Comparison of Prostaglandin F$_{2\alpha}$, Bimatoprost (Prostagamide), and Butaprost (EP$_2$ Agonist) on Cyr61 and Connective Tissue Growth Factor Gene Expression*

Yanbin Liang, Chen Li, Victor M. Guzman, Albert J. Evinger III, Charles E. Protzman, Achim H.-P. Krauss, and David F. Woodward†

From Allergan, Inc., Irvine, California 92612

Connective tissue growth factor (CTGF) and Cyr61 (cysteine-rich angiogenic protein 61) are members of the CCN gene family that encode multifunctional, extracellular matrix-associated signaling proteins. Because the mechanism of action of certain anti-glaucoma drugs involves extracellular matrix remodeling of ocular ciliary muscle, with a resultant increase in drainage of aqueous humor from the eye, we compared the effects of three pharmacologically distinct ocular hypotensive agents on Cyr61 and CTGF gene expression. Thus, prostaglandin F$_{2\alpha}$ (PGE$_{2\alpha}$) (FP receptor agonist), Butaprost (EP$_{2}$ receptor agonist), and Bimatoprost (a prostamide) were compared. Using Affymetrix gene chip technology, we first identified that PGE$_{2\alpha}$ dramatically up-regulated Cyr61 and CTGF mRNA expression in HEK 293/EBNA cells (hFP-HEK 293/EBNA). Northern blot further confirmed the Cyr61 and CTGF up-regulation is in a dose- and time-dependent manner. PGE$_{2\alpha}$-induced up-regulation of Cyr61 appeared to exclusively involve the Rho pathway, and up-regulation of CTGF was via multiple intracellular pathways. Because prostamide receptors are, to date, defined only at the pharmacological level, Bimatoprost effects on Cyr61 and CTGF were studied in the isolated feline iris sphincter preparation, a tissue highly responsive to prostamides. Both PGE$_{2\alpha}$ and Bimatoprost up-regulated Cyr61 mRNA expression in the cat iris tissue. Only PGE$_{2\alpha}$ up-regulated CTGF mRNA expression in the cat iris. Therefore, PGE$_{2\alpha}$ and Bimatoprost appear to interact with different receptor populations in the cat iris, according to their markedly different effects on CTGF. Activation of prostaglandin EP$_2$ receptors (G$_{q}$-coupled) also up-regulated Cyr61 but not CTGF mRNA expression in the isolated cat iris. Similar data were observed in human primary ciliary smooth muscle cells. Thus, despite quite different signal transduction pathways, FP receptor stimulation up-regulates CTGF and Cyr61. The prostamide analog Bimatoprost and an EP$_2$-selective agonist affect only Cyr61.

Cyr61 (cysteine-rich angiogenic protein 61) was the first CCN family member to be identified in mouse fibroblasts upon growth factor stimulation (1). It contains an N-terminal secretory signal, four modular structural domains, and 38 cysteine residues that are largely conserved among all members of the CCN family. Cyr61 functions as a ligand for integrin receptors, through which it induces angiogenesis in vivo and supports cell adhesion, promotes cell migration, and enhances growth factor-stimulated mitogenesis in fibroblasts and endothelial cells (2).

Connective tissue growth factor (CTGF) is also a cysteine-rich, CCN family protein. CTGF is a fibrogenic cytokine, a growth factor that is required for the fibrotic response mechanism in tissues (3). Unlike Cyr61, it functions as an autocrine growth factor, which acts on the same cells that produce it, causing the cells to proliferate, differentiate, and produce more collagen (4). Thus, both Cyr61 and CTGF are multifunctional, extracellular matrix-associated signaling proteins that directly regulate cell adhesion, migration, proliferation, survival, and differentiation.

Cyr61 and CTGF may also play a role as secreted proteins involved in tissue remodeling. This may be of some importance for understanding the underlying mechanisms involved in ocular hypotension produced by certain drugs used as glaucoma therapy. The ocular hypotensive effects of prostaglandins, prostamides, and $\alpha_2$-adrenoreceptor agonists (exemplified by brimonidine) involve an increase in uveoscleral outflow of aqueous humor (5–8). Increases in uveoscleral outflow result from a remodeling of the ciliary body such that aqueous humor outflow is increased through widened interstitial spaces between ciliary muscle bundles (9–12).

