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Upregulation of orphan nuclear receptor Nur77 following $PGF_{2\alpha}$, Bimatoprost, and Butaprost treatments. Essential role of a protein kinase C pathway involved in EP_2 receptor activated Nur77 gene transcription

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1 Using gene chip technology, we first identified that $PGF_{2\alpha}$ (FP agonist) and Butaprost (EP_2 agonist) induced about a five-fold upregulation of Nur77 mRNA expression in hFP-HEK 293/EBNA and hEP_2-HEK293/EBNA cells. Northern Blot analysis revealed that $PGF_{2\alpha}$- and Butaprost-induced upregulation of Nur77 expression are dose- and time-dependent.

2 Both $PGF_{2\alpha}$ and Butaprost upregulated Nur77 gene expression through the protein kinase C (PKC) pathway. These data are the first showing a link between EP_2 receptor stimulation and protein kinase C activation. Calcineurin was found to be involved downstream of the PKC pathway in $PGF_{2\alpha}$-induced Nur77 expression, but not in Butaprost-induced Nur77 expression.

3 We also used Nur77 as a marker gene to compare the effects of $PGF_{2\alpha}$, Butaprost, and Bimatoprost (a prostamide) on Nur77 expression in human primary trabecular meshwork and ciliary smooth muscle (SM) cells, which are target cells for antiglaucoma drugs. The results showed that $PGF_{2\alpha}$ and Butaprost, but not Bimatoprost, induced upregulation of Nur77 expression in human TM cells. $PGF_{2\alpha}$, but not Bimatoprost, dramatically induced upregulation of Nur77 mRNA expression in human ciliary SM cells, whereas Butaprost slightly upregulated Nur77 mRNA expression in SM cells.

4 Nur77 promoter deletion analysis indicated that $PGF_{2\alpha}$, but not Bimatoprost, activated Nur77 promoter-luciferase reporter in hFP-HEK 293/EBNA cells. Butaprost was less efficacious in inducing Nur77 promoter-luciferase reporter activity in hEP_2-HEK293/EBNA cells relative to $PGF_{2\alpha}$ in the comparable assay. The data for Nur77 promoter functional analysis were matched to the Northern blot analysis.

5 It appears that $PGF_{2\alpha}$ and Butaprost activate Nur77 transcription mechanisms through the activation of FP and EP_2 receptor-coupled signaling pathways, whereas Bimatoprost stimulates neither FP nor EP_2 receptors.
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Keywords: Transcription regulation; Nur77; prostaglandin $F_{2\alpha}$; butaprost; bimatoprost; gene expression

Abbreviations: DMEM, Dulbecco's modified eagle's medium; EBNA, Epstein–Barr nuclear antigen; EP_2, prostaglandin EP_2 receptor; FP, prostaglandin FP receptor; HEK, human embryonic kidney; MAP, mitogen-activated protein; NFAT, nuclear factor of activation T cell; $PGF_{2\alpha}$, prostaglandin $F_{2\alpha}$; SM, smooth muscle; TM, trabecular meshwork

Introduction

Prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) is a product of cyclooxygenase-catalyzed metabolism of arachidonic acid (Smith *et al*., 1991). It has been identified to be an endogenous ligand of prostaglandin FP receptors. Activation of FP receptors initiated by ligand binding triggers Gαq protein-coupled mechanisms involved Ca^{2+} signaling, IP_3 turnover, and activation of protein kinase C (Toh *et al*., 1995). $PGF_{2\alpha}$ has diverse physiological actions that include causing smooth muscle (SM) contraction (Horton & Poyser, 1976), stimulating DNA synthesis and cell proliferation, and cardiac myocyte hypertrophy (Adams *et al*., 1996). Importantly, $PGF_{2\alpha}$ analogs have been used clinically to reduce ocular hypertension (Woodward *et al*., 1993a). Although the precise mechanisms involved remain unclear, the effects of $PGF_{2\alpha}$ analogs on intraocular pressure (IOP) principally involve an increase in uveoscleral outflow of aqueous humor. These events involve secretion of metalloproteinases by ciliary SM cells and remodeling of the extracellular matrix with a resultant widening of intermuscular spaces (Gaton *et al*., 2001; Weinreb & Lindsey, 2002; Richter *et al*., 2003).

In contrast to prostaglandin $F_{2\alpha}$, Prostamides (prostaglandin ethanolamides) were recently identified as a new class of compounds that were formed from anandamide *via* metabolic transformation sequentially catalyzed by cyclooxygenase-2 (Yu *et al*., 1997). Although their physiological actions have not been fully investigated, a synthetic prostamide analog (Bimatoprost) has been shown to be very potent in reducing IOP by increasing

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both uveosceral and trabecular outflow of aqueous humor (Woodward *et al*., 2001). The activities of prostamides as endogenous ligands at prostaglandin receptor(s) have been investigated, but have been shown to exert no meaningful activity (Berglund *et al*., 1999; Woodward *et al*., 2001; Ross *et al*., 2002; Matias *et al*., 2004). Studies on their metabolic rate clearly demonstrate that prostamides and their synthetic analog Bimatoprost exert their *in vitro* pharmacological effects (Matias *et al*., 2004) and the ocular hypotensive effects as the intact molecule (Woodward *et al*., 2003). Experimental evidence suggests that prostamides may act as endogenous ligands at their own receptors (Woodward *et al*., 2001; Ross *et al*., 2002).

Besides prostaglandin $F_{2\alpha}$ and Bimatoprost, Butaprost, an EP_2 selective ligand, was also found to be very potent in lowering IOP in experimental animal models (Woodward *et al*., 1995). Interaction of Butaprost with EP_2 receptors has been shown to initiate the cAMP/PKA pathway, which in turn modulates a variety of physiological processes, such as vasodilatation and uterus relaxation (Pierce *et al*., 1995). Despite the fact that FP and EP_2 receptors are coupled to different signal transduction mechanisms, both receptors may ultimately share common intracellular pathways that mediate a reduction of intraocular pressure (IOP). For this reason, the effects of $PGF_{2\alpha}$, the prostamide analog Bimatoprost, and the EP_2 agonist Butaprost were compared with respect to one $PGF_{2\alpha}$-sensitive pathway, namely upregulation of Nur77.

The immediate-early gene Nur77 (also called NGFI-B or TR3) encodes an orphan nuclear receptor, which is classified as a ligand-dependent transcriptional modulator protein (Mangelsdorf *et al*., 1995). It was originally cloned as a gene induced by Nerve Growth Factor (NGF) in the rat pheochromocytoma cell line PC12 (Milbrandt, 1988). A variety of stimuli involved in cell differentiation and proliferation were found to be capable of rapidly inducing Nur77 expression (Fahrner *et al*., 1990). The Nur77 binding element (NBRE) was identified by genetic selection in yeast (Wilson *et al*., 1991). Co-transfection of a reporter gene coupled to the NBRE demonstrated that Nur77 was a strong transcription activator in the cells examined (Davis *et al*., 1991; 1993; Paulsen *et al*., 1992). However, the exact function(s) of Nur77 in the regulation of physiological processes remains to be elucidated.

In this study, we have demonstrated that $PGF_{2\alpha}$ and Butaprost upregulate Nur77 mRNA expression and activate the Nur77 promoter in hFP-HEK 293/EBNA and hEP_2-HEK 293/EBNA cells. Using Nur77 as a marker gene, we further studied the differential mechanisms of $PGF_{2\alpha}$ (FP receptor), Butaprost (EP_2 agonist), and Bimatoprost (prostamide) in the regulation of Nur77 mRNA expression in human primary trabecular meshwork (TM) and ciliary SM cells. These are cellular targets for designing ocular hypotensive drugs. The information gained from this study will help us in the understanding of the molecular mechanisms of prostaglandin analogs (FP and/or EP_2 agonist) and the prostamide analogs in the treatment of glaucoma.

