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Optimization of RNA isolation from human ocular tissues and analysis of prostanoid receptor mRNA expression using RT-PCR

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Purpose: The isolation and analysis of human ocular RNA is problematic due to variables such as rapid degradation, tissue composition, and melanin contamination. The purpose of this work was to optimize an extraction protocol for the isolation of intact total RNA from a variety of diverse human ocular tissues and to employ RT-PCR to assess the expression of mRNA coding for all eight prostanoid receptors.
Methods: Total RNA was extracted from human iris, ciliary body, choroid, and retina using an RNeasy® Midi Kit. Total RNA was extracted from human cornea, sclera, and optic nerve using Tri-Pure® Isolation Reagent. 1.0 µg of total RNA was reverse transcribed into cDNA and subsequently amplified by PCR (35 cycles) using primers designed against each of the human prostanoid receptor cDNAs. PCR products were analyzed by gel electrophoresis and endonuclease digestion.
Results: The total yield and quality of RNA derived from each tissue varied according to tissue composition and the isolation method employed. RT-PCR analysis revealed that each tissue expressed all prostanoid receptor mRNAs, however, 50 cycles of PCR was required to visualize FP receptor expression in scleral tissue. In all cases, prostanoid receptor mRNA expression was significantly lower than in human nonpregnant myometrium, which was used as the positive control.
Conclusions: The different cellular composition of each ocular tissue ultimately dictated the methodology to be employed for the isolation of total RNA. Thus, two extraction protocols were optimized for the isolation of intact high quality RNA from a variety of human ocular tissues. The identification of all prostanoid receptor mRNAs in a diverse set of human ocular tissues suggests potential mechanisms for prostanoid-based therapeutics aimed at IOP reduction and stimulates speculation as to additional physiological and or pathophysiological roles mediated by prostanoids.

Glaucoma describes a group of potentially blinding ocular disorders that involve progressive optic neuropathy of unknown etiology, frequently associated with elevated intraocular pressure (IOP)[1]. Open angle glaucoma (OAG), which represents the most prevalent form of glaucoma, is commonly treated by long term medical management of IOP [2,3]. Presently, the most common classes of drugs used for the management of OAG are topical forms of β-adrenergic antagonists (β-blockers), adrenomimetics, miotics, and carbonic anhydrase inhibitors. However, because of factors such as tolerance, contraindications, and occasional intolerable side effects, many of these drugs are unable to adequately control IOP [4-6].

The prostanoid analogues are the newest class of ocular hypotensive agents to become available for the treatment of OAG. Prostanoids, a family of compounds comprising prostaglandins (PGs) and thromboxanes (Txs) are local mediators of numerous ocular effects, including dose-dependent angiogenic, vasodynamic, miotic, anti-/pro-inflammatory, and hypo-/hyper-tensive actions [7-9]. The naturally occurring, biologically active prostanoids, which are considered to be PGD_2, PGE_2, $PGF_{2\alpha}$, PGI_2, and TxA_2, exert their biological actions by interacting with specific membrane bound receptors. Current pharmacological classification includes five types of prostanoid receptors on the basis of sensitivity to PGD_2, PGE_2, $PGF_{2\alpha}$, PGI_2, and TxA_2 [9]. These receptors are termed P receptors, with a preceding letter indicating the natural prostanoid to which each receptor is most sensitive. Thus, the receptors are termed DP, EP, FP, IP, and TP respectively. Furthermore, EP is subdivided into four subtypes; EP_1, EP_2, EP_3, and EP_4 on the basis of their responsiveness to various selective agonists and antagonists [9].

Presently, there are two prostanoid-based drugs commercially available: Xalatan® (latanoprost) and Travatan® (travoprost), all of which are thought to mimic the actions of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$). Taken together, prostanoid analogues appear to possess at least equal efficacy to that of current gold standard therapies such as timolol [10-12]. In addition, significant advantages of prostanoid analogs compared to other classes of anti-glaucoma medications are the minimal side effect profile and once-daily dosing regime associated with their use.

