYGRTTT-3′ (1 = inosine, R = G/A, D = G/A/T, Y = T/C, T) by AmpliTaq buffer, 3 mM MgCl<sub>2</sub>, and 0.5 mM dNTPs in a total reaction volume of 100 μl. The samples were subjected to a “Hot Start” as described previously (37), followed by the addition of 0.5 units of AmpliTaq DNA polymerase. The reaction was then subjected to denaturation at 99 °C for 3 min, annealing at 50 °C for 2 min, and extension at 72 °C for 3 min for 5 cycles, followed by denaturation at 99 °C for 1 min, annealing at 55 °C for 2 min, and extension at 72 °C for 3 min for 5 cycles. PCR products electrophoresed on a 1% agarose gel revealed the presence of a ~369-bp band, which generated a positive signal when subjected to Southern blot hybridization with a 32P-labeled FP-specific oligonucleotide, 5′-GACTGGGAGAATGATATTTTAT-3′ (18). ~180–bp PCR product was gel-purified using a 359-amino acid protein with seven putative membrane-spanning domains, belonging to the superfamily of G-protein coupled receptors.

Northern Blot Analysis—Tissue distribution of the human FP mRNA was analyzed on human multiple tissue Northern blots from CLONTECH (Palo Alto, CA) using a ~650 bp BamHI/HindIII fragment of the full-length FP clone random primed with [32P]dCTP to a specific activity of 3.1 × 10<sup>8</sup> cpm/µg. The blots were hybridized in Rapid-hyb buffer at 65 °C for 3 h and washed initially with 50 ml of 5 × SSC, 0.1% SDS at room temperature for 30 min and then with four washes of 50 ml of 0.2 × SSC, 0.1% SDS at 65 °C for 30 min. The blots were then autoradiographed overnight at ~80 °C.

Stable Expression in HEK 293 Cells—A 1.8-kilobase pair EcoRI fragment from the full-length hcb-FP cDNA was subcloned into pcDNA3 (Invitrogen, San Diego, CA). The orientation of the insert was verified by restriction digestions. This expression construct (pcDNA3-FP) was then used to transfect HEK 293 cells using Dotap under standard conditions. HEK 293 cells were also transfected with pcDNA3 to serve as a control. The medium was replaced after 6 h with fresh medium containing 1.5 mg/ml G418. Stable transfectants were selected on medium containing 1.5 mg/ml G418 and screened for the expression of the FP by binding to [3H]PGF<sub>2α</sub> and second messenger (inositol phosphate; InsP<sub>3</sub>) generation. One clone (HEK-FP), out of 19 clones selected, was chosen for further characterization.

Measurement of [3H]inositol Phosphate Formation—To study the signal transduction properties of the hcb-FP, confluent cultures of HEK-FP cells in 12-well plates were labeled to equilibrium with myo-[2-3H]inositol (2 μCi/ml) for 16–24 h in serum-free DMEM containing 20 μM HEPS, pH 7.5, and 0.5% Albumax. Cells were preincubated in this medium with 20 μM LiCl for 15 min at 37 °C and then stimulated directly by replacement with 5–10 μM of InsP<sub>3</sub> formation was measured as described previously (38). Briefly, InsP<sub>3</sub> formation was stopped by aspiration of the medium, addition of 0.75 ml of 10 μM formic acid and incubation at room temperature for 30 min. The solution containing the extracted InsP was neutralized and diluted with 3 ml of 10 μM NH<sub>4</sub>OH (yielding a final pH of 8–9) and then applied directly to a column containing 0.7 ml of the anion exchange resin, AG 1-X8. The column was washed with 4 ml of 40 mM ammonium formate, pH 5.0, to remove the free inositol and the glyceroinositol. Total InsPs were eluted with 4 ml of 2 M ammonium formate, pH 5.0. One ml of the eluate was counted with 9 ml of scintillation fluid. Results presented are an average of three to five independent experiments.

