Localization of EP_1 and FP Receptors in Human Ocular Tissues by In Situ Hybridization

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Purpose. To examine the expression and localization of EP_1 and FP receptor mRNAs in normal human ocular tissues by in situ hybridization.

Methods. Digoxigenin-labeled human EP_1 and FP receptor antisense and sense riboprobes were used for in situ hybridization on paraffin sections of normal human eye tissue.

Results. In situ hybridization revealed the presence of high levels of both EP_1 and FP receptor mRNA transcripts in the blood vessels of iris, ciliary body, and choroid. Both the endothelial and smooth muscle cells of blood vessels demonstrated intense hybridization signals corresponding to EP_1 receptor mRNA transcript. EP_1 receptor hybridization signals were present in all the muscle fibers of the ciliary body. In the retina, hybridization signals for EP_1 receptors were observed in photoreceptors and both nuclear layers and in ganglion cells. The hybridization signals corresponding to FP receptor transcript were similar to those of EP_1 receptors in the iris tissues. In the ciliary muscle, FP receptor mRNA transcript was predominantly present in the circular muscle and in the collagenous connective tissues; no hybridization signal for this receptor was observed in the retina.

Conclusions. The wide distribution of EP_1 and FP receptor mRNAs in human ocular tissues appears to be localized in the functional sites of the respective receptor agonists. Selective localization of FP receptor mRNA in the circular muscles and collagenous connective tissues of the ciliary body suggests their involvement in the increased uveoscleral outflow of aqueous humor by $PGF_{2\alpha}$. (*Invest Ophthalmol Vis Sci.* 2001;42: 424–428)

Biologically active arachidonic acid metabolites, prostaglandins (PGs), mediate diverse physiological actions and have a large number of pharmacological actions in vascular beds, gastric mucosa, corpus luteum, kidney, eye, and immune system.[1–3] For example, PGE_2 is cytoprotective in gastric mucosa and a potent vasodilator in almost all tissues. In the eye, PGE_2 and $PGF_{2\alpha}$ induce vasodilation, increase vascular permeability, cause miosis, and reduce intraocular pressure.[4,5]. These actions are also shared by thromboxane A_2 and to some extent by other PGs.[6,7] All these and other actions of PGs are mediated by their specific cell surface receptors coupled to G protein. PGE_2-specific EP receptors have four subtypes: EP_1, EP_2, EP_3, and EP_4.[8] To date, expression of FP receptor subtypes has not been reported; only isoforms have been identified in ovine corpus luteum.[9] Physiological role or the impact of the stimulation of EP_3 receptors as cytoprotective and that of FP receptors in corpus luteal functions are well known.[10,11] In the eye, the activation of EP_1, EP_4, and FP receptors by their selective agonists reduces intraocular pressure and causes pupil constriction.[12]

We have previously reported that EP_1, EP_4, and FP receptors exist in human ciliary muscle cells as demonstrated by second-messenger generation and mRNA expression.[13–15] However, the precise cellular localization of PG receptors in the ocular tissues is unknown. The purpose of the present study was to examine the distribution and localization of EP_1 and FP receptor mRNAs in the human ocular tissues by in situ hybridization.

Methods

Tissue Preparation

The human eye was obtained from the Department of Pathology, University of Louisville. This eye was enucleated because of orbital cancer and was fixed immediately. The eye was bisected equatorially, then fixed in 4% neutral buffered paraformaldehyde, and embedded in paraffin. Five-micrometer sections of the embedded tissue were mounted on the slides precoated with 2% APTES (3′-aminopropyltriethoxysilane; Sigma, St. Louis, MO). Some of the sections were stained with hematoxylin and eosin for histologic examination.

Preparation of Probe

Riboprobes were synthesized from pcDNA I (Invitrogen, San Diego, CA) plasmid vectors containing dual SP6 and T7 promoters and the full-length human EP_1 or FP receptor cDNA. Merck Frosst Canada (Quebec), generously provided these plasmids. EP_1 antisense and sense probes were transcribed from the plasmid linearized with *Fsp*I (Gibco/BRL, Rockville, MD) using digoxigenin (DIG) RNA labeling kit (Boehringer Mannheim, Indianapolis, IN). The transcription reaction was carried out with SP6 (antisense) or T7 (sense) polymerases according to the manufacturer's instruction. Briefly, 1 μg linearized plasmid DNA, 2 μl DIG RNA labeling mix, and 2 μl 10× transcription buffer were mixed to a final volume of 18 μl. RNA polymerase (2 μl; SP6 or T7) was added to the reaction mixture and incubated for 2 hours at 37°C. DNAase I (2 μl) was used to remove template DNA. The labeled probes were precipitated and purified from DNA and unincorporated DIG-UTP. The labeling efficiency was determined semiquantitatively using a standard DIG-labeled control RNA of known concentration. Approximately 90% of DIG-UTP were incorporated into the probe. Antisense and sense probes for the FP receptor were prepared from another plasmid containing the full-length human FP receptor cDNA, linearized with *Nco*I using the above labeling procedure.

