Uteroglobin Inhibits Prostaglandin F₂α Receptor-mediated Expression of Genes Critical for the Production of Pro-inflammatory Lipid Mediators*

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Prematurity is one of the leading causes of infant mortality. It may result from intrauterine infection, which mediates premature labor by stimulating the production of inflammatory lipid mediators such as prostaglandin F₂α (PGF₂α). The biological effects of PGF₂α are mediated via the G protein-coupled receptor FP; however, the molecular mechanism(s) of FP signaling that mediates inflammatory lipid mediator production remains unclear. We reported previously that in the human uterus, a composite organ in which fibroblast, epithelial, and smooth muscle cells are the major constituents, an inverse relationship exists between the levels of PGF₂α and a steroid-inducible anti-inflammatory protein, uteroglobin. Here we report that, in NIH 3T3 fibroblasts and human uterine smooth muscle cells, FP signaling is mediated via multi-kinase pathways in a cell type-specific manner to activate NF-κB, thus stimulating the expression of cyclooxygenase-2. Cyclooxygenase-2 is a critical enzyme for the production of prostaglandins from arachidonic acid, which is released from membrane phospholipids by phospholipase A₂, the expression of which is also stimulated by PGF₂α. Most importantly, uteroglobin inhibits FP-mediated NF-κB activation and cyclooxygenase-2 gene expression by binding and most likely by sequestering PGF₂α into its central hydrophobic cavity, thereby preventing FP-PGF₂α interaction and suppressing the production of inflammatory lipid mediators. We propose that uteroglobin plays important roles in maintaining homeostasis in organs that are vulnerable to inadvertent stimulation of FP-mediated inflammatory response.

Uteroglobin (UG), a also known as Clara cell 10-kDa protein (CC10), is a founding member of the secretoglobin superfamily (1). This protein is secreted by the mucosal epithelial of all mammalian organs that communicate with the external environment. In the uterus, UG gene expression is induced by progesterone (2), and this induction is further augmented by prolactin (3). In the respiratory system, the tracheobronchial epithelia constitutively express UG at a very high level and may be further stimulated by corticosteroids. During the past two decades it has been established that UG is a multifunctional protein with potent anti-inflammatory and anti-chemotactic properties (reviewed in Ref. 4). Recently, it has been reported that this protein may have receptor-mediated functions (5, 6). Furthermore, UG has been reported to bind hydrophobic ligands such as progesterone (7) and polychlorinated biphenyls (8). Structurally, UG is a homodimer in which the two 70-amino acid subunits are joined in antiparallel orientation by two interchain disulfide bridges forming a large central hydrophobic cavity, which may harbor hydrophobic ligands (9). Each subunit of UG forms four α-helices, and the active site of its anti-inflammatory properties appears to reside in a nonapeptide region (residues 39 – 47) of the α-helix 3 (10).

We reported previously that in the human uterus (11) and in fetal rabbit lungs (12) an inverse relationship exists between the levels of UG and prostaglandin F₂α (PGF₂α). Prostaglandins (PGs) are a family of biologically active lipids that mediate numerous physiological and pathological processes (13) via heterotrimeric G protein-coupled receptors. Cyclooxygenases (COXs) (14) are the key enzymes responsible for PG synthesis from arachidonic acid, which is released from membrane phospholipids by phospholipase A₂ (PLA₂) catalysis (15). One of the PGs, PGF₂α, plays critical roles in important biological processes such as inflammation, uterine smooth muscle contraction, cervical ripening, and initiation of parturition (reviewed in 16). During normal pregnancy, the strong contractions of uterine smooth muscles (i.e. myometrium) do not occur until term. It has been reported that PGF₂α mediates uterine smooth muscle contractility via its receptor, FP (17), and that mice in which the FP gene has been inactivated by gene targeting fail to initiate parturition (18). Moreover, it has been reported that inhibitors of COX-2, such as non-steroidal anti-inflammatory agents, may adversely affect fertilization, decidualization, implantation, and, consequently, delay the initiation of parturition (19). Although these important biological functions of PGF₂α may be mediated via FP (20), neither the molecular mechanisms of FP signaling nor the mechanism(s) by which the maternal organisms prevent inadvertent stimulation of PGF₂α-induced pro-inflammatory lipid mediator production in the uterus are clear. This is an important question, as it has been reported that intra-uterine infection is a major cause of premature labor and birth (reviewed in 21) that may be induced by PGs (22), although the molecular mechanisms are unknown.