Prostaglandins are the products of cyclooxygenase-catalyzed metabolism of arachidonic acid (13). Prostaglandin receptors have been classified into eight subtypes, FP, DP, IP, TP, EP$_1$, EP$_2$, EP$_{3}$, and EP$_4$, and receptors trigger G$_{q}$-protein-coupled mechanisms involving Ca$^{2+}$ signaling, inositol 1,4,5-trisphosphate turnover, and activation of protein kinase C (14), whereas EP$_2$, EP$_{3}$, EP$_{4}$, DP, and IP receptors trigger G$_{q}$-protein coupled to adenylyl cyclase and activation of protein kinase A. FP or EP$_2$ receptors activate different intracellular mechanisms and result in the reduction of intraocular pressure in experimental animal models (15, 16).

In addition to prostaglandin FP and EP$_2$ receptor selective ligands, prostamides, a novel class of compounds where the COOH typical of prostaglandins is replaced by an amide group, were identified as highly efficacious ocular hypotensives in animal models and human subjects (17, 18). Prostaglandides are formed from anandamide via metabolic transformation catalyzed by cyclooxygenase-2 (19). To date, the physiological actions of prostamides have not been fully investigated, and little is known about the mechanism of action of prostaglandins in vivo. Therefore, a detailed understanding of the effects of prostaglandins and their metabolic derivatives on ocular tissues is required.

*The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† To whom correspondence should be addressed: Dept. of Biological Science, Allergan, Inc., 2525 Dupont Dr., Irvine, CA 92612. Tel.: 714-246-5490; Fax: 714-246-5578; E-mail: Woodward_David@Allergan.com.
Comparison of PGF$_{2\alpha}$, Bimatoprost, and Butaprost

**Fig. 1.** PGF$_{2\alpha}$- and Butaprost-induced up-regulation of Cyr61 and CTGF mRNA expression is time- and dose-dependent. hFP-HEK293/EBNA cells were treated with 10$^{-9}$ to 10$^{-6}$ M PGF$_{2\alpha}$ for 6 h and treated with 10$^{-7}$ M PGF$_{2\alpha}$ for 0.5, 1, 6, and 24 h. Arrows indicate CTGF mRNA levels (a and b, top panels) and Cyr61 mRNA levels (c and d, top panels). hEP$_2$-HEK293/EBNA cells were treated with 10$^{-9}$ to 10$^{-7}$ M Butaprost for 6 h (e, top panel) and treated with 10$^{-7}$ M Butaprost for 0.5, 1, 6, and 24 h (f, top panel). Arrows indicate Cyr61 mRNA levels. The data represent mean ± S.D. of three independent experiments (a–f, bottom panels). *, $p < 0.01$ versus control. The intensities of the 28 and 18 S rRNA bands were used to normalize the RNA loading differences.
is known about their mechanisms of actions. The activities of prostanides at prostaglandin receptors have been investigated, but, compared with their corresponding natural prostaglandins, have been shown to exert only very weak activity (17, 20, 21). Experimental evidences suggest that prostanides may interact with their own receptors (17, 21).
Using a gene chip technology, we first identified that PGF₂α dramatically up-regulated Cyr61 and CTGF mRNA expression. Because both Cyr61 and CTGF play important roles in ECM remodeling, we further compared the effects of prostaglandin FP receptor stimulation (PGF₂α), an EP₂-selective agonist (Butaprost), and a synthetic prostamide analog (Bimatoprost) on the regulation of Cyr61 and CTGF mRNA expression. In this study, we report differential regulation of Cyr61 and CTGF mRNA expression following PGF₂α, Butaprost, or Bimatoprost treatments in cultured human primary trabecular meshwork (TM) and ciliary smooth muscle (SM) cells, which are cells critical in aqueous humor drainage from the eye.

**MATERIALS AND METHODS**

**Cell Cultures**—HEK 293/EBNA cells stably expressing the human FP receptor or EP₂ receptor (hFP-HEK293/EBNA or hEP₂-HEK293/EBNA cells) were a gift from Dr. John W. Regan (University of Arizona) (22, 23). Both hFP-HEK 293/EBNA and hEP₂-HEK293/EBNA cell lines were routinely maintained in DMEM (Invitrogen) with 10% fetal bovine serum, 1% glutamine, 0.5% penicillin/streptomycin, 250 μg/ml G418, and 200 μg/ml hygromycin and were kept in humidified 5% CO₂, 95% air at 37°C.

Human ciliary SM cells were isolated from a 69-year-old male donor eye (National Disease Research Interchange, Philadelphia) and cultured in DMEM with 10% fetal bovine serum and 0.5% penicillin/streptomycin according to the method reported previously by Wolde-Mussie et al. (24).