Despite their clinical success, the physiological mechanism(s) underlying the hypotensive effect of these prostanoid analogues has yet to be definitely described, although it is presumed to be mediated by FP receptors [13,14]. A more pronounced issue is the fact that the development of additional prostanoid-based ocular therapeutics has been significantly hindered by the near absolute lack of understanding

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of prostanoid receptor distribution and pharmacology, facts directly owing to the lack of appropriate molecular and pharmacological probes. Thus, before any advancement can be made towards a mechanistic understanding of any prostanoid analogue or in the development of superior prostanoid-based ocular therapeutics, a detailed characterization of human ocular prostanoid receptors must be established. To aid in this endeavor, we have studied the distribution of mRNAs coding for all human prostanoid receptors in a variety of ocular tissues by RT-PCR. In order to complete this objective, two obstacles associated with the isolation of high quality RNA from human ocular tissues had to be addressed. First, many ocular tissues contain melanin, which co-purifies with RNA and has been shown to be an inhibitor of *Taq* [15]. Previously, this problem has been dealt with through RNA purification by column chromatography [16], acid-precipitation [17], isolation of mRNA [18], or addition of proteins such as bovine serum albumin or dry milk in the RT-PCR reaction [15,19,20]. However, these methods are laborious and time consuming and often cannot remove all melanin leading to inefficient PCR. Second, tissues such as cornea, sclera, and the optic nerve possess a high collagen or adipose content that can lead to incomplete homogenization, yielding RNA of low concentration and quality. To overcome these obstacles, we first optimized two extraction protocols for the isolation of intact, high quality RNA from a variety of human ocular tissues. Using these procedures, we were able to assess the mRNA expression of human prostanoid receptors in a diverse array of ocular tissues.

METHODS

Materials: Tri-Pure® Isolation Reagent was purchased from Roche Molecular Biochemicals (Laval, PQ, Canada) and RNeasy® Midi Kit was purchased from QIAGEN Inc. (Mississauga, ON, Canada). SuperScript® II Reverse Transcriptase and 100 bp DNA molecular weight ladder were purchased from Invitrogen Life Technologies (Burlington, ON, Canada). AmpliTaq Gold® *Thermus aquatics* (*Taq*) DNA polymerase was purchased from Roche Molecular Biochemicals (Laval, PQ, Canada). Restriction enzymes were purchased from Roche Molecular Biochemicals (Laval, PQ, Canada) or Amersham Pharmacia Biotech (Baie d'Urfe, PQ, Canada). All other reagents were of the finest quality available and were obtained from either Sigma Chemical Co. (St. Louis, MO, USA) or Fisher Scientific (Nepean, ON, Canada).

Collection of donor eyes: Human globes were collected within 24 h post mortem from the Eye Bank of Canada (Ontario Division). Globes were individually wrapped in gauze soaked in saline solution and stored on ice during dissection. Individual tissues (iris, ciliary body, retina, choroid, optic nerve, sclera, and cornea) were dissected in saline solution on ice and rapidly placed in 2 ml eppendorf microcentrifugation tubes. Tissues were frozen in liquid nitrogen and either used immediately to isolate total RNA or stored at -70 °C until further processing. The Office of Human Research Ethics Committee at the University of Waterloo approved the tissue collection protocol.

Isolation of total RNA: Preliminary experiments compared two different commercially available total RNA extraction kits: Tri-Pure® Isolation Reagent and RNeasy® Midi Kit, for each human ocular tissue under study. Following optimization, tissues that possessed a high collagen and/or adipose content (cornea, optic nerve, and sclera) were isolated using Tri-Pure® Isolation Reagent. For all remaining human ocular tissues the RNeasy® Midi Kit was employed. In each case, tissues were homogenized using a Polytron PT10/35 homogenizer (Brinkmann; Westbury, NY, USA), for 60 s at a rheostat setting of 10 and incubated for 5 min at room temperature. Subsequent extraction steps for both isolation procedures were carried out at room temperature and were conducted according to manufacturer's instructions except for the following modifications. For the Tri-Pure® Isolation protocol, RNA pellets were resuspended in 75% ethanol, incubated at -20 °C for 4 h then centrifuged at 7500 x g for 10 min at 4 °C. Ethanol was removed by aspiration and the pellets were dried for no more then 5 h in a dessicator. The RNA pellets were resus-