Here we report that FP signaling is mediated via multi-kinase pathways in a cell type-specific manner, leading to the activation of NF-κB. The activation of NF-κB stimulates the expression of COX-2, critical for the generation of arachidonic acid from membrane phospholipids by
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EXPERIMENTAL PROCEDURES

Materials—The NIH 3T3 and human uterine smooth muscle cells were obtained from the American Type Culture Collection (Manassas, VA) and Cambrex, Inc. (Walkersville, MD), respectively. PGs were purchased from Cayman Chemical Co. (Ann Arbor, MI). RNAzol B was purchased from Tel Test Inc. (Friendswood, TX). [α-32P]dCTP and the Hybrid N + membrane were obtained from Amersham Biosciences. [3H]PGF2α was purchased from American Radiolabeled Chemicals, Inc. (St Louis, MO). A protein kinase C inhibitor (bisindolyl maleimide III), A p38 MAPK inhibitor (SB203580), NF-κB-specific inhibitors (pyrrolidinedithiocarbamate and NF-κB SN50), and a p44/42 inhibitor (PD98059) were purchased from Calbiochem. Fetal bovine serum (FBS) (Biofluids (Rockville, MD)). ExpressHyb solution was purchased from Amersham Biosciences. Materials—The NIH 3T3 and human uterine smooth muscle cells were obtained from the American Type Culture Collection (Manassas, VA) and Cambrex, Inc. (Walkersville, MD), respectively. PGs were purchased from Cayman Chemical Co. (Ann Arbor, MI). RNAzol B was purchased from Tel Test Inc. (Friendswood, TX). [α-32P]dCTP and the Hybrid N + membrane were obtained from Amersham Biosciences. [3H]PGF2α was purchased from American Radiolabeled Chemicals, Inc. (St Louis, MO). A protein kinase C inhibitor (bisindolyl maleimide III), A p38 MAPK inhibitor (SB203580), NF-κB-specific inhibitors (pyrrolidinedithiocarbamate and NF-κB SN50), and a p44/42 inhibitor (PD98059) were purchased from Calbiochem. Fetal bovine serum (FBS) (Biofluids (Rockville, MD)). ExpressHyb solution was purchased from Clontech. Mouse monoclonal anti-cPLA2 and goat polyclonal anti-COX-2 antibodies were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). An FP receptor antibody was purchased from Cayman chemical company (Ann Arbor, MI). The recombinant human uteroglobin (hUG) was produced in Escherichia coli and purified as described previously (23, 24).

Cell Culture and Treatment—Human uterine smooth muscle (hUSM) cells were grown in their specified SmGM-2 medium (Biowhitaker, Inc., Walkersville, MD). At 70% confluence, the cells were washed one time with smooth muscle basal medium (without the growth factors and serum) and then cultured in smooth muscle basal medium with PGF2α (100 nM), HUG (10–150 nM), and BSA (150 μM) for the indicated periods of time (Fig. 1A). The NIH 3T3 cells were grown in Dulbecco’s modified essential medium supplemented with 10% heat-inactivated fetal calf serum, 2 mM L-glutamine, penicillin (100 units/ml), and streptomycin (0.1 mg/ml) at 37 °C with 5% CO2. Cells were grown in their respective recommended growth medium to 70–80% confluence, washed once with the Opti-Mem-1 medium (Invitrogen) containing 2.5% FBS and then treated with PGF2α for indicated time intervals in Opti-Mem-1 medium containing 2.5% FBS. Cell lysates were prepared in presence of protease inhibitors. Total proteins in the samples were estimated by using Bio-Rad protein assay dye (Bio-Rad Laboratories) using BSA as the standard. Equal amounts (40 μg) of the total protein from each sample were loaded in the gel and resolved by electrophoresis using 7.5 or 12% SDS-polyacrylamide gels under denaturing and reducing conditions. Proteins were then electrotransferred to polyvinylidene fluoride membrane (Immobilon P; Millipore Corporation, Bedford, MA), blocked with 5% BSA in 1× phosphate-buffered saline buffer containing 0.1% Tween 20 and 0.1% Triton X-100 at 4 °C overnight, and then subjected to immunoblot analysis using mouse monoclonal anti-cPLA2/goat polyclonal anti-COX-2 or rabbit polyclonal FP antibodies. This was followed by incubation with anti-mouse/goat or rabbit IgGs conjugated with horseradish peroxidase and detection of chemiluminescence by an ECL system (Amersham Biosciences) according to the manufacturer’s instructions.