Human TM cells were a gift from Dr. J. Polansky (University of California, San Francisco, CA). The human TM cells were derived from a 30-year-old male donor eye and cultured in DMEM with 10% fetal bovine serum and 0.5% penicillin/streptomycin in humidified 8% CO₂, 92% air at 37°C. Both human primary TM and SM cells were grown to confluence before addition of the appropriate compounds.

Stock solutions of PGF₂α, Butaprost, and Bimatoprost were prepared in Me₂SO. The treated cells were incubated with graded concentrations of PGF₂α, Butaprost, or Bimatoprost, and the control cells received equivalent vehicle treatment.

**Cat Iris Tissue Bath Study**—Cat eyes were enucleated immediately following intravenous euthanization with 1 ml of Eutha-6 (a mixture of barbiturates) and placed on ice. Eyes were then hemisected along the ora serata to expose the posterior chamber of the anterior segment. After removing the lens by clipping the zonules, the iris was peeled away from the sclera, cut equally into two pieces (for treatment and control), and placed in a 10-ml jacketed organ bath containing Krebs buffer containing NaCl 118 mM, KCl 4.7 mM, KH₂PO₄ 1.2 mM, CaCl₂ 1.9 mM, MgSO₄ 1.18 mM, NaHCO₃ 25 mM, glucose 11.7 mM, Indomethacin 1 μM. Krebs buffer was gassed with 95% O₂/5% CO₂ to obtain a pH of 7.4, and the temperature was maintained at 37°C. After an equilibration period of 15 min, the tissue specimens were incubated for 6 h in the presence or absence of test compounds. All animals were managed in accordance with the Association for Research in Vision and Ophthalmology (ARVO) resolution on the Use of Animals in Ophthalmic and Vision Research.

**Plasmids and Luciferase Reporter Assay**—A DNA fragment containing the CTGF promoter region (−2047 to +65) (25) was cloned from human genomic DNA (Clontech). The fragment was subcloned into a pGL₃ luciferase expression vector (Promega), creating pGL₃-CTGF-LUC plasmid. The Cyr61 promoter region (−885 upstream of transcription start site (ATG) to −31 from ATG) (26) was isolated from human genomic DNA and subcloned into pGL₃ luciferase expression vector (Promega) at KpnI/XhoI site to create a pGL₃-Cyr61-LUC plasmid.

Luciferase reporter plasmids were transfected into HEK 293/EBNA cells stably expressing human FP receptors using the FuGENE 6 transfection method (Roche Diagnostics), according to manufacturer’s instructions. In brief, the cells were washed twice and resuspended in 1 ml of DMEM. 0.2 μg of plasmid DNA in 1 ml of DMEM containing 0.6 μl of FuGENE 6 solution was mixed with the cell suspension, and the cells were cultured for 24 h at 37°C. PGF₂α or Bimatoprost at concentrations ranging from 10⁻¹¹ to 10⁻⁷ M were added to the culture, and 6 h later, the cells were harvested and lysed in 100 μl of lysis buffer (Promega). 20 μl of soluble extracts were assayed for the luciferase activity. The luciferase assay was performed with a Promega assay kit at room temperature using an Autolumat LB 953 (EG&G, Berthold, Germany).
Luciferase content was measured by calculating the light emitted during the initial 10 s of the reaction. Relative luciferase activity was expressed as -fold values of ratio compared with control. The luciferase assay results shown in figures are representative of experiments independently repeated at least three times.

RNA Preparation and Northern Blot Analysis

Total RNA was isolated from cells and cat iris tissue using an RNase kit (Qiagen) according to its manufacturer’s instruction. RNA concentrations were determined by a UV spectrophotometer (Beckman DU640) at A260 nm, and stored at −80°C.

Total RNA (10 μg) was denatured at 65°C in RNA loading buffer (Ambion, Inc.) for 15 min and separated on 1.2% agarose gels containing 0.66 M formaldehyde. RNA loading was assessed by ethidium bromide staining of 28S and 18S ribosomal RNA bands. The relative intensities of 28S and 18S rRNA bands were used as internal controls to normalize the hybridizations. Human 0.4-kb CTGF (bases 11001-1326; GenBankTM accession number M92934) or 1.4-kb Cyr61 (bases 11001-1459; GenBankTM accession number AF003594) gene-specific DNA fragment was radiolabeled using [α-32P]dCTP and Klenow (Ambion, Inc.). The blots were hybridized with the gene-specific probes in 50% formamide, 4× SSC, 1× Denhardt’s solution, 50 mM sodium phosphate, pH 7.0, 1% SDS, 50 μg/ml yeast tRNA, and 0.5 mg/ml sodium pyrophosphate at 65°C for 16 h. The blots were washed with two 5× SSC washes. The blots were exposed to film (Hyperfilm MP, Amersham) with an intensifier screen for exposure times ranging from 10 min to 18 h.