Desensitization experiments were performed essentially as described by Opperman et al. (39). Briefly, after incubation with 20 μM LiCl, cells were preincubated with PGF<sub>2α</sub>, 12-iso-PGF<sub>2α</sub>, or PBS (control) for 5 min at 37 °C, followed by immediate aspiration of the medium. The cells were then washed twice with 1 ml of 50 mM glycine, 150 mM NaCl, pH 3.0. The cells were then restimulated with PGF<sub>2α</sub>, 12-iso-PGF<sub>2α</sub>, or PBS (control) for 5 min at 37 °C, followed by a 1-h incubation with fluorescein isothiocyanate-labeled anti-rabbit IgG (1:5000 dilution). Antigen-antibody complexes were visualized by chemiluminescence.

Generation of FP Antibodies—Polyclonal peptide antibodies were raised in rabbits to the sequence GINGNHSLETCET corresponding to the third extracellular loop of the human FP receptor by Research Genetics Inc (Huntsville, AL). The antisera were tested by immunoblotting, using membranes from HEK-FP cells. HEK-FP membranes were prepared from confluent 100-mm dishes as follows. Briefly, cells were washed twice with PBS and scraped into 20 ml Tris, pH 7.4, containing 4 mM EDTA, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 0.2 μM phenylmethylsulfonyl fluoride. Cells were lysed by sonication on ice, and membrane fractions were collected by centrifugation at 115,000 × g for 1 h at 4 °C. The resulting pellet was resuspended in the same buffer. Membrane proteins (100 μg/lane) were resolved on a 10% SDS-polyacrylamide gel and transferred to a nitrocellulose membrane. The immunoblot was first blocked with 5% milk in TBS-T. FPs were visualized by treating the immunoblots with 1:5000-dilution of the crude peptide antisera (in 5% milk/TBS-T) for 1 h at room temperature, followed by horseradish peroxidase-conjugated anti-rabbit IgG (1:5000 dilution). Antigen-antibody complexes were visualized by chemiluminescence.

For immunocytochemistry, cells were grown on chamber slides (Nunc, Napierville, IL) and fixed with 70% methanol, 30% acetic at -20 °C for 10 min, followed by incubation at room temperature for 5 min. The cells were blocked with 2% BSA/PBS and then treated with 1:2000 dilution of anti-FP antisera in 0.5% BSA/PBS for 1 h at room temperature, followed by a 1-h incubation with fluorescein isothiocyanate-labeled anti-rabbit IgG (1:500) in 0.5% BSA/PBS. Between each step, slides were washed three times for 10 min each with PBS. Slides were mounted in Vectashield (Vector Laboratories, Burlingame, CA) and examined by fluorescence microscopy with a Nikon Microphot FXA microscope.

Assay for DNA Synthesis—We measured [methyl-3H]thymidine incorporation into DNA by the method of Nakamura et al. (40) with slight modifications. NIH 3T3 cells were subcultured into 12-well plates.
Characterization of the FP Receptor

RESULTS

The hcb-FP encodes an open reading frame of 359 amino acids that is identical to the uterine FP. Northern blot analysis reveals the FP mRNA to be ~5 kilobase pairs in size and highly expressed in the human heart > pancreas > liver, placenta > skeletal muscle > uterus > kidney > small intestine (Fig. 1).

We generated a mammalian expression construct of the hcb-FP cDNA in pcDNA3 and used this to transfect HEK 293 cells. One of the stable transfectants (HEK-FP), was chosen for detailed characterization.

Activation of the FP by PGF$_{2\alpha}$ leads to an increase in InsP formation in HEK 293 cells in a dose-dependent manner, reaching a plateau around 1 μM PGF$_{2\alpha}$. The EC$_{50}$ for PGF$_{2\alpha}$-induced InsP formation is 10 ± 1.5 nm (Fig. 2).