Electrophoretic Mobility Shift Analysis—Cells were grown previously in their respective growth medium to 70–80% confluence, washed once with the Opti-Mem-1 medium (Invitrogen) containing 2.5% FBS and then treated with 20 ml of Opti-Mem-1 medium containing 2.5% FBS and other effectors as indicated for 1 h at 37 °C with 5% CO2. Cells were then harvested, washed in 1× phosphate-buffered saline buffer, and used for nuclear extract preparation following a supplied protocol of GENEKA Biotech Inc. (Montreal, Canada). Electrophoretic mobility shift analyses (EMASs) were performed using the nuclear extracts (20 μg protein) on a non-denaturing 5% polyacrylamide gel following a supplied protocol of GENEKA Biotech Inc. (Montreal, Canada) with the oligonucleotides 5′-GAGAGGTTAGGGGTACCCGTATGGAC-3′ (wild type NF-κB) and 5′-GAGAGGTTAGGGGTACCCGTATGGAGCCCTT-AGTTAGGGGTACCCGTATGGAC-3′ (mutated NF-κB). NF-κB double-stranded oligonucleotides were generated by annealing sense and antisense oligonucleotides without and with mutations (boldface residues). Specificity of protein-DNA complexes was verified either by competing with non-radioactive wild type NF-κB/murine NF-κB oligos or by immunoreactivity with polyclonal antibodies specific for p65/p50 subunits of NF-κB.

UG-[3H]PGF2α Binding Assay—An assay for the detection of the UG-[3H]PGF2α complex was performed as described previously (9). Briefly, in 20 μl of reaction mixture 50 pmol of [3H]PGF2α were incubated with 25 pmol of recombinant UG in the absence and presence of varying concentrations of non-radioactive PGF2α. After incubation for 1 h at 4 °C, aliquots were resolved by electrophoresis using non-denaturing and non-reducing 15% polyacrylamide gel. The gel was dried, and autoradiographs were obtained by using a BAS-15 phosphorimaging device (Fuji).
Molecular Modeling—The two-dimensional chemical structure of PGF₂α was converted to a three-dimensional structure using the CONCORD module of Tripos software (Tripos Inc., St. Louis, MO). Docking of one molecule of PGF₂α into the hydrophobic cavity of hUG (25) was difficult, as there were no hydrogen bonds between the COOH and OH groups in the five-membered ring and the Tyr-21 residues in the two monomers. On analyzing the docking modes, we found that the cis double bond in the A chain of PGF₂α makes the chain shorter and does not interact symmetrically with Tyr-21 residues in the dimer. A high-resolution crystal structure of PGF₂α has been published and is available in the Cambridge Data Base (26). This compound was crystallized as a dimer in which the hydrophobic A and B chains interact and form a structure such that the COOH groups are in the opposite side of the dimer. Because of this stacking interaction, two molecules of PGF₂α could interact symmetrically with Tyr-21 in both monomers, which is similar to the interaction found in the reported crystal structure of the UG-polychlorinated biphenyl complex (27). We docked two PGF₂α molecules into the central hydrophobic cavity of the reported crystal structure of hUG (25), which was energy minimized using the cpp91 force field of the DISCOVER module of InsightII molecular simulation software (Accelrys Inc., San Diego, CA). A distance-dependent dielectric constant was used for the calculation of the electrostatic interaction.

RESULTS

PGF₂α Stimulates COX-2 Gene Expression—During the past decade it has been increasingly evident that two COX enzymes, COX-1 and COX-2, play critical roles in the production of PGH₂, the first step in eicosanoid biosynthesis. It was further realized that COX-1 is essential for the production and maintenance of physiological levels of prostanoids and that COX-2 is responsible for the production of prostanoids that are found in sites of disease and inflammation. Thus, COX-2 has been the prime target of anti-inflammatory drug development (reviewed in Ref. 28).

It has been reported that COX-2 gene expression is inducible by various agonists (13, 14, 29, 30). Therefore, we sought to determine whether PGF₂α stimulates COX-2 gene expression in hUSM and in NIH 3T3 cells. Accordingly, we treated hUSM and NIH 3T3 cells with PGF₂α at varying concentrations and for varying lengths of time and analyzed the expression of COX-2 mRNA by Northern blot analyses. We found that PGF₂α, in a time-dependent manner, stimulates COX-2 mRNA and COX-2 protein expression in both hUSM (Fig. 1A) and NIH 3T3 (Fig. 1B) cells. The ethanol-treated control cells did not show any elevation of COX-2 expression over basal levels. Taken together, these results suggest that in hUSM and NIH 3T3 cells PGF₂α stimulates COX-2 gene expression.