Comparison of PGF2α, Bimatoprost, and Butaprost

Transcriptional regulation is involved in PGF2α and Butaprost-induced up-regulation of Cyr61 and CTGF mRNA expression. hFP-HEK293/EBNA cells were pretreated with 10−6 M cyclosporin A (CsA), 10 μg/ml actinD, and 10 μg/ml cycloheximide (CHX) for 30 min and continued 10−7 M PGF2α (a and b, top panels) and 10−7 M Butaprost (c, top panel) treatment for an additional 6 h. The data represent mean ± S.D. of three independent experiments (a–c, bottom panels). *, p < 0.01 versus control; **, p < 0.01 versus PGF2α alone; ***, p < 0.01 versus Control and Butaprost alone. The intensities of 28S and 18S rRNA bands were used to normalize the RNA loading differences.
Fig. 5. Regulation of Cyr61 and CTGF mRNA expression in cat iris following PGF$_{2\alpha}$, Butaprost, and Bimatoprost treatment. Isolated cat iris from each eye was cut equally into two pieces. One (treated) was incubated with 10$^{-7}$ M PGF$_{2\alpha}$, Bimatoprost, or Butaprost in a tissue bath for 6 h, and another piece (control) received equivalent vehicle treatment. Arrows indicate Cyr61 (a and c) and CTGF (b) mRNA levels. The data represent mean ± S.D. of three independent experiments (d). *$, p < 0.01$ versus control. The intensities of 28 and 18 S rRNA bands were used to normalize the RNA loading differences.

RESULTS

Kinetics of the Regulation of the Cyr61 and CTGF mRNA Expression—Using Affymetrix gene chip technology, a 10 to 20-fold up-regulation of Cyr61 and CTGF mRNA expression was identified in the PGF$_{2\alpha}$-treated hFP-HEK 293/EBNA cells. To further confirm the Affymetrix data and compare with Butaprost treatment, a kinetic study was performed to determine the time course and dose response of PGF$_{2\alpha}$ and Butaprost-induced up-regulation of Cyr61 and CTGF mRNA expression in the hFP- and hEP2-HEK 293/EBNA cells. For time course studies, the cells were treated with PGF$_{2\alpha}$ or Butaprost at time ranging from 0.5 to 24 h. Several dose response studies were determined by treating cells for 6 h with PGF$_{2\alpha}$ or Butaprost at concentrations ranging from 10$^{-10}$ to 10$^{-6}$ M. PGF$_{2\alpha}$, dramatically up-regulated both CTGF (Fig. 1, a and b) and Cyr61 (Fig. 1, c and d) mRNA expression in a time- and dose-dependent manner. Cyr61 and CTGF mRNA induction reached a peak at 6 h after PGF$_{2\alpha}$ treatment and stayed at peak for up to 24 h (24 h is maximum observation) (Fig. 1, b and d). Cyr61 and CTGF mRNAs were induced to the maximum level at 10$^{-7}$ M PGF$_{2\alpha}$. hEP$_2$-HEK293/EBNA cells showed higher background level of Cyr61 mRNA expression. Compared with PGF$_{2\alpha}$ treatment, Butaprost is a much less potent inducer of Cyr61 mRNA expression (Fig. 1, e and f) and did not up-regulate CTGF expression (data not shown). Cyr61 mRNA induction reached to a peak at 6 h after Butaprost treatment and was back to basal level at 24 h. The dose response of Butaprost-induced Cyr61 mRNA expression appeared to be similar to that of PGF$_{2\alpha}$.