In Situ Hybridization

For in situ hybridization, the sections were rehydrated, permeabilized in 0.2% Triton X-100, washed in PBS, and then digested with 1 μg/ml proteinase K for 30 minutes at 37°C. The sections were postfixed in 4% paraformaldehyde for 10 minutes, and washed in PBS and then in 2×

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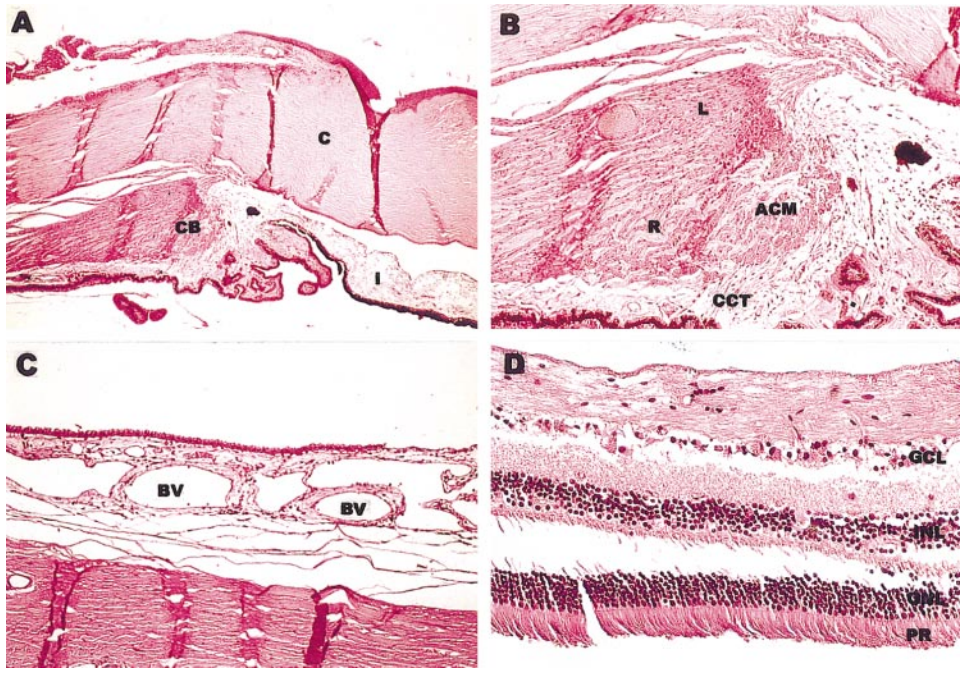

FIGURE 1. H&E-stained sections of human ocular tissues. (**A**) Anterior segment. C, cornea; I, iris; CB, ciliary body. Magnification, ×140. (**B**) Ciliary body. L, longitudinal muscles; ACM, anterior circular muscle; R, radial muscle; CCT, collagenous connective tissue. Magnification, ×350. (**C**) Choroid. BV, blood vessels. Magnification, ×350. (**D**) Retina. GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer; PR, photoreceptor. Magnification, ×700.

SSC (0.3 M NaCl, 30 mM sodium citrate, pH 7.4). Hybridization was carried out in 50% formamide, 10% dextran sulfate, 5× Denhardt's solution, 100 μg/ml denatured salmon sperm DNA, 0.1% SDS, and 3 μl DIG-labeled antisense or sense probes. Hybridization solution (25 μl) was applied to each tissue section. The prehybridization and the hybridization were performed at 42°C for 1 hour and overnight, respectively. The slides were washed with five changes of 2× SSC with 0.1% SDS at 48°C and then briefly rinsed in PBS. The block reagent was added to tissue sections, incubated for 2 hours at room temperature, and finally washed by PBS. The sections were incubated with anti-DIG–AP (alkaline phosphatase) conjugate for 0.5 hour, covered with a coverslip, incubated for another 1 hour, and then rinsed in PBS. The tissue sections were incubated with the AP substrate nitro-blue tetrazolium/5-bromo-4-chloro-3-indolyl-phosphate (NBT/BCIP) for 2 hours in the dark to develop color. The slides were examined under a light microscope. Photomicrographs were taken on Ektar, 200 ASA film (Eastman Kodak, Rochester, NY).