Experimental procedures

Cell cultures

HEK 293/EBNA cells stably expressing the human FP receptor or EP_2 receptor (hFP-HEK293/EBNA or hEP_2-HEK 293/EBNA cells) were a gift from Dr John W. Regan (University of Arizona) (Fujino *et al*., 2000; 2002). Both hFP-HEK 293/EBNA and hEP2-HEK293/EBNA cell lines were routinely maintained in DMEM (Life Technology, Inc., Rockville, MD, U.S.A.) with 10% fetal bovine serum, 1% glutamine, 0.5% penicillin/ streptomycin, 250 $\mu g\,ml^{-1}$ G418, and 200 $\mu g\,ml^{-1}$ hygromycin, and were kept in humidified 5% CO_2 and 95% air at 37°C.

Human ciliary SM cells were isolated from a 69-year-old male donor eye (NDRI, Philadelphia) and cultured in DMEM with 10% fetal bovine serum and 0.5% penicillin/streptomycin according to a method previously reported by Woldemussie *et al*. (1993).

Human TM cells were a gift from Dr J. Polansky (University of California, San Francisco, U.S.A.). 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% CO_2, 92% air at 37°C. Both human primary TM and SM cells were grown to confluence before addition of test compounds.

Stock solutions of $PGF_{2\alpha}$, Butaprost, and Bimatoprost were prepared in DMSO. The treated cells were incubated with graded concentrations of $PGF_{2\alpha}$, Butaprost or Bimatoprost, and the control cells received equivalent vehicle treatment.

Plasmids and luciferase reporter assay

A DNA fragment containing the Nur77 promoter (−960 bp to +67 bp; gene bank: U17590) (Uemura *et al*., 1995) was isolated from human genomic DNA, and subcloned into pGL_3-luciferase vector (Promega, Inc) and a pGL_3-N-960 plasmid was created. A series of deletion constructs of the Nur77 promoter (−960 to +67 bp) were created and are described as follows: pGL_3-N-496 plasmid (−496 to +67 bp); pGL_3-N-334 plasmid (−334 to +67 bp); pGL_3-N-181 plasmid (−181 to +67 bp); and pGL_3-N-114 plasmid (−114 to +67 bp). NFAT-Luciferase plasmids were purchased from Invitrogen, Inc.

Luciferase reporter plasmids were transfected into HEK 293/EBNA cells stably expressing human FP receptor (hFP-HEK293/EBNA) or human EP_2 receptor (hEP_2-HEK293/EBNA) using the Fugene 6 method (Roche Diagnostics Corp., Inc), according to manufacturer's instructions. In brief, the cells were plated in 24-well plates overnight, and then the 24-well plate cells were washed twice and resuspended in 1 ml of DMEM. In all, 0.2 μg of plasmid DNA in 100 μl of DMEM containing 0.6 μl Fugene 6 solution was mixed with the cell suspension and added into each well. The plates were cultured for 24 h at 37°C. $PGF_{2\alpha}$, Butaprost, or Bimatoprost at a concentration ranging from 10^{-11} to 10^{-6} M were added to the culture, and 6 h later the cells were harvested and lysed in 100 μl of 25 mM Tris-phosphate buffer (pH 7.5) containing 1% Triton X-100. A volume of 20 μl of soluble extracts was assayed for the luciferase activity. The luciferase assay was performed with a Promega assay kit (Promega, Inc.) at room temperature using an Autolumat LB 953 (EG&, Berthold). 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 to control. The luciferase assay results shown in the figures are representative of experiments independently repeated at least three times.

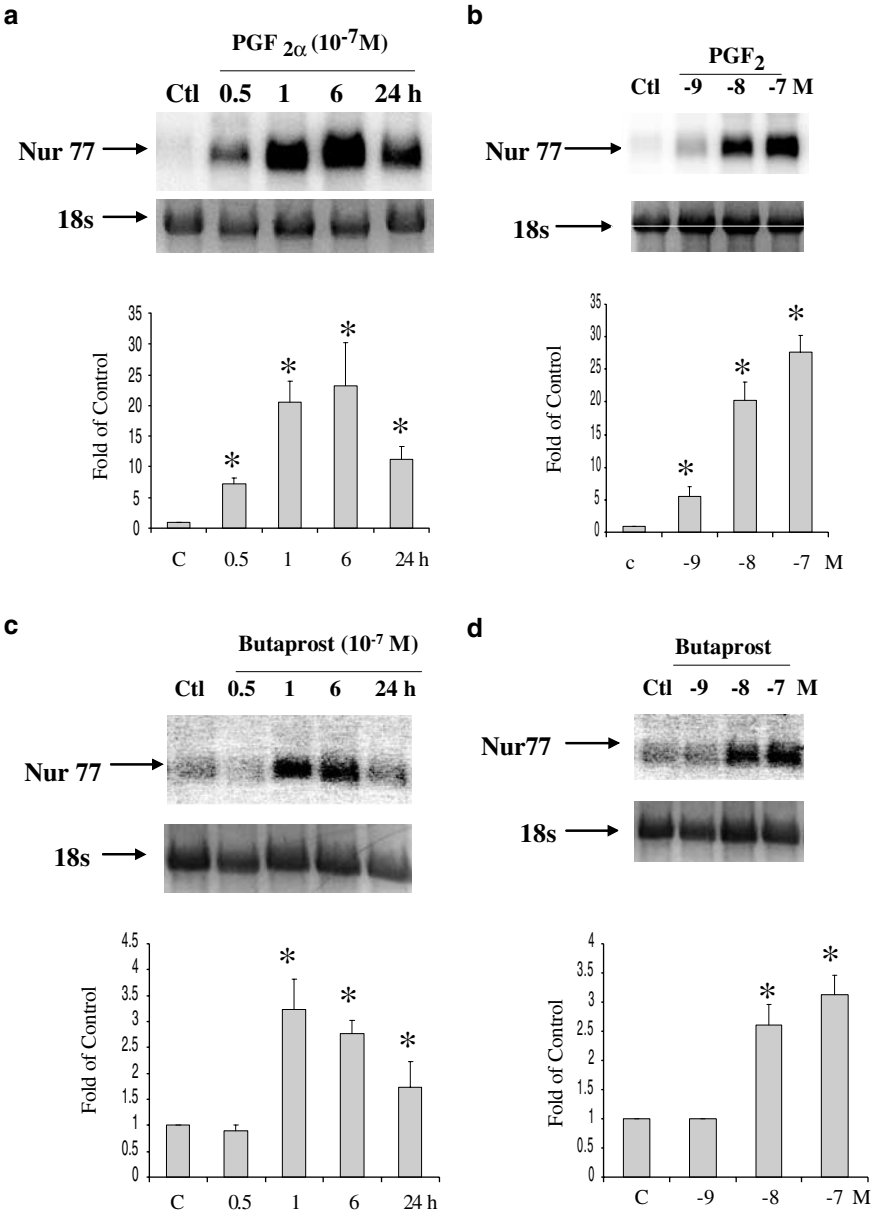

Figure 1 Upregulation of Nur77 mRNA expression following $PGF_{2\alpha}$ and Butaprost treatment in hFP- and hEP_2-HEK 293/EBNA cells. hFP-HEK 293/EBNA cells treated with 10^{-7} M $PGF_{2\alpha}$ for 0.5, 1, 6, 24 h (a), and treated with 10^{-9} to 10^{-7} M $PGF_{2\alpha}$ for 6 h (b). hEP_2-HEK293/EBNA cells treated with 10^{-7} M Butaprost for 0.5, 1, 6, 24 h (c), and treated with 10^{-9}–10^{-7} M Butaprost for 6 h (d). Arrows indicate mRNA levels of Nur77 and 18s rRNA (a–d, top panels). The intensity of 18s rRNA bands was used as an internal control to normalize the RNA loading differences. The data represented mean ± s.d. of three independent experiments (a–d, bottom panels). $^*P<0.01$ vs Control.