Table 1. Oligonucleotide primer sequence information

Primer Name	Receptor cDNA Sequence	Reference Location		Annealing Temperature (°C)	Product Size (bp)	Restriction Endonuclease Enzyme	Digestion Products (bp)
DP-S	5' ATG GTC CAC GAG GAG GGC TCG CTG 3'	623-643	[50]	58	531	Pst I	140/394
DP-AS	5' AAA ACA CTG TCA CAG ACT GGA TTC 3'	1438-1415	[50]	--			
EP1-S	5' ACG CGG CCG CTG CTC CAC GCC 3'	418-438	[51]	65	669	Sma I	134/33/502
EP1-AS	5' CAG TTG GCG CAG CAC GGC CTG 3'	1086-1065	[51]	--			
EP2-S	5' CGG ACC GCT TAC CTG CAG CTG 3'	740-757	[52]	48	432	Sma I	159/273
EP2-AS	5' TAA TGA AAT CCG ACA ACA GAG 3'	1164-1144	[52]	--			
EP3-S	5' AGC AGC GTT GGG AGC ACA TCG 3'	183-200	[53]	54	597	Sma I	264/333
EP3-AS	5' GCA GTG CTC AAC TGA TGT CTG 3'	1130-1107	[53]	--			
EP4-S	5' ATC GAC TGG ACC ACC AAC GTG ACG 3'	364-384	[52]	58	513	Sma I	204/309
EP4-AS	5' TCT ATT GCT TTA CTG AGC ACT GTC 3'	936-916	[52]	--			
FP-S	5' TGG TGT TTC TAC AAC ACA GAA GAC 3'	160-183	[54]	56	396	Bgl I	169/227
FP-AS	5' ATA GAG ATT CTT AAG GAC AGC CTT 3'	948-925	[54]	--			
IP-S	5' AGC TCC CTG CTG GGC CTG GCC 3'	235-255	[55]	65	690	Eae I	179/134
IP-AS	5' GCA GAC CCA GAG CTT GAG TCG CTG 3'	1107-1087	[55]	--			
TPα-S	5' GAG ATG ATG GCT CAG CTC CT3'	237-260	[56]	61	313	Xho II	445/245
TPα-AS	5' CTT CCT ACT GCA GCC CGG AGC GCT G 3'	1012-1036	[56]	--			
G3P-S	5' TGA AGG TCA GAG TCA ACG GAT TTG GT 3'	71-96	[57]	65	983	--	--
G3P-AS	5' CAT GTG GGC CAT GAG GTC CAC CAC 3'	1030-1053	[57]	--			

pended in 150 µl of RNA solubilization buffer (1 mM EDTA; 0.1% SDS; 10 mM Tris-HCL, pH 7.5), 15 µl of 2 M sodium acetate (pH 5.2) and 412.5 µl of absolute ethanol and stored at -70 °C for a maximum of 2 years. For the RNeasy® Midi Kit protocol, the second elution step was performed using the first elute, as opposed to an additional 150 µl of RNase-free water for iris and ciliary body samples only. All RNA isolates were stored in RNase-free water at -70 °C for a maximum of 2 years. Pharmacological [21] and molecular studies (data not shown) suggest that human non-pregnant myometrium possesses all of the currently defined prostanoid receptors. As such, total non-pregnant myometrial RNA was employed in all RT-PCR experiments to serve as a positive control. This was made possible through a generous gift from Dr. Denis Crankshaw (McMaster University, Department of Obstetrics and Gynecology).

Assessment of RNA quality and concentration: The concentration and purity of total RNA was determined by UV light absorption using a GeneQuant *pro* RNA/DNA calculator (Biochrom Ltd; Cambridge, England). Preparations were discarded if they had a ratio of optical densities at 260 nm / 280 nm that was lower than 1.6 [22]. To assess the presence of intact RNA, 5 µg of total RNA from each sample was loaded onto 1% agarose-formaldehyde gels and subjected to electrophoresis. Following ethidium bromide staining, RNA isolates were considered intact if the UV fluorescence of the 28S rRNA band was twice as intense as the 18S rRNA band and when no UV fluorescence was detected below the 18S rRNA band.