PGF₂α-stimulated COX-2 Expression Is Mediated via FP—To determine whether PGF₂α stimulates COX-2 expression via its receptor, FP, we determined the expression of the FP protein in hUSM and in NIH 3T3 cells. Accordingly, we treated hUSM and NIH 3T3 cells with PGF₂α at varying concentrations and for varying lengths of time and analyzed the expression of FP protein by Western blot analyses. We found that PGF₂α, in a time-dependent manner, stimulates the expression of FP protein in hUSM and NIH 3T3 cells treated with PGF₂α mediasation for varying lengths of time and analyzed the expression of COX-2 expression. As expected, the expression of FP and COX-2 proteins was not inhibited in untreated and FP sense S-oligo-treated cells (Fig. 2B, upper and middle sections, second and third lanes from the left). These results demonstrate that both NIH 3T3 and hUSM cells express FP and that PGF₂α-mediated COX-2 gene expression requires FP.

PGF₂α Stimulates cPLA₂ Expression—The biological effects of PGF₂α on uterine smooth muscle contraction, cell proliferation, luteolysis, and parturition are suggested to be mediated via pro-inflammatory lipid mediator production (17, 32–37). Thus, in the present study we sought to determine whether PGF₂α stimulates the expression of genes that play critical roles in the production of pro-inflammatory lipid mediators (e.g. PGs, leukotrienes, and the platelet-activating factor). The rate-limiting substrate for the generation of these mediators is arachidonic acid (AA), the production of which from membrane phospholipids is catalyzed by PLA₂ (15). Because cPLA₂ expression is reported to be stimulated by various agonists (38) and because NIH 3T3 cells express FP (our results and Refs. 39 and 40), we first determined if cPLA₂ expression in NIH 3T3 cells is stimulated by PGF₂α. Accordingly, we treated NIH 3T3 cells with PGF₂α for varying lengths of time and analyzed the expression of cPLA₂ mRNA and protein by Northern and Western blot analyses, respectively. Our results show that PGF₂α stimulates the expression of cPLA₂ mRNA (Fig. 3A) and protein (Fig. 3B) suggesting that FP signaling may regulate AA release from cellular phospholipids mediated by cPLA₂ catalysis.

FIGURE 1. PGF₂α stimulates COX-2 expression in hUSM and NIH 3T3 cells. A, upper sections, Northern blot analysis performed to determine the expression of COX-2 mRNA in hUSM cells treated with PGF₂α or ethanol (control) for the indicated periods of time. Loading of equal amounts of total RNA (40 μg/lane) was standardized by hybridization with a human GAPDH CDNA probe. Lower sections, Western blot analysis of total proteins from cells treated with either PGF₂α or ethanol (control). B, upper sections, Northern blot analysis using total RNA from NIH 3T3 cells treated with either ethanol (left) or PGF₂α and probed with mouse COX-2 probe to determine the COX-2 mRNA expression. mRNA loading was standardized by hybridization of the blots with mouse GAPDH probe. Lower sections, Western blot analysis of total protein from NIH 3T3 cells treated with either ethanol (left) or PGF₂α for the indicated periods of time, using goat polyclonal anti-COX-2 antibodies.
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FP Signaling Is Mediated via Multiple Kinase Pathways in a Cell Type-specific Manner—Prostaglandins are known to exert their biological effects via heterotrimeric G protein-coupled receptors. To delineate the intracellular mechanism(s) of signaling via the PGF2α receptor, FP, we examined various signaling pathways using specific pharmacological inhibitors of kinases. We found that PGF2α-mediated stimulation of COX-2 mRNA expression was strongly inhibited by a specific PKC-inhibitor, bisindolylmaleimide III, in NIH 3T3 (Fig. 4A) cells. In contrast, treatment of hUSM cells with inhibitors of p38 MAPK (SB203580) and p44/42 MAPK (PD98059) as well as PKC caused marked inhibition of PGF2α-stimulated COX-2 mRNA expression (Fig. 4B, lanes 2, 3, and 4). These results indicate that FP signaling is mediated via PKC, p38 MAPK, and p44/42 MAPK in a cell type-specific manner.