RNA isolation and Northern blot analysis

Total RNA was isolated from cells and human ocular tissues using RNeasy Kit (Qiagen, Inc) according to the manufacturer's instruction. RNA concentrations were determined by UV Spectrophotometry (Beckman DU640) at *A* 260 nM, and stored at −80°C.

10 μg of total RNA 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 the 28s and 18s ribosomal RNA bands were used as internal controls to normalize the hybridizations. Human 690 bp Nur77 (+1332 to +2022 bp; gene bank: L13740.1) specific DNA fragment was radiolabeled using α-^{32}P dATP and Klenow (Ambion, Inc.). The blots were hybridized with gene-specific probes in 50% formamide, 4 × SSC, 1 × Denhardt's solution, 50 mM sodium phosphate, pH 7.0, 1% SDS, 50 μg ml^{-1} yeast tRNA, and 0.5 mg ml^{-1} sodium pyrophosphate at 42°C overnight, and washed with 2 × SSC and 0.1% SDS twice at 42°C and 0.1 × SSC and 0.1% SDS twice at 42°C. The hybridized blots were exposed to phosphor screens, and the exposed screens were analyzed in a PhosphorImager (Molecular Dynamics, Inc).

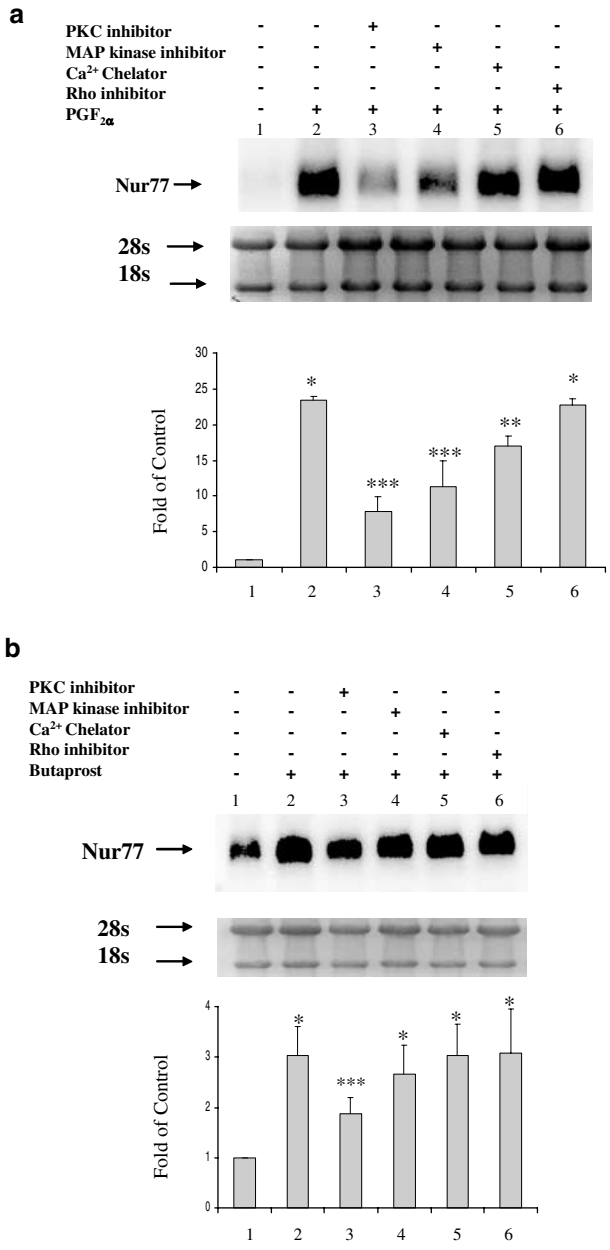

Figure 2 Activation of PKC is involved in $PGF_{2\alpha}$ and Butaprost-induced upregulation of Nur77 mRNA expression. hFP-HEK 293/EBNA cells or hEP_2-HEK 293/EBNA cells were pretreated with a Rho inhibitor (Clostridium difficile Toxin B, 100 ng ml^{-1}), a PKC inhibitor (GF109203 X, 2.5 μM), an MAPK inhibitor (PD 98059, 20 μM), or a Ca^{2+} chelator (BAPTA, 2.5 μM) for 30 min, followed by continued incubation with 10^{-7} M $PGF_{2\alpha}$ (a) or Butaprost (b) for 6 h. Arrows indicate Nur77 mRNA levels and 28s and 18s rRNA (a and b, top panels). The intensity of 28s and 18s rRNA bands was used as an internal control to normalize RNA loading differences. The data represent mean $\pm$ s.d. of three independent experiments (a and b, bottom panels). $^*P<0.01$ vs control; $^{**}P<0.05$ vs $PGF_{2\alpha}$ alone; $^{***}P<0.01$ vs $PGF_{2\alpha}$ or Butaprost alone.

Results

Kinetics of $PGF_{2\alpha}$- and Butaprost-induced upregulation of Nur77 mRNA expression

A kinetic study was performed to determine the time course and dose response of $PGF_{2\alpha}$- and Butaprost-induced upregulation of Nur77 mRNA expression in hFP- and hEP_2-HEK 293/EBNA cells. For time course studies, the cells were treated with 10^{-7} M $PGF_{2\alpha}$ or Butaprost at times ranging from 0.5 to 24 h. Dose response studies were determined by treating cells for 6 h with $PGF_{2\alpha}$ or Butaprost at concentrations ranging from 10^{-9} to 10^{-7} M. $PGF_{2\alpha}$ dramatically upregulated Nur77 mRNA expression in a time- and dose-dependent manner (Figure 1a, b). Nur77 mRNA induction reached a peak at 6 h after $PGF_{2\alpha}$ treatment, and thereafter declined at 24 h when the final observation was made. Nur77 mRNA was induced to the maximum level by 10^{-7} M $PGF_{2\alpha}$. The pattern of Butaprost-induced Nur77 mRNA expression appeared to be similar to that of $PGF_{2\alpha}$, but it was less active (Figure 1c, d). Nur77 mRNA induction reached a peak at 1 h after Butaprost treatment, and declined to near basal levels at 24 h. A kinetic study was also performed using Bimatoprost treatment in hFP- and hEP2-HEK293/EBNA cells. Bimatoprost did not induce Nur77 mRNA expression in both hFP- and hEP2-HEK293/EBNA cell lines (data not shown).

$PGF_{2\alpha}$- and Butaprost-induced upregulation of Nur77 mRNA expression occurs via *the activation of protein kinase C*

To clarify the signal transduction pathways involved in $PGF_{2\alpha}$- and Butaprost-induced Nur77 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, 100 ng ml^{-1}, GF109203 X, 2.5 μM, PD 98059, 20 μM, BAPTA, 2.5 μM) for 30 min and the incubation was then continued in the presence of 10^{-7} M $PGF_{2\alpha}$ or Butaprost for an additional 6 h. The PKC inhibitor (GF 109203) decreased both $PGF_{2\alpha}$- and Butaprost-induced Nur77 mRNA expression (Figure 2a, b). However, Butaprost-induced Nur77 expression appeared less dependent on the PKC pathway compared to $PGF_{2\alpha}$. The MAP kinase inhibitor (PD 98059) and the Ca^{2+} chelator (BAPTA) attenuated $PGF_{2\alpha}$-induced Nur77 mRNA expression. The Rho inhibitor (Toxin B) did not alter $PGF_{2\alpha}$- or Butaprost-induced Nur77 mRNA expression (Figure 2a, b). The protein kinase A (PKA) inhibitor (KT5720) was also used in this study and seemed to potentiate both $PGF_{2\alpha}$- and Butaprost-induced Nur77 mRNA expression (data not shown). These results suggested that both $PGF_{2\alpha}$- and Butaprost-induced Nur77 mRNA expression occurs *via* the PKC pathway.