**Fig. 1.** Tissue distribution of human FP. Northern blot analysis of 2 μg of human mRNA from heart, brain, placenta, lung, liver, skeletal muscle, kidney, pancreas, spleen, thymus, prostate, testis, uterus, small intestine, colon, and peripheral blood leukocytes. The multiple tissue Northern blot was hybridized with a ~650-bp BamHI/HindIII fragment from the hcb-FP cDNA as described under “Experimental Procedures.” The blots were washed with 0.2% SSC, 0.1% SDS at 60 °C, followed by autoradiography.

Confluent cultures were washed three times with PBS and then incubated in serum-free DMEM for 10 h. The quiescent cultures were then washed twice with serum free DMEM and stimulated with agonist in the serum-free DMEM for 24 h at 37 °C. [3H]Thymidine (0.5 Ci/ml) was added to the medium in the last 2 h of incubation. The cells were washed twice with ice-cold PBS at the end of the 24-h period and incubated with 1 ml of ice-cold 10% trichloroacetic acid for 10 min on ice to remove the intracellular pool of unincorporated [3H]thymidine. After the removal of the trichloroacetic acid solution, the cells were incubated with 1 ml of 0.5 N sodium hydroxide for 10 min at room temperature. The sodium hydroxide-soluble sample was counted with 9 ml of scintillation fluid. Results presented are an average of three independent experiments.

8-isoprostanes are isomers of PGF$_{2\alpha}$ and have been divided into four structural classes (41–43). To test their possible affinity for the FP, we selected members of the first (IPF$_{2\alpha}$-II), third (IPF$_{2\alpha}$-III), and fourth classes (8-iso-PGF$_{2\alpha}$, 12-iso-PGF$_{2\alpha}$, and 9β,11β-PGF$_{2\alpha}$) (Fig. 3). As seen in Fig. 4, only 12-iso-PGF$_{2\alpha}$ among these isoprostanes, caused significant activation of the hcb-FP, as observed by InsP formation in HEK-FP cells. To explore the specificity of 12-iso-PGF$_{2\alpha}$ for the FP, these compounds were also tested for their ability to activate other prostanoid receptors, namely, the IP and the two cloned isoforms of the thromboxane receptor (TPα and TPβ). Iloprost (a prostacyclin analog) induced a ~2.6 ± 0.4-fold increase in InsP formation in HEK 293 cells stably expressing the human IP receptor (44). However, none of the isoprostanes mimicked this response. On the other hand, when tested on HEK 293 cells stably expressing the TP receptor isoforms (45), 12-iso-PGF$_{2\alpha}$ resulted in 1.6–1.8-fold increase in InsP formation (Fig. 4). In comparison, equimolar concentrations of U46619, a thromboxane agonist, caused 9–10-fold stimulation of InsP formation in these cells. The 12-iso-PGF$_{2\alpha}$-induced InsP formation via the TP was abolished by the TP antagonist, SQ29548. As demonstrated previously (33), 8-iso-PGF$_{2\alpha}$ also activates TPs, and this response was also abolished by SQ29548.

Isoprostane-induced InsP formation via the prostanoid receptors is dose-dependent. The EC$_{50}$ for InsP formation by 12-iso-PGF$_{2\alpha}$ via the FP is 5 ± 0.7 μM, whereas that for 8-iso-PGF$_{2\alpha}$ is 20 ± 3.4 μM (Fig. 5, Table I). However, the maximal response to 12-epi-PGF$_{2\alpha}$ is greater than 500% over control, similar to the maximal response to PGF$_{2\alpha}$, whereas that for 8-iso-PGF$_{2\alpha}$ is less than 200% of control. By contrast, TPs appear to favor 8-iso-PGF$_{2\alpha}$ over 12-iso-PGF$_{2\alpha}$ as a ligand. The EC$_{50}$ of 8-iso-PGF$_{2\alpha}$ on the TP receptors is ~2.5–5 μM, whereas that of 12-iso-PGF$_{2\alpha}$ is greater than 50 μM. Furthermore, the maximal response of the TP to 8-iso-PGF$_{2\alpha}$ is ~700% over control, similar to their response to the thromboxane analog, U46619 (700–900%), whereas their maximal response to 12-iso-PGF$_{2\alpha}$ is ~400% over control.