Oligonucleotide PCR primers: Sense and antisense-specific primers were synthesized at the Central Facility of the Institute of Molecular Biology and Biotechnology at McMaster University and are detailed in Table 1. All primers were designed to span intron-exon boundaries to distinguish between amplification of mRNA and genomic DNA and were based on published human cDNA sequences.

cDNA synthesis: RNA was converted into cDNA in a 10 µl reverse transcription reaction containing 1.0 µg of total RNA from human ocular tissue or 0.5 µg of total RNA from human myometrial tissue; 1X first strand buffer (75 mM KCl; 50 mM Tris-HCl, pH 8.3; 3.0 mM $MgCl_2$); 1.7 mM $MgCl_2$; 1 mM each dNTP; 10 mM dTT; 2.5 mM oligo $(dT)_{18}$ and 5 U / µl of SuperScript® II Reverse Transcriptase. Reactions were incubated at 42 °C for 60 min, heated at 95 °C for 5 min, then cooled at 4 °C for a minimum of 5 min and a maximum of 30 min.

Polymerase chain reaction: PCR was performed on 5 µl of cDNA preparation, to which was added 44 µl of a PCR master mix containing 1X PCR buffer (55 mM KCl; 13 mM Tris-HCl, pH 8.3); 1 mM $MgCl_2$; 10% dimethylsulphoxide (DMSO); 1.25 U / 50 µl Ampli*Taq* Gold® with GeneAmp® DNA polymerase and 0.2 mM each sense and antisense primer in a total volume of 50 µl. A "hot start" PCR method was performed in a GeneAmp® PCR System 2400 thermocycler (Perkin Elmer; Norwalk, CA, USA) using the following parameters: an initial denaturing step of 10 min at 95 °C; denaturing at 94 °C for 30 s; annealing at the optimal temperature (Table 1) for 30 s; extending at 72 °C for 1 min. The final polymerization step was extended an additional 7 min. Unless otherwise specified, 35 cycles of PCR was performed. 50 cycles of PCR was performed in the case where no amplification product was seen at 35 cycles. Precautions were taken to avoid product contamination. PCR set-up, amplification, and product processing were performed using dedicated equipment in separate rooms. In addition, several control reactions were routinely run in parallel during RT-PCR analysis including RT reactions run in the absence of the reverse transcriptase

Table 2. Comparison of total RNA derived from a variety of human ocular tissues using two different isolation methods

Tissue	Method	Number of Samples	Average Ratio (A_{260}/A_{280})	Average Sample (mg)	Average RNA Yield (µg)	Average Total RNA Yield (µg/mg)
Iris	Tri-Pure® Isolation Reagent	5	1.23	49	48	0.979
	RNeasy® Midi Kit	4	1.87	111	14	0.126
Ciliary body	Tri-Pure® Isolation Reagent	5	1.38	505	54	0.107
	RNeasy® Midi Kit	4	1.95	237	23	0.097
Choroid	Tri-Pure® Isolation Reagent	3	1.43	313	70	0.223
	RNeasy® Midi Kit	4	1.90	459	93	0.203
Retina	Tri-Pure® Isolation Reagent	5	1.40	602	50	0.083
	RNeasy® Midi Kit	5	1.94	613	221	0.360
Optic nerve	Tri-Pure® Isolation Reagent	6	1.93	197	14	0.071
	RNeasy® Midi Kit	4	1.33	522	5	0.009
Sclera	Tri-Pure® Isolation Reagent	7	1.84	306	14	0.046
	RNeasy® Midi Kit	3	1.50	257	2.52	0.009
Cornea	Tri-Pure® Isolation Reagent	9	1.83	175	19	0.108
	RNeasy® Midi Kit	7	N/A	352	0	0