To further differentiate downstream of PKC pathways after the treatment with $PGF_{2\alpha}$ and Butaprost, cyclosporin A (CsA) was used as inhibitor of calcineurin (Clipstone & Crabtree, 1992), a PKC downstream mediator. Both hFP- and hEP2-HEK 293/EBNA cells were pretreated with CsA at concentrations ranging from 10^{-7} to 10^{-5} M for 30 min and the incubation was then continued in the presence of 10^{-7} M $PGF_{2\alpha}$ or Butaprost for an additional 6 h. CsA attenuated $PGF_{2\alpha}$-induced upregulation of Nur77 mRNA expression in a dose-dependent manner, but did not attenuate Butaprost-induced Nur77 mRNA expression (Figure 3a, b). This result was consistent with the NFAT luciferase reporter (Invitrogen, Inc) assay. NFAT is a substrate of calcineurin: de-phosphorylation of NFAT by calcineurin initiates NFAT nuclear translocation, and activates transcription (Crabtree, 2001). $PGF_{2\alpha}$ activated NFAT-luciferase reporter in hFP-HEK 293/EBNA in a dose-dependent manner, whereas Butaprost did

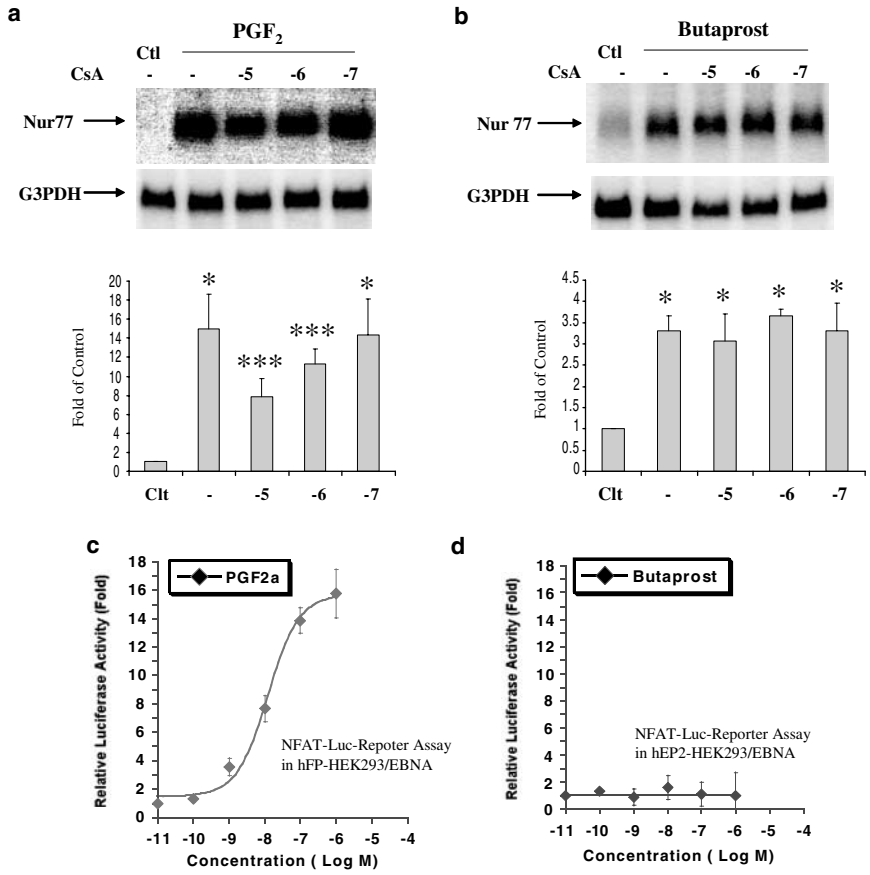

Figure 3 Calcinurin is involved in $PGF_{2\alpha}$-induced upregulation of Nur77 mRNA expression, but not in Butaprost-induced upregulation of Nur77 mRNA expression. hFP-HEK293/EBNA cells or hEP_2-HEK293/EBNA cells were pretreated with cyclosporine A (CsA) at concentrations ranging from 10^{-7} to 10^{-5} M for 30 min, followed by continued incubation with 10^{-7} M $PGF_{2\alpha}$ (a) or 10^{-7} M Butaprost (b) for an additional 6 h. Arrows indicate mRNA levels of Nur77 and 18s rRNA (a and b, top panels). The intensity of 18s rRNA bands was used as an internal control to normalize the RNA loading difference. The data represent mean ± s.d. of three independent experiments (a and b, bottom panels). $*P<0.01$ vs control; $**P<0.05$ vs control. NFAT-luciferase reporter plasmids were transfected into hFP-HEK293/EBNA cells (c) or hEP_2-HEK293/EBNA cells (d). The transfected cells were treated with $PGF_{2\alpha}$ or Butaprost at a concentration ranging from 10^{-11} to 10^{-6} M. Relative luciferase activity was expressed as fold changes of ratio compared with control. The luciferase assay results shown in figures are representative of experiments independently repeated at least three times.

not activate NFAT-luciferase reporter in hEP_2-HEK293/EBNA (Figure 3c, d). These data further supported calcineurin involvement in $PGF_{2\alpha}$-induced Nur77 mRNA expression. Calcineurin did not appear to be involved in Butaprost-induced Nur77 mRNA expression. These data suggest that $PGF_{2\alpha}$ and Butaprost may activate different isoforms of protein kinase C through which they differentially regulate Nur77 upregulation.

Novel transcript synthesis is required for $PGF_{2\alpha}$- and Butaprost-induced Nur77 mRNA expression

Upregulation of Nur77 mRNA in $PGF_{2\alpha}$- and Butaprost-treated hFP- and hEP_2-HEK 293/EBNA cells may be due to 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 ($10\,\mu g\,ml^{-1}$, actinD), was used to pretreat the cells for 30 min with continued incubation with 10^{-7} M $PGF_{2\alpha}$ or Butaprost for 6 h. ActinD, at a concentration of $10\,\mu g\,ml^{-1}$, completely prevented $PGF_{2\alpha}$- and Butaprost-induced upregulation of Nur77 mRNA expression (Figure 4a, b). Thus, it is most likely that Nur77 mRNA transcription rates are increased by $PGF_{2\alpha}$ and Butaprost.

To determine if $PGF_{2\alpha}$- and Butaprost-induced upregulation of Nur77 mRNA requires *de novo* protein synthesis, a protein synthesis inhibitor, cycloheximide ($10\,\mu g\,ml^{-1}$, CHX), was used to pretreat the cells for 30 min with continued incubation in the presence of 10^{-7} M $PGF_{2\alpha}$ or Butaprost for 6 h. CHX did not block $PGF_{2\alpha}$- or Butaprost-induced upregulation of mRNA expression, suggesting that $PGF_{2\alpha}$- and Butaprost-induced upregulation of Nur77 mRNA expression did not require *de novo* protein synthesis (Figure 4a, b).