FP Signaling Activates NF-κB—COX-2 is an agonist-inducible gene, and in the 5′-promoter region of this gene there are several transcriptional regulatory elements such as the cAMP-response element-binding protein (CREBP), AP2, SP1, GATA box, the nuclear factor of interleukin 6 (NF-IL6), and NF-κB (reviewed in 41). Among these elements, NF-IL6 and NF-κB act as positive regulatory elements for COX-2 gene expression in some cell lines (42). The results of our present experiments suggest that COX-2 expression by FP signaling is mediated via PKC, p38 MAPK, and p44/42 MAPK in a cell type-specific manner. This prompted us to examine the activation of NF-κB by PGF2α, as it has been reported that PKC activates this nuclear factor, required for COX-2 expression, in NIH 3T3 cells (43) as well as in other cell types (44). Accordingly, we examined the effects of the NF-κB inhibitors pyrrolidinedithiocarbamate and NF-κB SN-50 on PGF2α receptor-mediated induction of COX-2 mRNA expression. We found that PGF2α receptor-mediated COX-2 mRNA expression is inhibited by specific inhibitors of NF-κB (Fig. 4C), suggesting that PGF2α, via multiple kinase pathways, mediates NF-κB activation, which stimulates COX-2 gene expression.

Uteroglobin Inhibits PGF2α-mediated Stimulation of COX-2 Gene Expression—UG is a low molecular weight anti-inflammatory protein secreted by the mucosal epithelia of virtually all mammalian organs (4), including the respiratory and reproductive organs. It has been previously reported that there is an inverse relationship between the levels of PGF2α and uteroglobin in the human uterus (11) and the fetal rabbit lungs (12). The lungs and the uterus are composite organs consisting predominantly of epithelial, smooth muscle, and fibroblast cells, which have the capability to initiate and propagate inflammatory responses. Thus, we sought to determine the effects of purified recombinant human UG on FP-mediated stimulation of COX-2 expression in NIH 3T3 and hUSM cells. We found that FP-mediated stimulation of COX-2 mRNA expression in NIH 3T3 (Fig. 5A) and hUSM (Fig. 5B) cells is drastically suppressed by UG in a dose-dependent manner. The inhibitory effects of UG on PGF2α-stimulated COX-2 expression may suggest that UG inhibits FP signaling by suppressing the activation of NF-κB, which is essential for COX-2 gene expression (41).

Uteroglobin Inhibits PGF2α-mediated NF-κB Activation—Because NF-κB is an inducible positive transcriptional regulator of COX-2 (45, 46), we examined whether PGF2α stimulates COX-2 expression via NF-κB activation in NIH 3T3 cells. We then examined the effects of UG on PGF2α-mediated activation of NF-κB by an EMSA using PGF2α-treated and untreated cells. We found that whereas FP-mediated activation of NF-κB (Fig. 6A, second lane from left) is completely inhibited by cold wild type NF-κB-specific oligonucleotides (Fig. 6A, fifth lane from left), the nonspecific mutant oligonucleotide had no effect (Fig. 6A, fourth lane from left). Most importantly, UG markedly inhibited NF-κB activation (Fig. 6A, third lane from left). The use of p65-antibody resulted in a super-shift of the band (Fig. 6A, far right lane). It has been reported that a cyclopentenone PG, PGA1, which manifests anti-inflammatory activity, inhibits NF-κB activation (47). Because UG also possesses potent anti-inflammatory activity and because the results of our
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FIGURE 4. Multiple kinase pathways of FP-mediated COX-2 expression. A and B, PGF$_{2\alpha}$, receptor-mediated stimulation of COX-2 mRNA expression in NIH 3T3 (A) and hUSM (B) cells in the absence (−) and presence (+) of specific inhibitors of p38 MAPK (SB203580, p44/42 MAPK (PD98059) or PKC (bisindolyl maleimide III (BIM)). Note the marked inhibitory effect of PGF$_{2\alpha}$ receptor-mediated stimulation of COX-2 mRNA expression. C, expression of COX-2 mRNA expression in NIH 3T3 cells treated with PGF$_{2\alpha}$ in the absence (−) and presence (+) of inhibitors of NF-κB activation, pyrrolidinedithiocarbamate (PDTC) and NF-κB SN 50. Note the inhibition of COX-2 expression by these inhibitors. Murine GAPDH was used to standardize the amount of total RNA loaded into each lane. DMSO, dimethyl sulfoxide.

FIGURE 5. UG inhibits PGF$_{2\alpha}$-stimulated COX-2 mRNA expression. A, inhibition of FP-mediated stimulation of COX-2 mRNA expression by uteroglobin in NIH 3T3 cells. Cells were treated with PGF$_{2\alpha}$ in the absence (−) and presence (+) of varying concentrations of recombinant UG. BSA was used as a nonspecific control for UG. B, inhibition of FP-mediated stimulation of COX-2 mRNA expression by uteroglobin in hUSM cells. Cells were treated with PGF$_{2\alpha}$ in the absence (−) and presence (+) of varying concentrations of recombinant UG. BSA was used as a nonspecific control for UG.