enzyme to confirm the absence of genomic DNA and/or cDNA contamination and RT reactions without RNA to check for reagent contamination. As well, PCR amplification of 1.5 ng of human genomic DNA served as a negative control. PCR amplification reactions were evaluated through electrophoresis of 12 µl of PCR product on 1.5% agarose gels containing 1 µg/ml ethidium bromide and visualized by UV transillumination on a GeneGenius Imager (Synoptics Ltd, Cambridge, England). Initial product identification was made by comparison to the myometrial control and the molecular weight ladder. Endonuclease digestion was used to confirm product identity. Briefly, digestion of each prostanoid receptor mRNA amplification product was performed using the appropriate restriction endonuclease enzyme (Table 1) in a final reaction volume of 25 µl. Following digestion, products were resolved by 2.5 h of electrophoresis at 90 V on 2.0% agarose-TBE gels stained with 1 µg/ml ethidium bromide. Gels were visualized and photographed by GeneGenius and GeneSnap software. Confirmation of appropriate splice products was made by comparison to the molecular weight ladder and to the myometrial control. All RT-PCR experiments were carried out at least three times.

Statistical analysis: All data are expressed as mean ±standard deviation (S.D.). Statistical comparisons of total RNA isolated using Tri-Pure® Isolation Reagent protocol and RNeasy® Midi Kit protocol were performed using a Students' t-Test. In each case, data were considered to be significant when $P < 0.05$.

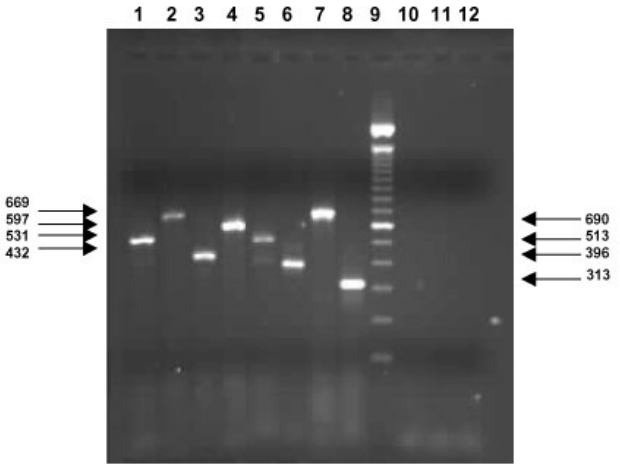

Figure 1. Survey of prostanoid receptor mRNA expression in human choroidal tissue. Representative ethidium bromide staining of prostanoid receptor mRNA amplification products obtained from 1.0 µg of total RNA from human choroidal tissue run at 35 cycles of PCR and resolved by electrophoresis. RT-PCR amplification products representing: Lane 1: DP receptor mRNA (531 bp); Lane 2: EP_1 receptor mRNA (669 bp); Lane 3: EP_2 receptor mRNA (432 bp); Lane 4: EP_3 receptor mRNA (597 bp); Lane 5: EP_4 receptor mRNA (513 bp); Lane 6: FP receptor mRNA (396 bp); Lane 7: IP receptor mRNA (690 bp); Lane 8: TP receptor mRNA (313 bp); Lane 9: 100 bp DNA molecular weight ladder; Lane 10: representative negative control where the RT reaction was carried out in the absence of reverse transcriptase enzyme; Lane 11: representative negative control where the RT reaction was carried out in the absence of total RNA; Lane 12: representative PCR amplification of 1.5 ng of human genomic DNA. Arrows mark the approximate position of each prostanoid receptor mRNA amplification product. Representative of 3 experiments.

RESULTS

Comparisons made between total RNA isolation protocols: To optimize an extraction protocol for isolating total RNA from human ocular tissues, preliminary experiments were designed to compare the quality and yield of total RNA obtained from both the RNeasy® Midi Kit and the Tri-Pure® Isolation Reagent (Table 2). Quality of total RNA isolated from tissues with high collagen and/or adipose content such as cornea, sclera, and optic nerve was significantly lower (<0.05), as judged by comparing 260 nm / 280 nm ratios, when isolated using the RNeasy® Midi Kit protocol compared to the Tri-Pure® Isolation Reagent protocol. Furthermore, isolation of total RNA from cornea was unsuccessful using the RNeasy® Midi Kit, while isolation with Tri-Pure® Reagent produced high quality RNA. In contrast, the quality of total RNA from tissues pigmented with melanin was significantly higher ($P < 0.05$) when isolated using the RNeasy® Midi Kit. Lastly, only the RNeasy® Kit generated melanin-free RNA from pigmented ocular tissues (iris, ciliary body, and choroid).