Identification of the Nur77 gene promoter regions that confer the regulatory responses to $PGF_{2\alpha}$ and Butaprost treatments

To further study the transcription mechanisms of hFP- and hEP_2-mediated Nur77 mRNA expression, Nur77 promoters (−960 to +67 bp; Gene Bank: U17590) were isolated from a human genomic DNA library (Clontech) and subcloned into a

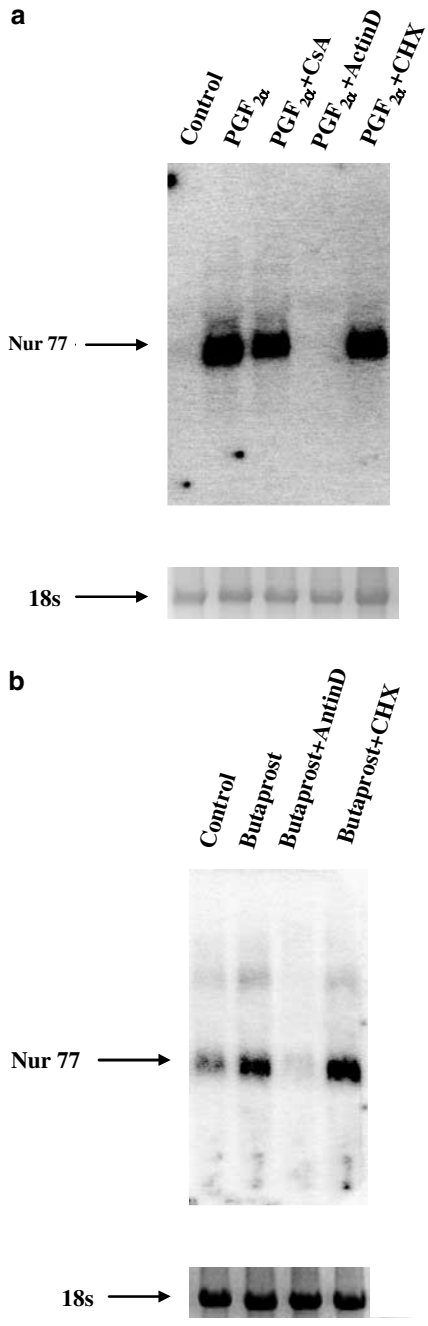

Figure 4 Transcriptional regulation is involved in $PGF_{2\alpha}$- and Butaprost-induced upregulation of Nur77 mRNA expression in hFP- and hEP_2-HEK293/EBNA cells. hFP-HEK293/EBNA cells or hEP_2-HEK293/EBNA cells were pretreated with 10 μg ml^{-1} actinomycin D (actinD) or 10 μg ml^{-1} cycloheximide (CHX) for 30 min, with continued 10^{-7} M $PGF_{2\alpha}$ (a) or 10^{-7} M Butaprost (b) for an additional 6 h. Arrows indicate mRNA levels of Nur77 and 18s rRNA. The intensity of 18s rRNA was used as an internal control to normalize the RNA-loading differences.

luciferase reporter plasmid, pGL_3, at the *Xho*I/*Hind*III site. Nur77 promoter luciferase reporter plasmids were transfected into both hFP-HEK 293/EBNA cells and hEP_2-HEK 293/EBNA cells and then treated with $PGF_{2\alpha}$ vs Bimatoprost or Butaprost vs Bimatoprost at concentrations ranging from 10^{-11} to 10^{-6} M. Both $PGF_{2\alpha}$ and Butaprost, but not Bimatoprost, activated the Nur77 promoter in a dose-dependent manner (Figure 5a, b). These results suggested that Bimatoprost did not activate either the prostaglandin FP receptor or EP_2 receptor. These data are matched to Northern blot analyses (Figure 6a, b).

A 1 kb 5′ flanking fragment of the human Nur77 gene was subcloned in front of a luciferase gene for studying transcriptional regulation of the human Nur77 gene. When pGL_3-N-960 plasmid was transfected into the hFP-HEK 293 or hEP2-HEK 293/EBNA cells, a remarkable increase of luciferase activity was observed compared to that of pGL_3 basic without promoter (Figure 5c), which indicated that the 1 kb 5′ flanking fragment of human Nur77 gene contains a functional promoter that directs Nur77 gene expression. Deletion analysis of the Nur77 promoter in hFP- and hEP2-HEK 293/EBNA cells (Figure 5c) showed that a deletion from −960 to −496 slightly decreased luciferase reporter activity. The region from −960 to −496 may contain negative regulatory elements for Nur77 expression. Further deletion from −496 to −334 dramatically decreased luciferase activity about 30-fold as compared to pGL_3-N-496, suggesting that the region from −496 to −334 may contain strong enhancers. Further deletion from −181 to −114 dramatically increased luciferase activity about 30-fold compared to pGL_3-N-181. The region from −181 to −114 may contain strong suppressors. The region from −114 to +1 contains a basic functional promoter with multiple SP-1 and AP-1 elements for transcription activity of the human Nur77 gene in HEK 293/EBNA cells (Uemura *et al*., 1995).

To further identify which regions in the Nur77 promoter are responsive to $PGF_{2\alpha}$ and Butaprost treatments, a series of Nur 77 promoter deletion constructs (Figure 5c) were transfected into hFP-HEK293/EBNA cells (Figure 5d) or hEP_2-HEK293/EBNA 293 cells (Figure 5e), and then treated with 10^{-7} M $PGF_{2\alpha}$ or Butaprost for 6 h. Relative luciferase activity was calculated as fold changes of ratio compared with untreated. These results suggested that a region from −960 to −496 contains regulatory elements that negatively regulate Nur77 expression in response to both $PGF_{2\alpha}$ and Butaprost treatments. The regions from −496 to −334 contain regulatory elements that positively and dramatically induced Nur77 expression in response to $PGF_{2\alpha}$ and Butaprost treatments. This region (−496 to −334) contains two putative IL-6-RE and a SP-1 elements (Uemura *et al*., 1995), which may contain novel *cis*-acting elements that are responsive to $PGF_{2\alpha}$ and Butaprost treatments. The basic Nur77 promoter from −114 to +1 does not contain essential *cis*-acting elements that regulated Nur77 expression in response to $PGF_{2\alpha}$ and Butaprost treatments. The overall patterns of the Nur77 regulatory regions that responded to $PGF_{2\alpha}$ are similar to those of Butaprost, suggesting that $PGF_{2\alpha}$- and Butaprost-induced Nur77 expression may share the same regulatory mechanisms at transcriptional levels. Bimatoprost did not activate any of the Nur77 deletion promoters in either hFP- or hEP_2-HEK 293/EBNA cells, which further supported the view that Bimatoprost does not interact with either hFP or hEP_2 receptors.