Isolation of Total RNA and Northern Blot Analysis

Confluent HCM cells were collected by scraping in a guanidinium thiocyanate homogenization buffer (4 M guanidinium thiocyanate, 0.5% *N*-sodium lauryl sarcocinate, 25 mM sodium citrate, and 0.7% 2-mercaptoethanol) at pH 7.0. Total RNA was extracted according to the guanidinium thiocyanate method.[16] RNA concentration was quantified by UV absorption at 260 nm.

The EP_1 and FP riboprobes used in the Northern blot analysis were synthesized as described in preparation of probe, except that they were radiolabeled with [α-^{32}P]cytidine triphosphate (3000 Ci/mmol; DuPont-NEN, Boston, MA) using an in vitro transcription kit (Maxiscript Transcription Kit; Ambion, Austin, TX). Total RNA (25 μg) was separated by electrophoresis on a 1% denaturing agarose gel and was transferred to nylon membranes (Gene-Screen; NEN Research Products, Boston, MA). Membranes were hybridized with either a ^{32}P-labeled EP_1 or FP probe in High-Efficiency Hybridization Buffer (Molecular Research, Cincinnati, OH) containing 1% SDS and 0.1 M NaCl overnight at 60°C. Blots were washed three times in 1× SSC/0.1% SDS for 7 minutes at 55°C and developed by autoradiography.

RESULTS

In situ hybridizations with EP_1 and FP receptor riboprobes demonstrated the expression of their mRNAs in the anterior and posterior uveal tissues of a normal human eye. For easy identification of in situ hybridization signals in tissues, human ocular sections stained with hematoxylin and eosin are included (Figs. 1A through 1D).

EP_1 Receptor mRNA

In situ hybridization with EP_1 antisense riboprobe revealed the presence of a large amount of EP_1 receptor mRNA transcript in the iris vasculature, iris-sphincter muscles, and ciliary body (Fig. 2A). In the iris, strong hybridization signals were obtained in sphincter muscles and in blood vessels (Fig. 2B). Both the endothelial and smooth muscle cells of blood vessels, particularly the endothelium, showed intense hybridization signals reflecting the existence of EP_1 receptor transcripts. Also, there were positive signals in the iris root and ciliary body (Fig. 2C). In the posterior uveal tissues, choroidal vessels (Fig. 2D), photoreceptors, both nuclear layers, and ganglion cells showed hybridization signals corresponding to EP_1 receptor (Fig. 1E). Treatment of all the above tissues with the EP_1 sense riboprobe (negative control) demonstrated very weak or no signals for EP_1 receptor transcripts (Fig. 2F).

FP Receptor mRNA

Blood vessels, iris-sphincter, and ciliary body showed the expression of FP receptor mRNA after hybridization with the FP antisense riboprobe (Figs. 3A, 3B). Hybridization signals were found to be present in the anterior circular muscles and collagenous connective tissues of the ciliary body (Fig. 3C). Interestingly, in the longitudinal muscles, signals were weak, and the radial muscles did not show any signal. Choroidal vessels and retinal tissues did not show any hybridization signal. All the above tissues treated with the FP sense probe did not show any positive signals (Fig. 3D).

Northern Blot Analysis

Northern blot analysis with EP_1 and FP antisense probes demonstrated the presence of EP_1 and FP mRNAs in human ciliary muscle cells (Fig. 4). No signals were obtained by Northern blot analysis performed with EP_1 or FP sense probes.