The present experiment show that UG inhibits NF-κB activation, we sought to determine whether PGA$_1$, like UG, also suppresses PGF$_{2\alpha}$-stimulated COX-2 expression. Accordingly, we treated NIH 3T3 cells with PGF$_{2\alpha}$ in presence and absence of varying concentrations of PGA$_1$. Our results show that PGA$_1$, a known inhibitor of NF-κB activation, also inhibits COX-2 expression induced by PGF$_{2\alpha}$ in a dose-dependent manner (Fig. 6B). Taken together, these results suggest that both UG and PGA$_1$ suppress PGF$_{2\alpha}$-mediated stimulation of COX-2 expression by inhibiting NF-κB activation.

Uteroglobin Inhibits FP Signaling by Binding and Most Likely Sequestering PGF$_{2\alpha}$—To determine whether UG may interact with FP and block PGF$_{2\alpha}$ signaling, we suppressed FP expression in NIH 3T3 cells by transfecting them with FP antisense oligonucleotides. The results show no inhibition of $[^{3}H]$UG (data not shown). We then sought to determine whether UG binds $[^{3}H]$PGF$_{2\alpha}$, as UG is reported to bind hydrophobic ligands (7–9). Our results show that UG binds $[^{3}H]$PGF$_{2\alpha}$ (Fig. 7A) with high specificity, as unlabeled-PGF$_{2\alpha}$ competes with $[^{3}H]$PGF$_{2\alpha}$ for binding to UG. Consistent with these results, the molecular modeling studies show that two molecules of PGF$_{2\alpha}$ can be docked into the hydrophobic cavity of hUG dimer (Fig. 7B). This binding is unlike that of polychlorinated biphenyls, for which only one molecule appears bound in the hUG central cavity. Also, unlike progesterone and polychlorinated biphenyls, which are rigid, the A and B chains of PGF$_{2\alpha}$ are very flexible and could be easily packed without any expenditure of energy. In this model, one of the hydroxyl group in the five-membered ring of one molecule and the COOH group in the other molecule interact favorably by forming hydrogen bonds with the hydroxyl group of Tyr-21.

DISCUSSION

Premature birth is a leading cause of infant mortality in which intrauterine infection plays a major role (21, 22). It has been suggested that premature labor is induced by PGs, the production of which are stimulated by bacterial antigens (48). One of these PGs, PGF$_{2\alpha}$, is a known inducer of uterine smooth muscle contraction and labor (16–18). PGF$_{2\alpha}$ mediates its biological effects via the heterotrimetric, G protein-coupled receptor FP, stimulating the production of pro-inflammatory lipid mediators. However, the molecular mechanisms of FP signaling and the mechanism by which this eicosanoid induces the production of pro-inflammatory lipid mediators, until now, remained poorly understood. Because the uterus is a composite organ composed of epithelial, fibroblast, and smooth muscle cells, we used mouse fibroblasts (NIH 3T3) and hUSM cells to determine the molecular mechanism(s) of FP signaling. Using this system, we also determined the effects of a steroid-inducible anti-inflammatory protein, UG, on FP signaling. We uncovered the following four facts. (a) FP signaling was mediated via activation of PKC, p38 MAPK, and p44/42 MAPK in a cell type-specific manner—To determine whether UG may interact with FP and block PGF$_{2\alpha}$ signaling, we suppressed FP expression in NIH 3T3 cells by transfecting them with FP antisense oligonucleotides. The results show no inhibition of $[^{3}H]$UG (data not shown). We then sought to determine whether UG binds $[^{3}H]$PGF$_{2\alpha}$, as UG is reported to bind hydrophobic ligands (7–9). Our results show that UG binds $[^{3}H]$PGF$_{2\alpha}$ (Fig. 7A) with high specificity, as unlabeled-PGF$_{2\alpha}$ competes with $[^{3}H]$PGF$_{2\alpha}$ for binding...
manner. (b) FP signaling activated NF-κB and stimulated the expression of COX-2, a critical enzyme for the production of proinflammatory lipid mediators from AA. (c) PGF2α also stimulated cPLA2, which plays a pivotal role in generating AA from cell membrane phospholipids. (d) Most importantly, UG suppressed FP-mediated NF-κB activation and COX-2 gene expression.