Regulation of Nur77 mRNA expression in human TM and ciliary SM cells following treatment with $PGF_{2\alpha}$, Butaprost, and Bimatoprost

TM and ciliary SM cells are thought to be the major target cells in the aqueous humor outflow pathway for glaucoma

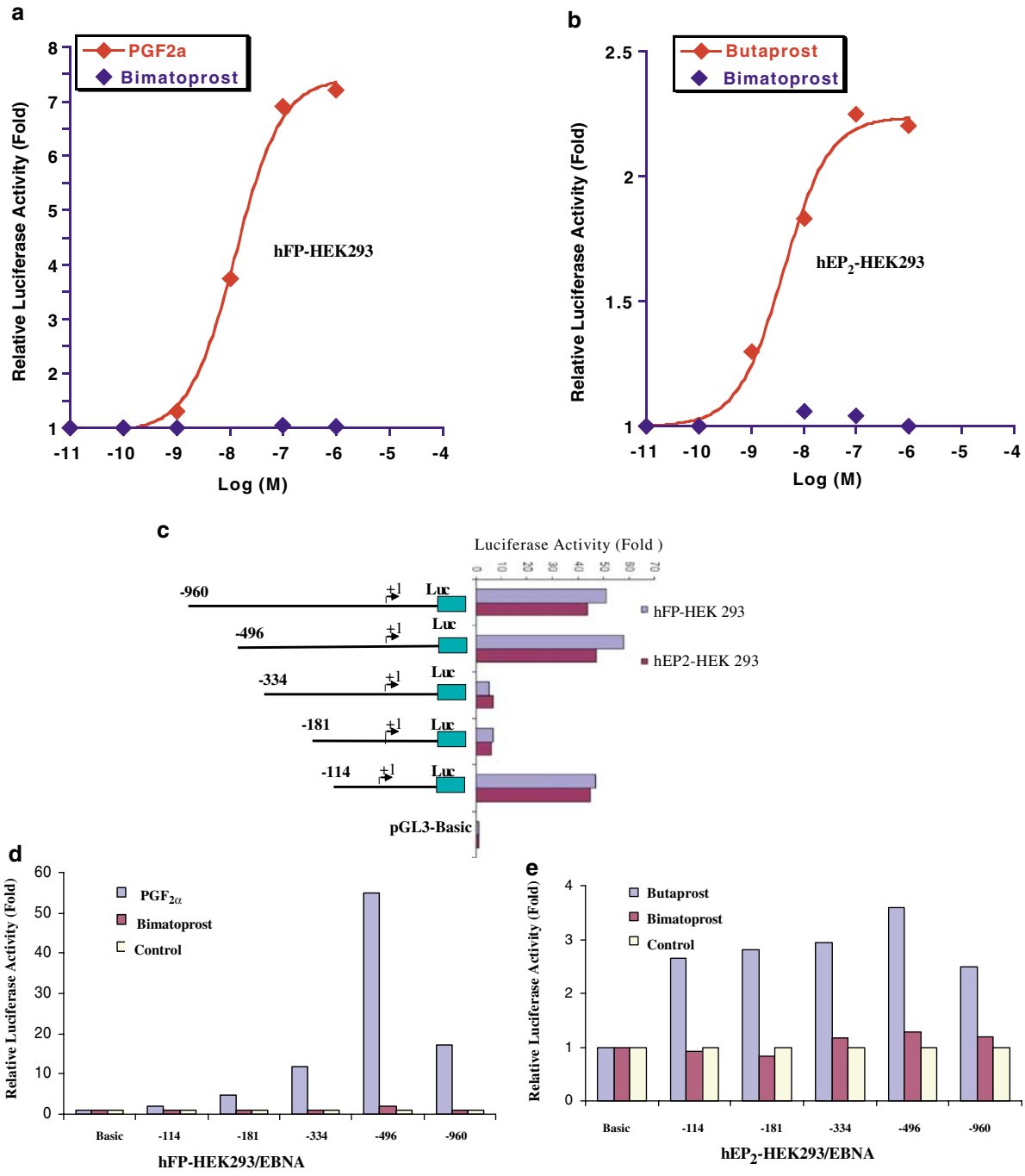

Figure 5 Identification of Nur77 promoter regions that are responsive to $PGF_{2\alpha}$ and Butaprost treatment. (a) pGL_3-N-960 plasmids were transfected in HEK 293/EBNA cells (a) or hEP_2-HEK 293/EBNA cells (b), and then the transfected cells were treated with $PGF_{2\alpha}$ or Butaprost at concentrations ranging from 10^{-11} to 10^{-7} M for 6 h. A variety of deletion constructs for Nur 77 promoter were transfected into hFP-HEK 293/EBNA cells or hEP_2-HEK 293/EBNA cells. The basal transcription activities of Nur77 promoter regions were determined (c). Relative luciferase activity was expressed as fold changes of ratio compared with control. The HEK 293/EBNA cells transfected with the deletion constructs were treated with 10^{-7} M $PGF_{2\alpha}$ vs Bimatoprost (d) or Butaprost vs Bimatoprost (e) for 6 h. Relative luciferase activity was expressed as fold changes of ratio compared with untreated. The luciferase assay results shown in the figures are representative of experiments independently repeated at least three times.

treatments. To further compare the mechanisms of $PGF_{2\alpha}$, Bimatoprost, and Butaprost in human ocular tissues, each of these compounds at a receptor effective (Abramovitz *et al*., 2000; Woodward *et al*., 2003) concentration of 10^{-7} M was used to treat cultured human TM and ciliary SM cells for 6 h. Northern blot analysis of Nur77 mRNA expression revealed that $PGF_{2\alpha}$ and Butaprost, but not Bimatoprost, induced upregulation of Nur77 mRNA expression in human TM cells (Figure 6a). $PGF_{2\alpha}$, but not Bimatoprost, dramatically induced upregulation of Nur77mRNA expression in human ciliary SM cells, whereas Butaprost slightly upregulated Nur77 mRNA expression (Figure 6b). The effect of Butaprost is significantly