Identification of prostanoid receptor mRNA expression in human ocular tissues: PCR amplification products indicative of all prostanoid receptor mRNAs were seen in all tissues studied (Table 3). Amplification products were identical to the size predicted from the published human prostanoid receptor cDNA sequences (Table 1). Further verification of PCR product identification was obtained through direct comparison to the myometrial control and through specific endonuclease digestion with the appropriate enzyme. Figure 1 and Figure 2 are representative of results obtained from the survey of prostanoid receptor mRNA expression in human choroidal and optic nerve tissue, respectively. Amplification of human scleral total RNA failed to yield an FP receptor mRNA amplification product at 35 cycles of PCR. The failure to see a product was not attributed to failed RT-PCR as amplification of G3PDH mRNA was successful as was amplification of FP receptor mRNA from human myometrial total RNA. In order to determine if in fact FP receptor mRNA was expressed in

Table 3. Survey of human prostanoid receptor mRNA distribution in a variety of human ocular tissues

Prostanoid Receptor	Iris	Ciliary Body	Choroid	Retina	Optic Nerve	Sclera	Cornea
DP	+	+	+	+	+	+	+
EP1	+	+	+	+	+	+	+
EP2	+	+	+	+	+	+	+
EP3	+	+	+	+	+	+	+
EP4	+	+	+	+	+	+	+
FP	+	+	+	+	+	+*	+
IP	+	+	+	+	+	+	+
TP	+	+	+	+	+	+	+

human scleral tissue, 50 cycles of amplification was performed in which case an FP receptor mRNA amplification product was detected. No amplification product was detected in negative control PCR reactions run at 50 cycles. Additionally, all negative control reactions failed to yield amplification products.

DISCUSSION

The purpose of this paper was two-fold: (1) to optimize an extraction protocol for isolation of intact high quality RNA from various human ocular tissues; and (2) to employ RT-PCR to assess the expression of mRNAs coding for all eight prostanoid receptors in a variety of human ocular tissues. In the past, isolation of RNA from human ocular tissues has been problematic due to variables such as rapid degradation, tissue composition, and melanin "contamination". Thus, an optimal extraction protocol to overcome these obstacles was required. One recent study described a preservation/isolation procedure whereby high quality RNA was obtained by first preserving human ocular globes in RNA*later*® Stabilization Reagent (Ambion, Austin, Texas), followed by the use of one of two commercially available RNA extraction kits, RNAqueous-4