We generated polyclonal peptide antibodies to the third extracellular loop of the FP. Immunoblot analysis of HEK-FP membranes with anti-FP antisera revealed FP to be a broad complex with a molecular weight ranging from 42 to 55 kDa (Fig. 6A). This signal appeared to be specific to FP inasmuch as it was not evident in pcDNA3 vector-transfected HEK 293 cells. Furthermore, the FP signal was competed away by preincubat-
ing the antisera with 10 μg/ml of the corresponding peptide. The human anti-FP antiserum also recognizes the native FP in mouse NIH 3T3 cells. Immunocytochemistry of HEK-FP cells reveals FP to be expressed predominantly at the cell surface (Fig. 6B).

We investigated the ability of FP to undergo agonist-induced rapid homologous desensitization. Although receptor desensitization is a common method of regulation among G protein-coupled receptors (46), there is very little information available on the regulation of FP function by desensitization. Pretreatment of HEK-FP cells with 1 μM PGF₂α for 5 min causes a significant dose-dependent reduction in InsP formation as compared with control cells (Fig. 7A), although membrane receptor protein (Fig. 7B) and whole cell binding (408.8 dpm/10⁶ cells in control group and 419.6 dpm/10⁶ cells in the pretreated group) remain essentially unchanged. The dose response for pretreatment revealed that attenuation of InsP formation was maximal when cells were pretreated with ~1 μM PGF₂α, resulting in
TABLE I

| Receptor | EC\textsubscript{50} for Inositol Phosphate Formation | Maximum responses (% control) |
|----------|-----------------------------------------------|-------------------------------|
|          | PGF\textsubscript{2\alpha} | 12-iso-PGF\textsubscript{2\alpha} | 8-iso-PGF\textsubscript{2\alpha} | U46619 |
| FP       | 10 ± 1.7 nM | 5 ± 0.8 μM | 20 ± 3.4 μM | ND |
| TP\textalpha | ND | 50 ± 8 μM | 2.5 ± 0.8 μM | 10 ± 1.3 nM |
| TPβ      | ND | 70 ± 14 μM | 5 ± 1.2 μM | 51 ± 8.6 nM |

= ND, not determined.

~60% desensitization (Fig. 7C). The ability of 12-iso-PGF\textsubscript{2\alpha} to cause FP desensitization was also tested on HEK-FP cells. When pretreated with PGF\textsubscript{2\alpha} or 12-iso-PGF\textsubscript{2\alpha}, FP undergoes rapid desensitization to both PGF\textsubscript{2\alpha} and 12-iso-PGF\textsubscript{2\alpha} (Fig. 8).

Both PGF\textsubscript{2\alpha} and 12-iso-PGF\textsubscript{2\alpha} induce InP formation in NIH 3T3 cells in a dose-dependent manner (Fig. 9A). The EC\textsubscript{50} for InP formation by PGF\textsubscript{2\alpha} is 50 ± 8.3 nm. This is comparable to the EC\textsubscript{50} of PGF\textsubscript{2\alpha} for InP formation in HEK-FP cells (Fig. 2) and also comparable to the EC\textsubscript{50} for InP formation reported by Nakao et al. (47) in NIH 3T3 cells (46 nm). In NIH 3T3 cells, PGF\textsubscript{2\alpha} causes a dose-dependent increase in mitogenesis, with an EC\textsubscript{50} of ~25 ± 3.8 nm (Fig. 9B). This response was also mimicked by 12-iso-PGF\textsubscript{2\alpha}. The mitogenic response parallels InP formation, resulting in a maximum of ~3.2-fold increase over basal values.