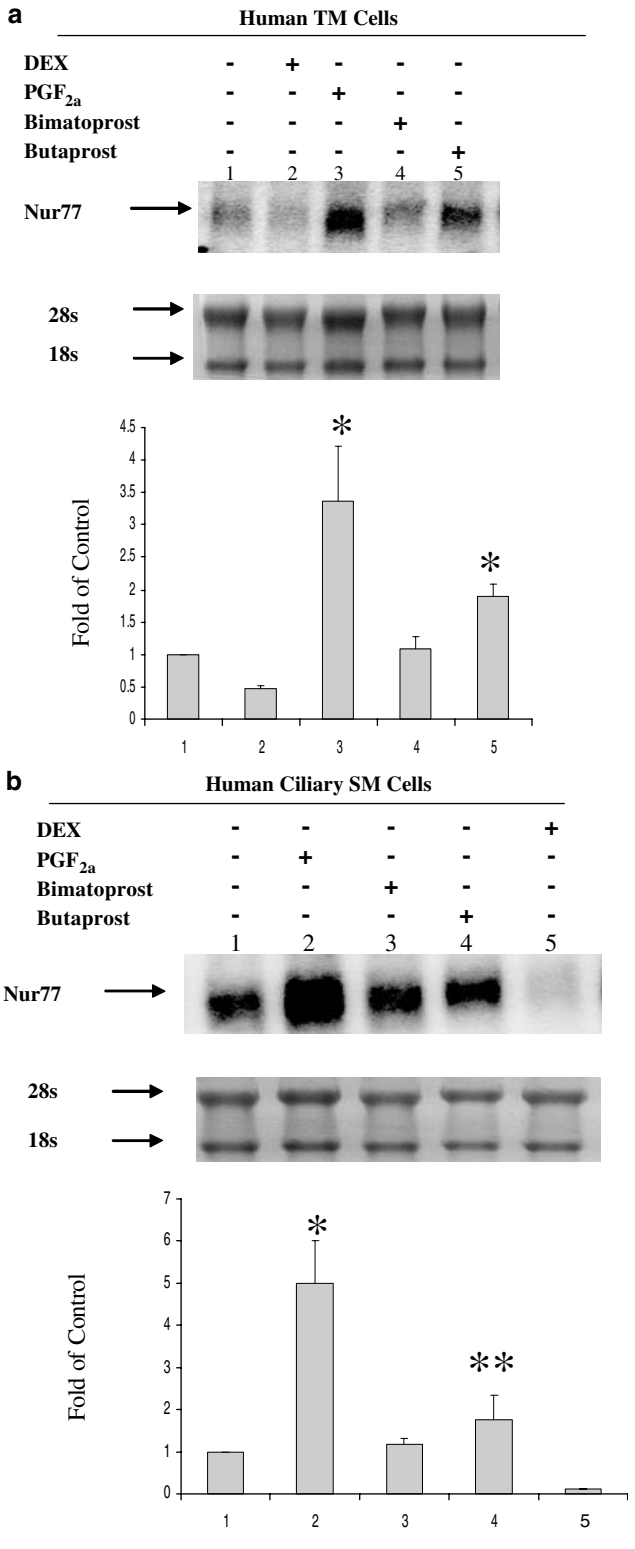

Figure 6 $PGF_{2\alpha}$ and Butaprost, but not Bimatoprost, upregulated Nur77 mRNA expression in human ciliary SM and TM cells. Human TM cells (a) and ciliary SM cells (b) were treated with 10^{-7} M of Dexamethasone (DEX), $PGF_{2\alpha}$, Bimatoprost or Butaprost for 6 h. Arrows indicate mRNA levels of Nur77 and rRNAs (a and b, top panels). The intensity of 28s and 18s rRNA bands was used as an internal control to normalize the RNA-loading differences. The data represent mean ± s.d. of three independent experiments (a and b, bottom panels). $^{*}P<0.01$ vs control, $^{**}P<0.05$ vs control.

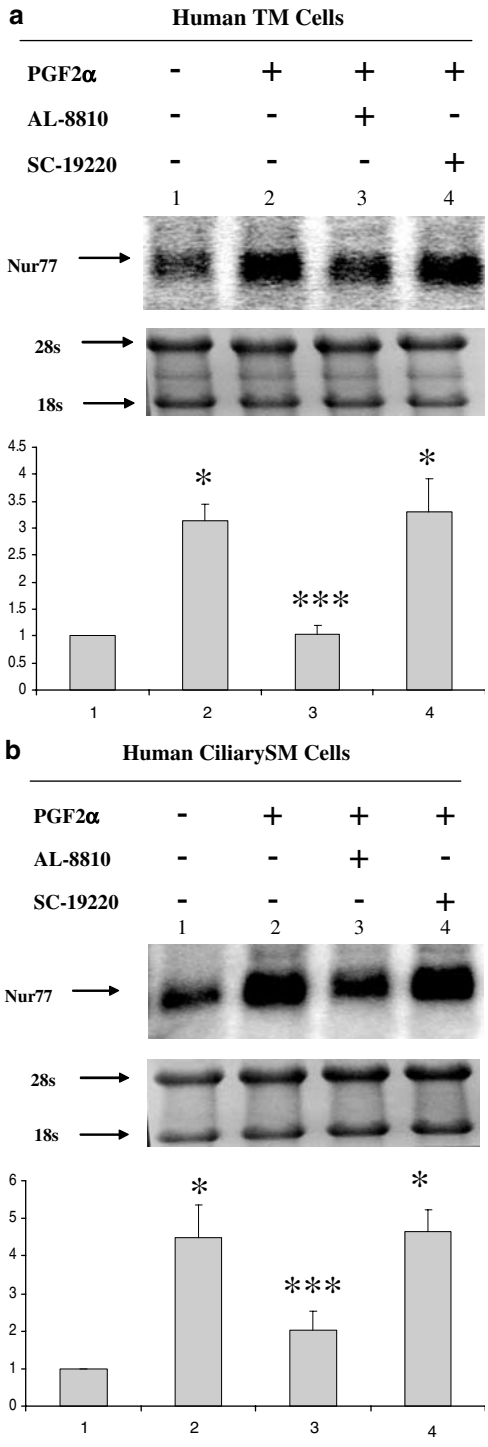

Figure 7 Prostaglandin FP receptor antagonist, AL-8810, blocked PGF2α-induced Nur77 mRNA expression in human ciliary SM and TM cells. Human TM cells (a) and Ciliary SM cells (b) were pretreated with 10^{-5} M of AL-8810 or SC-19220 for 30 min, and then incubated with 10^{-7} M $PGF_{2\alpha}$ for 6 h. Arrows indicate mRNA levels of Nur77 and rRNA (a and b, top panels). The intensity of 28s and 18s rRNA bands was used as an internal control to normalize the RNA-loading differences. The data represent mean ± s.d. of three independent experiments (a and b, bottom panels). $^{*}P<0.01$ vs control, $^{***}P<0.01$ vs PGF2α alone.

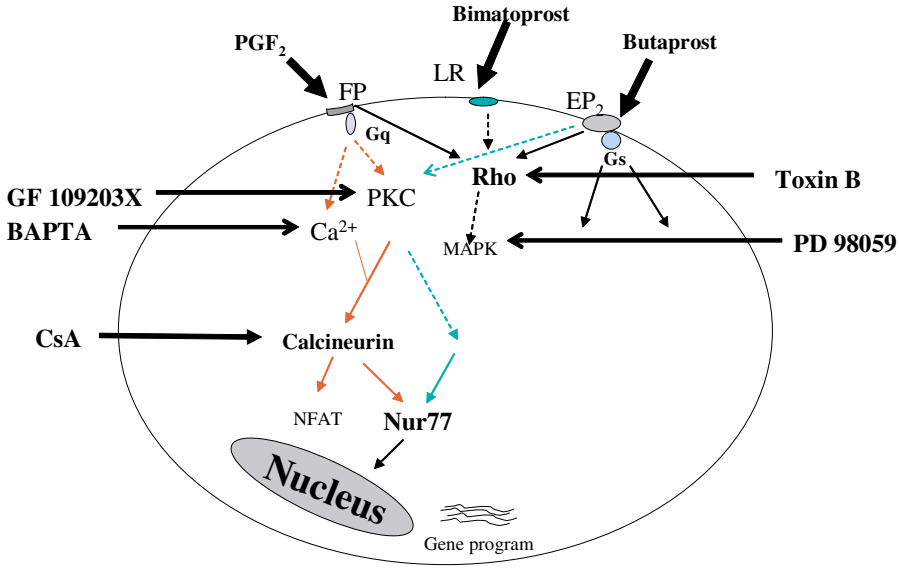

Figure 8 Diagrammatic representation of the second messenger pathways involved in the regulation Nur77 expression by $PGF_{2\alpha}$ (FP receptor agonist), Butaprost (EP_2 receptor agonist), and Bimatoprost (prostamide). Red and green arrows indicate FP- and EP_2-associated pathways, respectively.

different from control and Bimatoprost in the SM cells with *P*-value <0.05. Therefore, $PGF_{2\alpha}$ is more efficacious than Butaprost in inducing Nur77 mRNA upregulation in human TM cells and ciliary SM cells. The effects of dexamethasone (DEX) on Nur77 expression will be fully described as a separate research topic elsewhere. To further support that the induction of Nur77 mRNA is through the activation of FP receptors, a selective FP receptor antagonist, AL-8810, and a selective EP_1 antagonist, SC-19220, were used in the experiments (Figure 7a and b). AL-8810, but not SC-19220, blocked $PGF_{2\alpha}$-induced Nur77 mRNA expression in human TM and ciliary SM cells, suggesting that $PGF_{2\alpha}$-induced Nur77 mRNA expression is *via* the activation of prostaglandin FP receptors.