Multiple Signal Transduction Pathways Are Involved in the PGF$_{2\alpha}$- and Butaprost-induced Up-regulation of Cyr61 and CTGF mRNA Expression—To clarify the signal transduction pathways associated with PGF$_{2\alpha}$- and Butaprost-induced Cyr61 and CTGF mRNA expression, pathway-specific inhibitors were utilized to distinguish the intracellular mechanisms. Both hFP- and hEP$_2$-HEK 293/EBNA cells were pretreated with each of these inhibitors (Toxin B 1 ng/ml, GF 109203X 2.5 μM, PD 98059 2.5 μM, BAPTA 2.5 μM) for 30 min, and the incubation was continued with 10$^{-7}$ M PGF$_{2\alpha}$ or Butaprost for an additional 6 h. A Rho inhibitor (Toxin B) completely blocked PGF$_{2\alpha}$-induced Cyr61 mRNA up-regulation (Fig. 2a), whereas a protein kinase C inhibitor (GF 109203X), a MAP kinase inhibitor (PD 98059), and a Rho inhibitor (Toxin B) partially inhibited PGF$_{2\alpha}$-induced CTGF mRNA up-regulation (Fig. 2b). These results suggested that PGF$_{2\alpha}$-induced Cyr61 mRNA up-regulation is via the Rho pathway, but PGF$_{2\alpha}$-induced CTGF mRNA expression is through multiple pathways that involved the activation of protein kinase C, MAP kinase, and activation of small G protein, Rho. Butaprost-induced Cyr61 mRNA up-regulation appears different from that of PGF$_{2\alpha}$ (Fig. 3). Both MAP kinase and Rho inhibitors attenuated Butaprost-induced up-regulation of Cyr61 mRNA expression, suggesting not only the Rho pathway but also MAP kinase involvement.

Novel Transcript Synthesis Is Required for the Up-regulation of Cyr61 and CTGF mRNA Expression—Up-regulation of Cyr61 and CTGF mRNA in PGF$_{2\alpha}$- and Butaprost-treated hFP- and hEP$_2$-HEK 293/EBNA cells may be because of an increase in the rate of synthesis, a decrease in the rate of degradation, or a combination of both. To test these possibilities, a transcription inhibitor, actinomycin D (10 μg/ml; actinD), was used to pretreat the cells for 30 min with continued incubation with...
Fig. 6. Differential regulation of CTGF and Cyr61 following PGF$_{2\alpha}$, Bimatoprost, and Butaprost treatments. Human trabecular meshwork cells (a and c) or ciliary smooth muscle cells (b and d) were treated with $10^{-7}$ M PGF$_{2\alpha}$, Bimatoprost, and Butaprost for 6 h. Arrows indicate Cyr61 (a and b, top panels) mRNA levels and CTGF (c and d, top panels). The data represent mean ± S.D. of three independent experiments (a–d, bottom panels). *, $p < 0.01$ versus control. The intensity of 28 S rRNA bands was used to normalize the RNA loading differences.
imide did not block PGF$_2\alpha$ incubation with 10 nM, was used to pretreat the cells for 30 min with continued

cytokine synthesis, a protein synthesis inhibitor, cycloheximide (10 μM), completely prevented PGF$_2\alpha$-induced up-regulation of Cyr61 and CTGF mRNA expression (Fig. 4, a and b). ActinD not only prevented Butaprost-induced up-regulation of Cyr61 mRNA expression but also significantly decreased Cyr61 mRNA level below the basal line (Fig. 4c). Thus, it is most likely that the rates of Cyr61 and CTGF mRNA transcription are increased by PGF$_2\alpha$ and Butaprost.

To determine whether PGF$_2\alpha$- and Butaprost-induced up-regulation of Cyr61 and CTGF mRNA requires de novo protein synthesis, a protein synthesis inhibitor, cycloheximide (10 μg/ml), was used to pretreat the cells for 30 min with continued incubation with 10 nM PGF$_2\alpha$, or Butaprost for 6 h. Cycloheximide did not block PGF$_2\alpha$- or Butaprost-induced up-regulation of Cyr61 and CTGF mRNA expression (Fig. 4), suggesting that PGF$_2\alpha$- and Butaprost-induced up-regulation of Cyr61 and CTGF mRNA expression did not require de novo protein synthesis.

Differential Up-regulation of Cyr61 and CTGF mRNA Expression Following PGF$_2\alpha$, Butaprost, and Bimatoprost Treatments in Cat Iris Tissue and Cultured Human Trabecular Meshwork and Ciliary Smooth Muscle Cells—Using Cyr61 and CTGF mRNA as markers, we further studied the mechanisms of PGF$_2\alpha$ and Bimatoprost at the gene expression level in cat iris. This is an isolated tissue preparation that is sensitive to PGF$_2\alpha$-coupled, activation of the prostaglandin EP$_2$ receptor (G$_{\text{q}}$-coupled) with Butaprost also up-regulated Cyr61 (Fig. 5c) but not CTGF mRNA expression in the cat iris (data not shown). Bimatoprost and Butaprost seemed to be very similar in the pattern of Cyr61 and CTGF mRNA induction, although they caused opposing action in the cat iris contraction assay. Therefore, Bimatoprost appears to interact with a unique receptor that is neither FP nor the EP$_2$ receptor in cat iris.