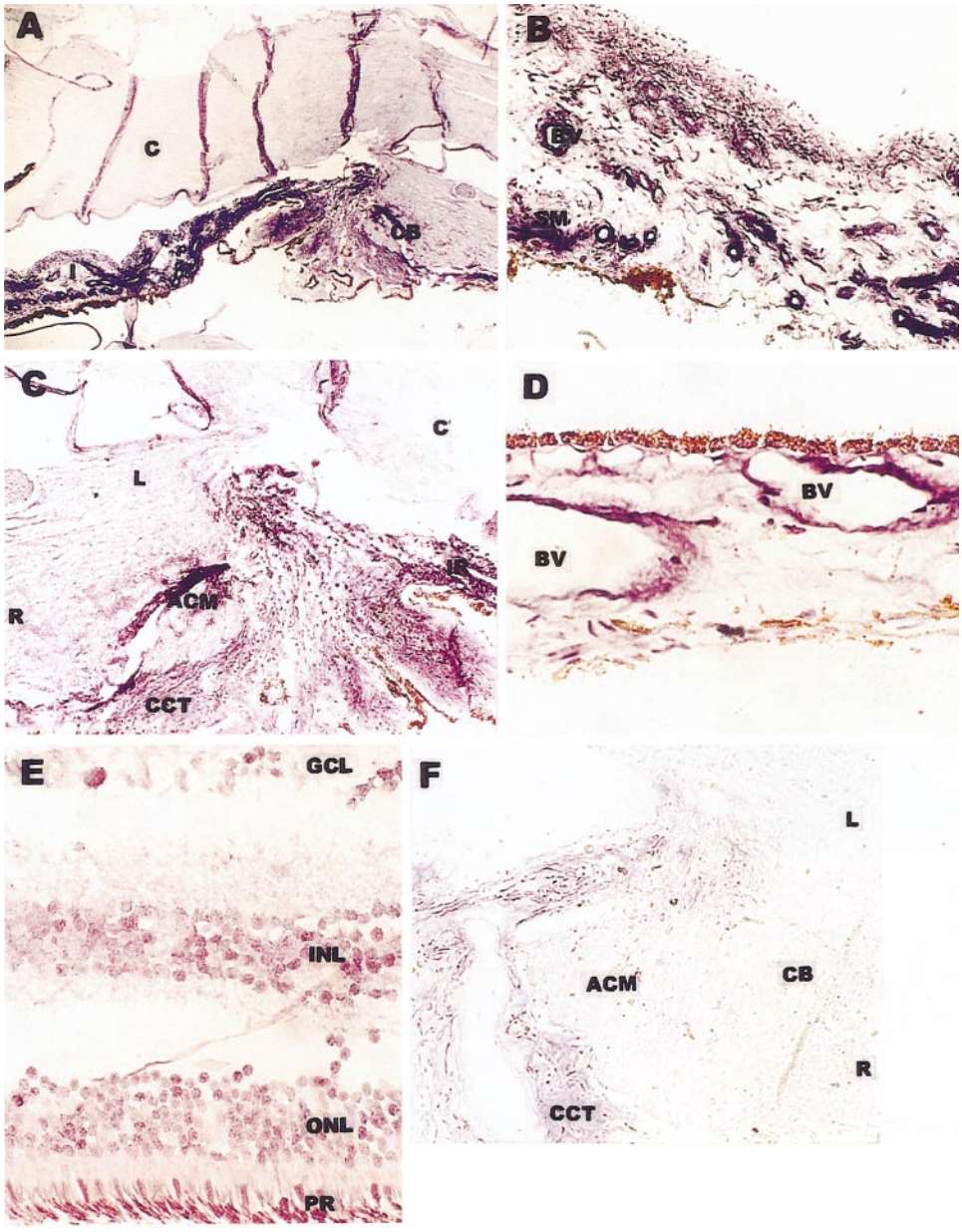

FIGURE 2. In situ hybridization for EP_1 receptor in human ocular tissue. *Purple,* positive signals for in situ hybridization of antisense riboprobes. (**A**) Anterior segment. C, cornea; I, iris; CB, ciliary body. Magnification, ×140. (**B**) Iris. BV, blood vessel. SM, iris-sphincter muscle. Magnification, ×350. (**C**) Iris and ciliary body. CCT, collagenous connective tissues; ACM, anterior circular muscle; IR, iris root; L, longitudinal muscle; R, radial muscle. Magnification, ×350. (**D**) Choroid. Magnification, ×700. (**E**) Retina. GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer; PR, photoreceptor. Magnification, ×700. (**F**) Negative control for in situ hybridization in which DIG-labeled sense probes were used. Magnification, ×350.