Our results, for the first time, define at least one of the molecular mechanisms of FP signaling and demonstrate a critical role of UG in maintaining homeostasis by suppressing NF-κB activation and COX-2 gene expression. Previous studies have shown that PGF2α mediates its biological effects via FP, although the molecular mechanism(s) of FP signaling, until now, remained unclear. We demonstrate that FP is expressed in both NIH 3T3 and hUSM cells and that inhibition of FP expression leads to the inhibition of PGF2α-stimulated COX-2 expression. Recently, two spliced variants of FP have been reported in humans (49). We confirmed these results in hUSM cells (data not shown). It has also been reported that via FP (B) variant PGF2α stimulates COX-2 gene expression (48, 50). Whether these variants are responsible for differential signaling in NIH 3T3 and hUSM cells remains to be determined. Furthermore, we uncovered for the first time that FP signaling is mediated via several kinase pathways activating NF-κB and that these path-

FIGURE 6. UG inhibits FP-mediated NF-κB activation and COX-2 gene expression. A, EMSA performed to determine the PGF2α-FP-mediated activation of NF-κB in NIH 3T3 cells treated without (−) or with (+) PGF2α in the absence (−) or presence (+) of 150 nM pure recombinant hUG for 1 h. EMSA was performed in a 5% non-denaturing polyacrylamide gel using the nuclear extract from NIH 3T3 cells. The binding assay was performed at 4°C with 32P-labeled double stranded NF-κB oligo probe (NF-κB-Probe) in the absence or presence of double stranded cold wild type NF-κB oligo (WT oligo) or cold mutated NF-κB oligo (Mut oligo). The super-shift assay was done using polyclonal p65-specific antibody (P65 Ab). B, treatment of NIH 3T3 cells with an inhibitor of NF-κB activation, PGA1, suppresses COX-2 mRNA expression. Cells were treated with PGF2α in the absence (−) and presence (+) of varying concentrations of PGA1, and COX-2 mRNA expression was detected by Northern hybridization with mouse COX-2 probe. Murine GAPDH was used to standardize RNA loading into each lane.

FIGURE 7. UG binding to [3H]PGF2α and molecular modeling. A, binding of UG with [3H]PGF2α. Formation of [3H]PGF2α-UG complexes is achieved by incubating PGF2α with purified recombinant UG (21) as described under “Experimental Procedures.” After incubation, aliquots of the mixture were resolved by electrophoresis using native polyacrylamide gels under non-denaturing and non-reducing conditions. Left lane, [3H]PGF2α-UG complex is readily detectable; lanes marked 10, 100, and 1000, non-radioactive PGF2α competes with [3H]PGF2α in a dose-dependent manner; lane marked MG, myoglobin, a nonspecific protein control, does not bind [3H-PGF2α; lane marked [3H-UG Std, [3H]UG standard. B, molecular modeling. Two molecules of PGF2α docked into the hydrophobic cavity of hUG, which is represented by a cyan ribbon. Tyrosine-21 (Y21) and tyrosine 21′ (Y21′) from the two UG-monomers are shown by a stick model. The picture was produced using the program CHIMERA (University of California, San Francisco).
ways are cell type-specific. It has been previously reported that the 5'-promoter region of the COX-2 gene contains binding site for NF-κB and that its activation leads to COX-2 gene expression (41, 42, 45, 46). The most important finding in this study is the inhibition of PGF$_{2\alpha}$-mediated stimulation of COX-2 expression by UG, a steroid-inducible, anti-inflammatory protein secreted by the mucus epithelia of all mammalian organs that communicate with the external environment (4). Interestingly, inhibition of NF-κB activation by UG bears a striking similarity to that elicited by PAG$_1$, a cyclopentenone PG with potent anti-inflammatory activity, that is also known to inhibit NF-κB activation (47). Taken together, our results suggest that UG counteracts the production of lipid mediators of inflammation. These phospholipid mediators have been implicated in the initiation and progression of human labor and delivery, especially in connection with premature labor induced by intrauterine infection (21, 48). In human gestational tissues both soluble PLA$_2$ and cPLA$_2$ have been suggested to play pivotal roles in the parturition process (52). Our results show that PGF$_{2\alpha}$-coordinately stimulates cPLA$_2$ and COX-2 expression in NIH 3T3 cells, suggesting the importance of this eicosanoid in the progression of lipid mediators of inflammation in organs such as the uterus in which both fibroblasts and smooth muscle cells are among the major constituents.