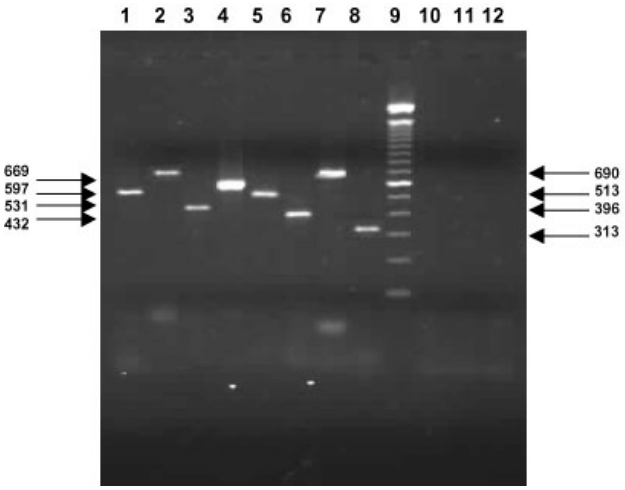

Figure 2. Survey of prostanoid receptor mRNA expression in human optic nerve tissue. Representative ethidium bromide staining of prostanoid receptor mRNA amplification products obtained from 1.0 µg of total RNA from human optic nerve tissue run at 35 cycles of PCR and resolved by electrophoresis. RT-PCR amplification products representing: Lane 1: DP receptor mRNA (531 bp); Lane 2: EP_1 receptor mRNA (669 bp); Lane 3: EP_2 receptor mRNA (432 bp); Lane 4: EP_3 receptor mRNA (597 bp); Lane 5: EP_4 receptor mRNA (513 bp); Lane 6: FP receptor mRNA (396 bp); Lane 7: IP receptor mRNA (690 bp); Lane 8: TP receptor mRNA (313 bp); Lane 9: 100 bp DNA molecular weight ladder; Lane 10: representative negative control where the RT reaction was carried out in the absence of reverse transcriptase enzyme; Lane 11: representative negative control where the RT reaction was carried out in the absence of total RNA; Lane 12: representative PCR amplification of 1.5 ng of human genomic DNA. Arrows mark the approximate position of each prostanoid receptor mRNA amplification product. Representative of 3 experiments.

PCR and ToTALLY RNA kit [23]. The advantage of this method is the flexibility it provides in scheduling tissue dissection time without concern for sacrificing RNA quality and quantity [23]. However, one potential draw back to this procedure is the potential "incompatability" that RNA*later*® may introduce. In other words, pre-treatment of whole globes and/or individual tissues with reagents such as RNA*later*® may not be feasible if tissues are shared amongst multiple researchers or used in multiple experimental procedures. Furthermore, RNA*later*® treated tissues cannot be processed with all commercially available RNA isolation kits, such as the Tri-Pure® Isolation Reagent (data not shown). In light of these observations, we decided to adopt an alternative method for preserving human ocular tissues prior to isolation of total RNA. Each donated globe was rapidly dissected on ice and each individual tissue was snap-frozen in liquid nitrogen as described in the methods section. Two commercially available extraction kits, the RNeasy® Midi Kit and the Tri-Pure® Isolation Reagent, were used to isolate high quality RNA. After comparing the quality and yield of total RNA isolated from the two different isolation methods (Table 2), we concluded that the different cellular composition of each ocular tissue ultimately dictated the methodology to be employed for the isolation of total RNA. Total RNA from tissues pigmented with melanin was best isolated using the RNeasy® kit, which uses a silica-based filter-binding procedure. A significant difference in RNA quality was seen with this procedure, in comparison to the Tri-Pure® Isolation Reagent; all traces of melanin contamination were removed. High quality RNA from tissues that possessed a high collagen and/or adipose content was easily obtained using the Tri-Pure® Isolation Reagent, which is a GITC based procedure. We found that the RNeasy® kit could not accommodate the fatty content of the optic nerve head and the high collagen content in both the cornea and sclera. Specifically, the spin column was clogged, thus preventing efficient binding of RNA to the RNeasy® membrane and therefore, significantly reducing yield. In light of these findings, the Tri-Pure® Isolation Reagent was used for the isolation of total RNA from optic nerve, sclera, and cornea and the RNeasy® Midi Kit was used for all remaining human ocular tissues.

Using RT-PCR, the presence of mRNA coding for all eight currently recognized prostanoid receptors was demonstrated in the iris, ciliary body, choroid, retina, optic nerve, sclera, and cornea. These results are consistent and expand on our earlier work detailing the molecular distribution of FP and TP receptor mRNA in various human ocular tissues [24]. The identity of prostanoid receptor mRNA amplification products was established by: (1) the size of the fragment stained by ethidium bromide, (2) comparison to the myometrial amplification product, and (3) restriction endonuclease digestion. By using primers annealing to two different exons, any artifactual amplification of contaminating DNA was precluded. This was also excluded by control experiments including performing the RT reaction in the absence of reverse transcriptase enzyme or total RNA and the PCR of human genomic DNA. It is tempting to speculate, based on the relative intensity of the bands, that the concentration of prostanoid receptor mRNAs present in

human ocular tissues is much lower compared to the myometrial control. Although previous studies have indicated low expression of prostanoid receptors in ocular tissues [25,26], future quantitative experiments are required to validate this observation before accurate statements regarding the relative abundance of ocular prostanoid receptor mRNAs in human ocular tissues can be made.