TM and ciliary SM cells are thought to be the major target cells in the aqueous humor outflow pathway for glaucoma treatments. To further compare the mechanisms of PGF$_2\alpha$, Bimatoprost, and Butaprost in human ocular tissues, each of the compounds at a concentration of 10 nM was used to treat the cultured human trabecular meshwork and ciliary smooth muscle cells for 6 h. Northern blot analysis of Cyr61 and CTGF mRNA expression revealed that Cyr61 and CTGF mRNA inductions by PGF$_2\alpha$, Bimatoprost, and Butaprost in human ciliary SM cells are very similar to the cat iris (major cell type of cat iris is smooth muscle cells). PGF$_2\alpha$, but not Butaprost and Bimatoprost, dramatically induced up-regulation of CTGF mRNA expression in human ciliary SM cells (Fig. 6d), whereas PGF$_2\alpha$, Butaprost, and Bimatoprost all up-regulated Cyr61 mRNA expression (Fig. 6b). PGF$_2\alpha$, and Bimatoprost are very similar in potency for inducing Cyr61 mRNA up-regulation, whereas Butaprost is much less potent (Fig. 6b, bottom panel). PGF$_2\alpha$, but not Bimatoprost and Butaprost, up-regulated Cyr61 and CTGF mRNA in human trabecular meshwork cells
(Fig. 6, a and c), suggesting that the properties of TM cells are much different from ciliary SM cells at the level of gene transcriptional regulation. All together, we conclude that the effects of PGF$_{2\alpha}$, Bimatoprost, and Butaprost on the Cyr61 and CTGF mRNA expression in human ciliary SM cells are very similar to those observed in the cat iris but are different in trabecular meshwork cells. PGF$_{2\alpha}$, Bimatoprost, and Butaprost differentially regulated Cyr61 and CTGF mRNA expression in smooth muscle cells, implying that Bimatoprost might exert its pharmacological actions through a unique mechanism in human ocular tissues, which is different from PGF$_{2\alpha}$ (FP) and/or Butaprost (EP$_2$).

**Functional Analysis of Cyr61 and CTGF Promoter in Response to PGF$_{2\alpha}$, Butaprost, and Bimatoprost Treatments in hFP-HEK 293/EBNA cells and hEP$_2$-HEK 293/EBNA Cells**

To further study the transcription mechanisms of FP- and EP$_2$-mediated Cyr61 and CTGF mRNA expression, Cyr61 and CTGF promoters were isolated from a human genomic DNA library and subcloned in a luciferase reporter plasmid, pGL3. Cyr61 promoter-luciferase reporter plasmids were transfected into hFP-HEK 293/EBNA cells and then treated with PGF$_{2\alpha}$ or Bimatoprost at concentrations ranging from $10^{-11}$ to $10^{-6}$ M. PGF$_{2\alpha}$, but not Bimatoprost, activated the Cyr61 promoter in a dose-dependent manner (Fig. 7a). This result suggested that Bimatoprost-induced up-regulation of Cyr61 mRNA expression (see Fig. 5a and Fig. 6b) is not because of the activation of the prostaglandin FP receptor but a different receptor. In the CTGF promoter-direct reporter assay, PGF$_{2\alpha}$, but not Bimatoprost, induced CTGF promoter activity in a dose-dependent manner (Fig. 7c). In a comparison study, Cyr61 or CTGF promoter was transfected into hEP$_2$-HEK293/EBNA cells and then treated with Butaprost at concentrations ranging from $10^{-11}$ to $10^{-6}$ M. Butaprost activated Cyr61 promoter (Fig. 7b) but not CTGF promoter in hEP$_2$-HEK 293/EBNA cells (Fig. 7d). These data are matched to Northern blot analysis (see Fig. 5b and Fig. 6, c and d).

**DISCUSSION**

A diverse variety of prostaglandins and fatty acid amides have been reported to lower intraocular pressure (15–18), and some are used in glaucoma therapy. The mechanisms of action reported to date all appear to involve remodeling of ciliary body tissue with a resultant increase in uveoscleral outflow of aqueous humor from the eye (27). The involvement of metalloproteases has been reported for PGF$_{2\alpha}$ and its derivatives (9–12). Beyond this, little is known regarding the events that occur between receptor stimulation and tissue remodeling. To further elucidate these mechanisms at the gene expression/second messenger level, we compared the activity of three pharmacologically distinct ocular hypotensive agents, PGF$_{2\alpha}$ (FP agonist), Butaprost (EP$_2$ agonist), and Bimatoprost (Prostamide).