DISCUSSION

The results of our studies demonstrated, for the first time, the localization and expression of EP_1 and FP receptor mRNAs in human ocular tissue by in situ hybridization. Northern blot analysis demonstrated the presence of EP_1 and FP transcripts in human ciliary muscle cells and also confirmed the specificity of EP_1 and FP antisense probes used in this in situ hybridization study. Previous studies[13,14] reported the presence of EP_2, EP_4, and FP receptor mRNAs in human ciliary nonpigmented epithelial and ciliary muscle cells by RT-PCR or by measuring intracellular calcium. Anthony et al.[17] reported the expression of FP receptors in human trabecular cells. $PGF_{2\alpha}$ and its analogue latanoprost lower intraocular pressure in the human eye.[18] Studies in animals and humans suggested that this ocular hypotensive action is due to the increased uveoscleral drainage of aqueous humor.[19,20] $PGF_{2\alpha}$ and latanoprost are FP receptor agonists, and the target of their ocular hypotensive action is thought to be ciliary muscles that are known to express FP receptors.[13–15,21] Our study demonstrated for the first time that FP receptor mRNAs are expressed in anterior circular but not in the radial and longitudinal muscles of the ciliary body. Also, connective tissues of the ciliary body express FP receptors. These observations suggest that $PGF_{2\alpha}$ or latanoprost acts on the circular and collagenous tissues to increase uveoscleral drainage of aqueous humor. It has been reported that $PGF_{2\alpha}$ increases the levels of matrix metalloproteinase-1 and -3 in human ciliary muscle cells.[22] It is possible that $PGF_{2\alpha}$ acts on collagenous connective tissue and anterior circular muscle cells to increase the activities of metalloproteinases. These would then degrade ciliary muscle extracellular cell matrix, leading to increased uveoscleral outflow. An earlier study on in situ hybridization of FP receptors by Ocklind et al.[23] reported positive in situ hybridization signals in monkey ocular tissues; these findings are broadly similar to those demonstrated in the present study. However, there are a few important differences in the results between the two studies that may be due to the species variation. These differences were as follows: (1) in human ciliary muscles, hybridization signals were localized in

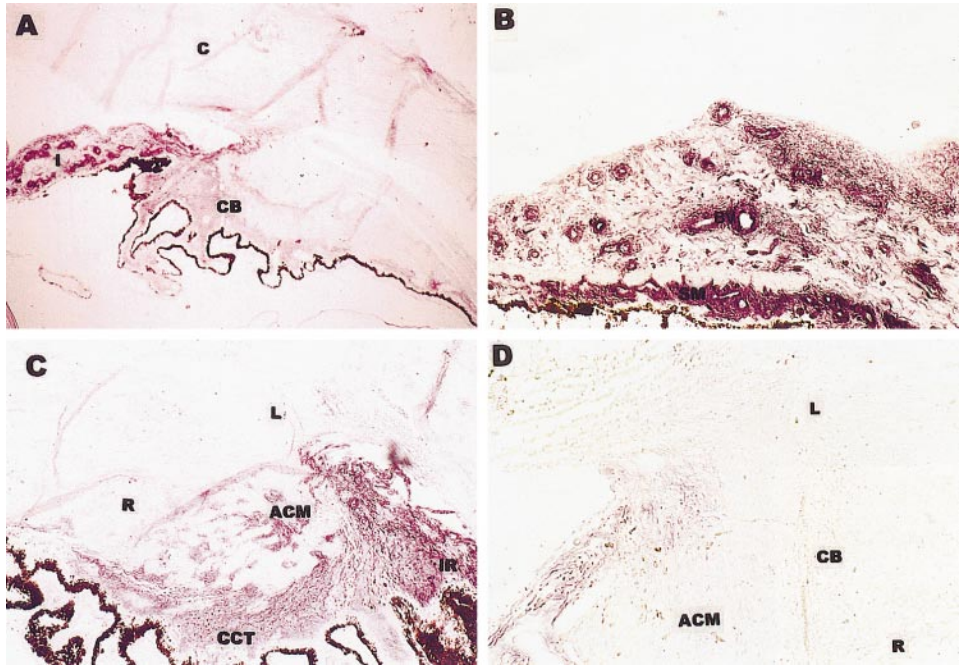

FIGURE 3. In situ hybridization for FP receptors in human ocular tissues. *Purple,* positive signals for in situ hybridization of antisense riboprobes. (**A**) Anterior segment. C, cornea; I, iris; CB, ciliary body. Magnification, ×140. (**B**) Iris. BV, blood vessel. Magnification, ×350. (**C**) Iris and ciliary body. CCT, collagenous connective tissues; ACM, anterior circular muscle; IR, iris root; L, longitudinal muscles; R, radial muscle. Magnification, ×350. (**D**) Negative control for in situ hybridization in which DIG-labeled sense probes were used. Magnification, ×350.