Discussion

A diverse variety of prostaglandins and prostaglandin ethanolamides (prostamides) have been reported to be highly effective in lowering intraocular pressure. Prostanoid FP and EP_2 receptor agonists and the prostamide bimatoprost are the most potent ocular hypotensives reported to date (Woodward *et al*., 1993a, b; 1995; 2001; Dubiner *et al*., 2001; Noecker *et al*., 2003). Their effects on intraocular pressure occur predominantly as an increase in aqueous humor outflow *via* the uveoscleral outflow pathway. The mechanisms that are involved in re-modeling of ciliary body tissue, with a resultant increase in uveoscleral outflow of aqueous humor from the eye, appear to involve the release of metalloproteinases and their modulation. Morphological studies on the ciliary body of monkey eyes treated for 1 year with the FP agonist prodrug Latanoprost, the EP_2 agonist AH13205, or the prostamide Bimatoprost revealed remarkably similar effects with respect to the formation of uveoscleral outflow channels (Richter *et al*., 2003). This was surprising as Latanoprost and AH 13205 act at distinctly different prostanoid receptors and exert their effects *via* different second messenger pathways (Pierce *et al*., 1995; Toh *et al*., 1995). Bimatoprost appears to be pharmacologically independent of FP and EP2 receptors (Woodward *et al*., 2001; 2003). This implicates a possible common pathway that mediates the effects of these drugs on ciliary muscle remodelling. The expression of genes that may coordinate this organized remodeling remains to be investigated. To this end, we have studied Nur77. The immediate-early gene Nur77 (also called NGFI-B) encodes an orphan nuclear receptor, a class of ligand-dependent transcriptional modulator proteins, which controls a program of gene expression that is involved in the regulation of cell differentiation and proliferation (Perlmann & Jansson, 1995; Maruyama *et al*., 1998). To further elucidate the mechanisms of Nur77 at the gene expression/second messenger levels, we compared the effects of three pharmacologically distinct ocular hypotensive agents $PGF_{2\alpha}$ (FP agonist), Butaprost (EP_2 agonist), Bimatoprost (prostamide), which appear to behave as mechanistically identical ocular hypotensives at the gross level, on the Nur77 gene expression.

Activation of FP receptors initiated by ligand binding triggers Gαq protein-coupled mechanisms involving activation of phospholipase C, initiation of the second messengers IP3 and diacylglycerol, and a resultant mobilization of intracellular Ca^{2+} and increased protein kinase C activity (Toh *et al*., 1995). Butaprost is a synthetic prostaglandin analog that is highly selective for EP_2 receptors; it triggers Gαs protein-coupled mechanisms involved in activation of adenylate cyclase, initiation of the cAMP formation with resultant activation of protein kinase A (Pierce *et al*., 1995). Despite different G protein signaling pathways, activation of both FP and EP2 receptors by their selective agonists induced Nur77 expression *via* protein kinase C pathways (Figure 8). This is the first observation that showed a link between EP_2 receptor stimulation and protein kinase C. Further studies suggested that the downstream PKC signaling initiated by FP receptor activation differs from that of EP_2 receptor activation, suggesting that EP_2 receptor stimulation may activate a different PKC isoform, linked to a different PKC downstream signaling pathway to trigger Nur77 gene expression. It has been reported that the PKC activator PMA induced only a low

level of Nur77 expression, but became highly induced by the addition of calcium ionophore to T-cell hybridoma (Woronicz *et al*., 1995). This may explain why EP_2 receptor stimulation only weakly induced Nur77 expression, whereas FP receptor stimulation strongly induced Nur77 expression. The induction of Nur77 expression by other stimuli has also been reported in other cell types. Nur77 expression was rapidly induced by Nerve Growth Factor (NGF) in PC 12 phenochromocytoma cells *via* Ca^{2+} (Katagiri *et al*., 1997; Milbrandt, 1988), by T-cell receptor activation (TCR) in T-cells *via* Ca^{2+} (Liu *et al*., 1994; Woronicz *et al*., 1994) and by serum in fibroblasts *via* AP-1 (Hazel *et al*., 1988). Thus, the regulation of Nur77 expression may be through diverse intracellular mechanisms that are dependent upon different stimuli.

Actinomycin D blocked Nur77 mRNA expression in response to $PGF_{2\alpha}$ and Butaprost treatment, which implied that transcription mechanisms were involved in $PGF_{2\alpha}$- and Butaprost-induced upregulation of Nur77 expression. The 5′-flanking region of Human Nur77 gene has been previously characterized to be a functional promoter for Nur77 transcription (Crabtree, 2001). Activation of Nur77 expression by phorbol esters requires multiple transcription elements between −126 to −72 of the promoter region (William & Lau, 1993). Activation of Nur77 expression by NGF and membrane depolarization involves a region between −60 to −30 of the promoter region (Yoon & Lau, 1993). A region of Nur77 promoter from −322 to −151 is required for T-cell receptor signalling-induced Nur77 expression (Woronicz *et al*., 1994). In this study, we first identified that a region from −496 to −334 contains positive regulatory elements (enhancers) that are responsive to $PGF_{2\alpha}$ and Butaprost treatment. A region from −960 to −496 contains negative regulatory elements that are responsive to $PGF_{2\alpha}$ and Butaprost treatment. We also identified that a region from −181 to −114 contains negative regulatory elements (suppressors) for Nur77 expression, but these are not responsive to $PGF_{2\alpha}$ and Butaprost treatment. Localization of discrete regions in human Nur77 promoters that respond to $PGF_{2\alpha}$ and Butaprost treatment provides a clue for further studies on the mechanisms of $PGF_{2\alpha}$- and Butaprost-induced upregulation of Nur77 gene expression at the transcriptional level.

In terms of ocular hypotensive activity resulting from ciliary muscle re-modelling, Nur77 may represent a common modulator associated with both FP and EP2 receptor activation. Bimatoprost represents a novel class of anti-glaucoma compounds where the -COOH typical of prostaglandins is replaced by an amide group (Woodward *et al*., 2001). Unlike $PGF_{2\alpha}$ and Butaprost, Bimatoprost did not upregulate Nur77 expression in human ciliary SM cells and TM cells and Nur77 would, therefore, be concluded to play no role in its ocular hypotensive effects. In our previous study, we found that Bimatoprost induced upregulation of Cyr61 gene expression in cat iris and human ciliary SM cells (Liang *et al*., 2003). In contrast to $PGF_{2\alpha}$, Bimatoprost did not regulate either CTGF or Nur77 upregulation in human ciliary SM and TM cells. These results suggest that Bimatoprost may exert its pharmacological actions through a unique mechanism to lower intraocular pressure, which is independent of Nur77.

The precise role of early response genes in mediating the ocular effects of the various PGs and Bimatoprost are not likely to be elucidated until an effective means of inhibiting their actions can be employed in living primates. Methods for measuring intraocular pressure in mice have recently been reported (Avila *et al*., 2001) and disruption of the Nur 77 gene could provide valuable insight into the importance of Nur 77 in mediating drug induced ocular hypotension. It is, however, important to realize that, between the various mammalian species, enormous variations in drug-induced ocular hypotension occur. For example, the rabbit and cat do not predict the effects of FP (Woodward *et al*., 1989; Stjernschantz, 2001) or DP (Crawford *et al*., 1992; Woodward *et al*., 1993b) agonists in primate eyes and are, therefore, unreliable therapeutic indicators. In addition to reducing intraocular pressure, negative consequences of Nur77 upregulation in the eye may possibly occur. Although this is clearly not the case in the anterior segment of the eye, PG-based drugs may achieve the retina in aphakic patients. In this case, Nur77 upregulation may accelerate the neurodegenerative aspects of glaucoma by influencing apoptotic events (Laabich *et al*., 2001).

In addition to glaucoma, efforts are directed towards prostanoid EP_2 and FP agonist therapies for treating osteoporosis (Paralkar *et al*., 2003). In this case, there is an established link between Nur77 upregulation and bone formation. Thus, PTH induces Nur77 expression in primary mouse osteoblasts and in primary mouse calvariae cultures (Pirih *et al*., 2003). The existence of a convergent pathway for EP_2 and FP agonists *via* Nur77 in bone remains to be determined.

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