The molecular distribution of EP_1, EP_2, EP_4, FP, and TP receptors in human iris, ciliary body, choroidal, and retinal tissue are similar, consistent, and complementary to previously published studies. The presence of EP_2, EP_4, and FP receptors was first demonstrated both at the molecular and pharmacological level, in human nonpigmented ciliary epithelium and ciliary muscle cell lines [27]. Secondly, EP_4 and FP receptors were found on human lens epithelial cells both at the molecular and pharmacological level. By in situ hybridization, EP_1 and FP receptors were localized in the blood vessels of human iris, ciliary body, and choroidal tissue [28]. Furthermore, high levels of specific binding sites have also been found for $PGF_{2\alpha}$ and PGE_2in areas of the ciliary muscle and iris sphincter muscle and at a lower level in the iris epithelium and the retina, using an in vitro ligand-binding technique and autoradiography [29]. Additionally, using phosphor-imaging autoradiography, FP receptors were localized in different human ocular structures including iris sphincter muscle, longitudinal ciliary muscle and retina [30]. The presence of TP receptors in human corneal epithelium, the ciliary processes and retinal tissues using film autoradiography, liquid emulsion autoradiography and in situ hybridization have also been reported [31]. Although no previous work has demonstrated the distribution and localization of human ocular DP receptors, the presence of DP receptor mRNA on the human iris, ciliary body, and retina presented here is in agreement with the work by Gerashchenko et al., (1998) [32], who demonstrated the presence of DP receptor mRNA in rat ocular tissues. Additionally, we have provided the first report indicating the presence of IP receptor mRNA in a variety of human ocular tissues and the presence of DP, EP_1, EP_2, EP_3, EP_4, FP, and TP receptor mRNA in human sclera, optic nerve, and corneal tissue.

The precise role of prostanoid receptors in different human ocular tissues is purely speculative. However, based on the widespread ocular distribution of prostanoid receptor mRNAs, one can assume a large and diverse variety of functions within the eye. EP_1 receptors are reported to be involved in prostanoid-induced conjunctival pruritus and allergic conjunctival itching [33]. Recently, the EP_1 receptor agonist 17-phenyl-trinor-PGE_2 was shown to lower intraocular pressure in cats and rabbits [34]. It has been reported that PGE_2may have a partial role in modulating cellular DNA synthesis in low-level UVB exposed rabbit and human lens epithelial cells [35,36]. The work by Krauss et al., 1997 [37], suggested that stimulation of TP receptors in beagle dog eyes resulted in a marked decrease in IOP. Thus, TP receptor agonists were hypothesized to be potentially potent and efficacious hypotensive agents. The $PGF_{2\alpha}$ analogues latanoprost and travoprost have recently been introduced as novel anti-glaucoma therapies in clinical practice [38-40]. FP receptors have been localized on cultured retinal pigmented epithelial and ciliary muscle cells from cynomolgus monkey eyes, thereby suggesting that $PGF_{2\alpha}$ analogues decrease IOP by increasing uveoscleral outflow in both monkeys [25,41-43] and humans [14,44]. Additionally, recent studies demonstrate the presence of all prostanoid receptors in the human trabecular meshwork, suggesting a potential role for prostanoid receptor agonists and antagonist on the outflow of aqueous humor through the trabecular meshwork for the management of glaucoma [45]. There is increasing evidence in the literature to suggest that both PGE_2 and PGI_2 exhibit neuroprotective roles in vitro [46-49]. However, extensive research is still required both in vitro and in vivo to confirm this function.

Further biochemical, molecular and pharmacological studies are required in order to elucidate both mechanistic and functional details of ocular prostanoid receptors. Such knowledge may then provide significant insight to aid the design of new therapeutic agents for use in IOP reduction and/or novel treatment strategies for glaucoma such as neuroprotection and enhanced blood flow. Additionally, based on the widespread distribution of ocular prostanoid receptor mRNA and limited functional evidence, one might speculate a potential role for prostanoid-based medications in the treatment of ocular allergies and inflammation.

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