DISCUSSION

We have cloned the FP from a hcb cDNA library, a likely target tissue for the efficacy of FP agonists in the treatment of glaucoma. Although the hcb-FP is identical to that cloned from the human uterus, the isolation of only two clones from the ocular source suggests that the FP is not expressed abundantly in the ciliary body. However, it does not rule out the existence of other FP isoforms (23) in other parts of the eye. Studies on the distribution of FP over a wide range of human tissues reveal its mRNA to be abundant in the human heart, in addition to reproductive tissues, as reported previously (19–21). This is particularly interesting in light of recent reports on the ability of PGF\textsubscript{2\alpha} to cause hyper trophy of cardiac myocytes (12).

Generation of HEK cells stably expressing the FP presents a tool for the detailed molecular characterization of FP. This is of importance, because there is a discrepancy between the rank order of potency of PGF\textsubscript{2\alpha} analogs in their ability to reduce IOP and their ability to bind FP in various membrane preparations (48). As with other prostanoid G protein-coupled receptors (45), the pattern of fluorescence observed with an FP-specific antibody suggests that FP is localized predominantly at the cell membrane. Availability of a human FP-specific antibody will facilitate determination of the pattern of FP receptor expression in the eye.

The molecular mechanisms of agonist-induced rapid FP receptor desensitization have not been elucidated to date. FPs are down-regulated in astrocytes after prolonged (>4 h) exposure to PGF\textsubscript{2\alpha} (49), and constriction of bovine sphincter muscle evoked by PGF\textsubscript{2\alpha} is down-regulated upon pretreatment of the preparation with the eicosanoid for 45 min (50). We now demonstrate that stimulation of HEK cells expressing FP, or of NIH 3T3 cells expressing endogenous FP, with PGF\textsubscript{2\alpha} results in rapid desensitization, initially without loss of receptor protein from the cell surface. The availability of these reagents is likely to facilitate investigation of the mechanism of action of PGF\textsubscript{2\alpha} analogs in ocular disease and of tachyphylaxis to FP agonists in the treatment of glaucoma.

F\textalpha isoprostanes are free radical-catalyzed products of arachidonic acid (28). Up to 64 different isomers may be formed theoretically, belonging to four structural classes (41–43). Initially, these compounds are formed in situ on the cell membrane, from which they may be cleaved by the action of phospholipases to circulate and, ultimately, be excreted in urine (28). Specific measurement of isoprostanes in affected tissues, circulating lipoproteins, and urine holds promise as an approach to study oxidative stress in vivo. A more controversial issue is whether F\textalpha isoprostanes, or indeed analogous isomeric forms of other eicosanoids (29), might mediate some of the functional consequences of free radical generation. It has been speculated that in their esterified form, they may contribute to free radical-catalyzed membrane injury (28).

The biological effects of isoprostanes have only recently been investigated. Much attention has been paid to one member of the class IV F\textalpha isoprostanes, 8-iso-PGF\textsubscript{2\alpha}. This has been shown to stimulate inositol phosphate formation and DNA synthesis in cultured rat aortic smooth muscle cells (32). It is also a potent vasoconstrictor, at least in the renal and pulmonary circulations (51). It also stimulates mitogenesis and modulates platelet function, facilitating aggregation by subthreshold concentrations of conventional platelet agonists, such as ADP and thrombin (33). These effects of 8-iso-PGF\textsubscript{2\alpha} are blocked by...
pharmacological TP antagonists. However, the concentration of 8-iso-PGF$_2\alpha$ needed to evoke these effects seem much greater than that which circulates in vivo (33). Furthermore, 8-iso-PGF$_2\alpha$, unlike other isoprostanes, may also be formed by a cyclooxygenase-dependent pathway (52).