Activation of FP receptors initiated by ligand binding triggers G$_{q\alpha}$ protein-coupled mechanisms involved intracellular Ca$^{2+}$ signaling, IP$_7$ turnover, and activation of protein kinase C (28). Butaprost is a synthetic prostaglandin analog that interacts with EP$_2$ receptors; it triggers G$_{s\alpha}$ protein-coupled mechanisms involved in activation of adenylate cyclase and initiation of the cAMP pathway with resultant activation of protein kinase A (29). Despite different intracellular mechanisms, it has been shown that activation of prostaglandin FP or EP$_2$ receptors resulted in lowering intraocular pressure (15, 16). The precise mechanisms by which PGF$_{2\alpha}$ and Butaprost reduce intraocular pressure (IOP) are not yet clear. The effects of PGF$_{2\alpha}$ and Butaprost on intraocular pressure lowering appear to be mediated through increasing uveoscleral outflow of aqueous humor by alteration of ciliary muscle bundles and remodeling extracellular matrix. Changes of gene expression follow-
ing PGF$_{2\alpha}$ or Butaprost treatment may be attributed to the molecular basis of prostaglandin actions.

To identify changes in gene expression that are regulated in response to PGF$_{2\alpha}$ or Butaprost stimulation, both hFP-HEK293/ENBA and hEP$_2$-HEK 293/ENBA cells were used to compare gene expression profiles in unstimulated cells versus PGF$_{2\alpha}$- or Butaprost-stimulated cells. It is shown herein that, with respect to activation of CCN early response genes, recombinant FP and EP$_2$ receptors expressed in HEK 293/EBNA cells are coupled to the same signal transduction pathways as in smooth muscle cells (30), fibroblasts (31), and ocular tissues (32–34). In unstimulated hFP-HEK 293/EBNA cells, PGF$_{2\alpha}$ rapidly induced both Cyr61 and CTGF expression within 30 min of stimulation. The expression levels of both genes reached a maximum 6 h after stimulation and stayed elevated for at least 24 h. This time course is similar to the kinetics of Factor VIIa and thrombin induction of human Cyr61 and CTG expression in human fibroblasts (35), serum induction of murine Cyr61 expression in fibroblasts (36), TGF-β induction of CTGF expression in human fibroblasts (37), and muscarinic receptor induction of rat Cyr61 in rat primary neurons and brain (38). Only Rho inhibition completely blocked PGF$_{2\alpha}$-induced Cyr61 expression, whereas PGF$_{2\alpha}$-induced up-regulation of CTGF expression could be attenuated by the pre-incubation of Rho, protein kinase C, and MAP kinase inhibitors. The signal mechanisms of FP-mediated up-regulation of Cyr61 and CTGF expression are different from mechanisms initiated by other stimulators. Protein kinase C and Ca$^{2+}$ are two principal signaling mechanisms that couple Cyr61 expression to muscarinic receptor activation (38). Factor VIIa and thrombin up-regulated Cyr61 through different signal mechanisms; Factor VIIa induced Cyr61 expression via phospholipase C and activation of MAP kinase pathways, whereas thrombin did not (35). Lysophosphatidic acid, serotonin, and TGF-β-induced CTGF up-regulation was dependent of the Rho pathway but independent of the MAP kinase protein kinase C pathways (39). In comparison with the G$_s$-coupled FP receptor, activation of the G$_s$-coupled EP$_2$ receptor by Butaprost also induced Cyr61 expression in a similar kinetic manner as PGF$_{2\alpha}$, but Butaprost did not induce CTGF expression. EP$_2$-mediated Cyr61 expression is via the Rho and MAP kinase pathways. Signal transduction pathways of FP- and EP$_2$-mediated Cyr61 and CTGF mRNA expression are summarized in a diagram in Fig. 8. Rho is a common signal mechanism by which FP and EP$_2$ activation is coupled to Cyr61 expression. Rho, protein kinase C, and MAP kinase pathways were coupled to FP-mediated CTGF expression, suggesting multiple mechanisms are involved in. Both Cyr61 and CTGF belong to the CCN gene family and regulate ECM remodeling through activation of matrix metalloproteinases (2). FP receptor activation up-regulates both Cyr61 and CTGF, whereas EP$_2$ receptor stimulation causes only Cyr61 up-regulation, which may reflect different mechanisms of FP- and EP$_2$ receptor-mediated ECM remodeling. This result further allowed us to use CTGF and Cyr61 gene expression as markers to further differentiate down stream signaling after treatment with Butaprotrost versus PGF$_{2\alpha}$.