the anterior circular and radial muscles, but in the monkey ciliary muscles, the hybridization signals were present in the longitudinal muscles; and (2) human ciliary processes showed that the signals were associated with highly vascular stroma but not with the epithelial cells. In contrast in monkey ciliary processes, signals were present in the epithelial cells and in the stroma. In our study, we observed hybridization of FP receptor transcript in the iris but not in the choroidal and retinal vasculature. Ocklind et al.[23] reported the presence of FP receptor protein but not the expression of mRNA of FP receptors in the monkey ocular blood vessels.

In the human eye, the expression of FP receptor mRNAs in the ocular vascular smooth muscle and endothelial cells suggests that FP receptors mediate vascular reactions of $PGF_{2\alpha}$. FP receptors increase intracellular $[Ca^{2+}]_i$ via the inositol phosphate pathway and thus are expected to cause contraction of vascular smooth muscle and endothelial cells. However, it is well established that $PGF_{2\alpha}$ causes either vasodilation or vasoconstriction, depending on the species and anatomic location of blood vessels.[24–27] In human eyes, $PGF_{2\alpha}$ causes conjunctival vasodilation, and in addition, it induces dilatation of iris vasculature in experimental animals. Therefore, it seems that stimulation of FP receptors results in the formation and release of a vasoactive substance. In fact, Chen et al.[25] and others[24,27] reported the release of vascular endothelial relaxing factor, NO in the endothelium by $PGF_{2\alpha}$. The mechanism for such a release is not clear. Sato et al.[28] reported that in the vascular endothelium, carbachol and histamine induced an increase in $[Ca^{2+}]_i$ and suggested that increased intracellular calcium stimulates the release of NO. It is possible that a similar mechanism exists for FP receptor–mediated vasodilation in the eye.

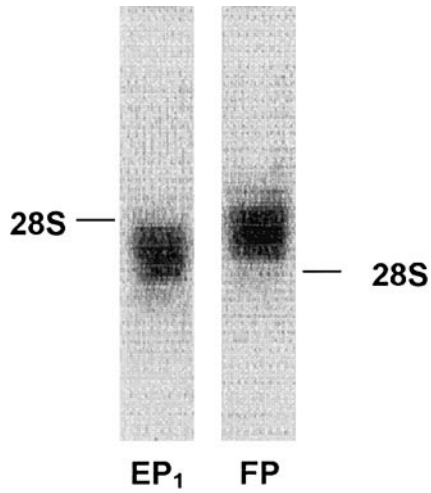

FIGURE 4. Northern blot analysis analyses of EP$_1$ and FP receptor mRNAs from human ciliary muscle cells. 28S, migration of the 28S ribosomal RNA band.

EP receptor subtype EP$_1$ is expressed in a number of tissues and cells. For instance, this receptor subtype is present in cultured myometrial cells,[29] amnion cells,[30] renal collecting tubules,[31] and central nervous system and human nonpigmented ciliary epithelial and ciliary muscle cells.[13–15] In all these tissues, EP$_1$ receptors appear to have functional significance. It has been reported that EP$_1$ receptors mediate PGE_2-dependent inhibition of Na^+ absorption in the collecting ducts of rabbits[31] and hyperthermia and interleukin-1β–induced fever in rats.[32] EP$_1$ receptors are also involved in the maintenance of tracheal smooth muscle tone in guinea pigs.[33] In the eye, EP$_1$ receptors are reported to be involved in PG-induced conjunctival pruritus and allergic conjunctival itching.[34] Recently, Bhattacherjee et al.[35] reported that EP$_1$ receptor agonist, 17-phenyl trinor PGE_2 lowers intraocular pressure in cats and rabbits. In the present study, we have observed that EP$_1$ receptor mRNA is expressed in vascular endothelium and smooth muscles. Probably, these receptors are involved in vasoconstriction because the stimulation of EP$_1$ receptors results in the mobilization of intracellular calcium. The ocular hypotensive action of EP$_1$ receptor agonists may be due to an increased outflow facility, because EP$_1$ receptors are expressed in the ciliary muscle. The significance of the expression of EP$_1$ receptor mRNA in the nuclear cell layers and ganglion cells of the retina is not yet known.

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