We have recently synthesized several F$_2$ isoprostane isomers (43, 53–55). One of these, 12-iso-PGF$_2\alpha$ (43), activates the FP in a specific and saturable manner. It seems likely that 12-iso-PGF$_2\alpha$ may be an abundant member of the F$_2$ isoprostane family, inasmuch as free radical cyclization rules predict that upon formation of a cyclopentane ring, after oxidative modification of arachidonic acid, the adjacent substituents formed are cis to each other. Thus, cyclization of the hydroperoxy radical derived from 11-hydroperoxyeicosatetraenoic acid would lead predominantly to the formation of cis products such as 8-iso-PGF$_2\alpha$ and 12-iso-PGF$_2\alpha$. Two reports actually predict the formation of 12-iso-PGF$_2\alpha$ type products in larger amounts than 8-iso-PGF$_2\alpha$, as a result of such free radical cyclization (56, 57).

Clearly, discrete isoprostanes might activate their own specific receptors. However, despite much speculation, no such receptors have been cloned to date and, save for the case of 8-iso-PGF$_2\alpha$ (which may also be formed enzymatically), specific receptors for the by-products of lipid peroxidation may seem unlikely. A more plausible concept is that isoprostanes act, in concert, as incidental ligands at prostanoid receptors. However, the comparative dose-response relationships for individual isoprostanes versus the natural prostanoid ligand, as exemplified in this report, reveals that highly concentrated forms of iso-

![Fig. 7](image1.png)

**Fig. 7.** A, agonist-induced rapid homologous desensitization of FP. Confluent cultures of HEK-FP cells in 12-well plates were labeled to equilibrium with myo-[2-3H]inositol (2 $\mu$Ci/ml) for 16–24 h in serum-free DMEM. Cells were treated with 20 mM LiCl for 15 min at 37 °C and then pretreated with vehicle, 25 nM PGF$_{2\alpha}$, or 25 nM 12-iso-PGF$_{2\alpha}$ for 5 min at 37 °C. Cells were then washed with 50 mM glycine, 150 mM NaCl, pH 3.0, and restimulated with 50 nM PGF$_{2\alpha}$ or 50 nM 12-iso-PGF$_{2\alpha}$ for 10 min at 37 °C. Total InsP formation was measured as described under “Experimental Procedures.”

![Fig. 8](image2.png)

**Fig. 8.** 12-iso-PGF$_{2\alpha}$-induced rapid desensitization of FP. Confluent cultures of HEK-FP cells in 12-well plates were labeled to equilibrium with myo-[2-3H]inositol (2 $\mu$Ci/ml) for 16–24 h in serum-free DMEM. Cells were treated with 20 mM LiCl for 15 min at 37 °C and then pretreated with vehicle, 1 mM PGF$_{2\alpha}$, or 25 nM 12-iso-PGF$_{2\alpha}$ for 5 min at 37 °C. Cells were then washed with 50 mM glycine, 150 mM NaCl, pH 3.0, and restimulated with 50 nM PGF$_{2\alpha}$ or 50 nM 12-iso-PGF$_{2\alpha}$ for 10 min at 37 °C. Total InsP formation was measured as described under “Experimental Procedures.”

Cells were then washed with 50 mM glycine, 150 mM NaCl, pH 3.0, and restimulated with varying concentrations of PGF$_{2\alpha}$ for 10 min at 37 °C. Total InsP formation was measured as described under “Experimental Procedures.”
The availability of these reagents will facilitate investigations into the molecular basis of action of PGF$_{2\alpha}$ analogs in reducing IOP. Furthermore, we have demonstrated that the FP may be activated and desensitized, not only by its natural ligand, PGF$_{2\alpha}$, but also by F$_2$ isoprostanes like 12-iso-PGF$_{2\alpha}$. These observations raise the possibility that the therapeutic response to PGF$_{2\alpha}$ analogs may be modulated by F$_2$ isoprostanes in syndromes of oxidant stress, such as glaucoma or congestive heart failure.

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