Bimatoprost represents a novel class of anti-glaucoma compounds where the -COOH typical of prostaglandins is replaced by an amide group (17). It has been clinically proven to be a very efficacious IOP lowering drug (18). The mechanisms of its actions have not been fully investigated, and little is known about its intracellular signal transduction pathways and its receptor system. Although the activities of prostamides at prostaglandin receptor(s) have been investigated, they have been shown to exert no meaningful activity (17). In this and previous isolated cat iris contraction studies, we found that both PGF$_{2\alpha}$ and Bimatoprost triggered cat iris smooth muscle contraction at very similar EC$_{50}$ (17). Thus, the isolated cat iris sphincter was used as a prostamide-sensitive pharmacological preparation and a model for comparison of gene expression for all three drug classes. Gene expression studies revealed that PGF$_{2\alpha}$, Bimatoprost, and Butaprost up-regulated Cyr61 mRNA expression in the cat iris. PGF$_{2\alpha}$, but not Bimatoprost, up-regulated CTGF mRNA expression in the cat iris. Similar data were also observed in the human primary ciliary smooth muscle cells treated with PGF$_{2\alpha}$ and Bimatoprost. Therefore, PGF$_{2\alpha}$ and Bimatoprost appear to interact with different receptors in cat iris and human ocular tissues. Bimatoprost failed to stimulate up-regulation of Cyr61 in human TM cells, suggesting either that these cells are lacking Bimatoprost-sensitive receptors or an insufficiency of signal mechanisms coupled to Cyr61 expression.

Actinomycin D blocked Cyr61 and CTGF mRNA expression in response to PGF$_{2\alpha}$ and Bimatoprost treatments, which implied that transcription mechanisms were involved in PGF$_{2\alpha}$- and Butaprost-induced up-regulation of Cyr61 and CTGF expression. Human Cyr61 and CTGF promoters (hCyr61 and hCTGF) have been identified previously (25, 26) to be functional promoters. hCyr61 promoter located at −885 bp to −31 bp of Cyr61 gene have been shown to contain a TATA box, CEBP-β, CREB, and CA repeat elements (26). In hCyr61 promoter reporter gene assays, PGF$_{2\alpha}$, and Butaprost, activated Cyr61 promoter in a dose-dependent manner in hFP and EP$_2$-HEK 293/EBNA cells. Bimatoprost did not activate Cyr61 promoter, because it did not activate either FP in HEK 293/EBNA cells (Fig. 7a). Bimatoprost may interact with its own receptor in human ciliary smooth muscle cells and mediate Cyr61 expression. The hCTGF promoter contained TATA box, AP-1, CEBP, and Smads binding elements (25). In hCTGF reporter gene assays, PGF$_{2\alpha}$, activated CTGF promoter in a dose-dependent manner in hFP-HEK 293/EBNA cells, but Butaprost did not activate the CTGF promoter in hEP$_2$-HEK 293/EBNA Cells. These data suggested that activation of FP receptor directly initiated and regulated CTGF expression through promoter-directed transcriptional mechanisms, and EP$_2$ receptor-mediated pathways may not couple to CTGF expression. The reporter gene assay was consistent with the Northern blot analysis in this study, and the data further support the contention that Bimatoprost does not interact with FP receptor but rather a different receptor.
Comparison of PGF$_{2\alpha}$, Bimatoprost, and Butaprost

27277
Comparison of Prostaglandin F₂α, Bimatoprost (Prostamide), and Butaprost (EP₂ Agonist) on Cyr61 and Connective Tissue Growth Factor Gene Expression
Yanbin Liang, Chen Li, Victor M. Guzman, Albert J. Evinger III, Charles E. Protzman, Achim H.-P. Krauss and David F. Woodward

J. Biol. Chem. 2003, 278:27267-27277.
doi: 10.1074/jbc.M301009200 originally published online April 30, 2003

Access the most updated version of this article at doi: 10.1074/jbc.M301009200

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 38 references, 16 of which can be accessed free at http://www.jbc.org/content/278/29/27267.full.html#ref-list-1