PGF$_{2\alpha}$ has been implicated in diverse physiological processes such as ovulation (53), luteolysis (32, 53), contraction of smooth muscles (16, 54–58), and initiation of parturition (34, 55). Recently, mice lacking the PGF$_{2\alpha}$ receptor (FP-null) were generated by targeted disruption of the FP gene in embryonic stem cells (34). Although these mice develop normally, they are unable to deliver fetuses at term (34). However, the FP-null mice show no abnormality in the estrus cycle, ovulation, fertilization, or implantation, indicating that PGF$_{2\alpha}$-FP interaction plays a critical role or roles specifically in the initiation of labor and parturition. Interestingly, the FP-null mice do not express COX-2 in the uterus at term (34), lending support to our observation that PGF$_{2\alpha}$-stimulated COX-2 gene expression is mediated via FP in addition to demonstrating the importance of PGF$_{2\alpha}$-stimulated COX-2 gene expression in parturition. Studies on parturition in COX-2-deficient mice are not possible because of their infertility (31, 51). The COX-2-deficient mice have an altered inflammatory response in addition to manifesting renal abnormalities (31) and multiple female reproductive failures (51), including ovulation, fertilization, implantation, and decidualization.

The FP-null mice, which are defective in parturition, have persistently high levels of progesterone and absence of COX-2 induction in the uterus (18). The ovaries of these animals do not undergo luteolysis, necessary for parturition, because of high levels of plasma progesterone. Withdrawal of local progesterone by ovarectomy in FP-null mice led to the induction of COX-2 expression and successful delivery (18). It has been reported that progesterone induces high levels of UF in the uterus (4). We propose that by stimulating UG production in the uterus progesterone may mediate the suppression of PGF$_{2\alpha}$-induced stimulation of COX-2 expression, thereby preventing inadvertent initiation of labor and parturition by PGF$_{2\alpha}$.

Our results show that, in NIH 3T3 cells, PGF$_{2\alpha}$ receptor-mediated stimulation of COX-2 mRNA expression is inhibited by the PKC-specific inhibitor but not by p38 MAPK or p44/42 MAPK inhibitors, suggesting an important role of PKC in the PGF$_{2\alpha}$ receptor-mediated signaling pathway for the regulation of inducible COX-2 expression in this cell type. Consistent with these results has been reported PKC-deficient cells are refractory to PGF$_{2\alpha}$-stimulated protein phosphorylation and DNA synthesis (58). Thus, PKC-mediated phosphorylation may play an important role or roles in the stimulation of COX-2 gene expression in NIH 3T3 cells. Interestingly, in hUSM cells inhibitors of PKC, p38 MAPK, and p44/42 MAPK lead to inhibition of COX-2 expression, suggesting that in this cell type all three kinase pathways are important for mediating FP-mediated COX-2 expression. Taken together, it appears that FP signaling may occur via multi-kinase pathways in a cell type-specific manner stimulating COX-2 gene expression.

The most important finding in our present study is the suppression of PGF$_{2\alpha}$ receptor-mediated stimulation of COX-2 gene expression by UG. Our results show that inhibition of FP-mediated stimulation of COX-2 expression is due to the binding and most likely the sequestering of PGF$_{2\alpha}$ by UG. Furthermore, these results are supported by those of molecular modeling studies indicating that two molecules of PGF$_{2\alpha}$ can be docked to the central hydrophobic cavity of hUG dimer. Recently, it has been reported that the cyclopentenone PG, PG$_{A}$, exerts its anti-inflammatory effects by inhibiting NF-κB activation (47). In this respect, the mechanism by which UG inhibits COX-2 gene expression bears at least some similarity to that of PG$_{A}$ in that both agents function via suppression of NF-κB activation.

In sum, we have shown that PGF$_{2\alpha}$ via FP coordinately stimulates the expression of cPLA$_2$ and COX-2, two of the most critical enzymes in the generation of pro-inflammatory lipid mediators in NIH 3T3 and hUSM cells. We also demonstrate that FP signaling stimulates the up-regulation of COX-2 expression via NF-κB activation mediated by multi-kinase pathways in a cell type-specific manner. AA, released from membrane phospholipids by cPLA$_2$, in response to agonist-bound FP activation, is converted by COX-2 to inflammatory lipid mediators. We propose that UG plays a critical role as a component of an innate mechanism to suppress inflammation in organs like the uterus that directly communicate with the external environment and are exposed to numerous infectious agents that can stimulate PGF$_{2\alpha}$ production, causing premature labor and parturition.

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