Localisation of thromboxane A₂ receptors and the corresponding mRNAs in human eye tissue

Zheng Chen, Shiv Prasad, Max Cynader

Abstract
Thromboxane A₂ (TxA₂) receptors in human eye sections were identified and localised using a potent TxA₂ specific agonist, 125I-iodinated 5-heptenoic acid 7-[3-[3-hydroxy-4-[4-(iodo-131I)phenoxyl]-1-butyl]-7-oxabicyclo[2.2.1]hept-2-yl]-[1S-[1a,2a,(Z),3B(1E,3S*),4a]-C23 H29 O5 (125I-BOP) in a binding assay. TxA₂ receptors were concentrated in several specific loci within ocular tissues, including the corneal epithelium, the ciliary processes, retina, and posterior ciliary arteries. In addition, we have used the method of in situ hybridisation to observe the distribution of TxA₂ receptor mRNA. The distributions of both receptor binding sites and receptor mRNAs showed a close correlation. These studies employed film autoradiography which does not permit cellular resolution. In order to obtain enhanced cellular resolution and more detailed information about the localisation of the receptors and their corresponding mRNAs, emulsion autoradiography was used after ligand binding and in situ hybridisation. This approach showed further that TxA₂ receptors are mainly concentrated on non-pigmented epithelial cells of the ciliary processes, on photoreceptors within the retina, and on endothelial cells of the posterior ciliary arteries. These results may be helpful for understanding the pathophysiological effects of TxA₂ in the human eye.

Department of Ophthalmology, University of British Columbia, Vancouver, Canada
Z Chen
S Prasad
M Cynader

Correspondence to:
Dr Zheng Chen, Department of Ophthalmology, 2550 Willow Street, Eye Care Center, University of British Columbia, Vancouver, BC, Canada V6Z 1N9.

IN VITRO LIGAND BINDING AND AUTORADIOGRAPHY
Human cadaveric eyes were obtained within 24 hours after death from the Eye Bank of British Columbia. The eyes from three different individuals were used: a 60-year-old woman who died of breast cancer, a 47-year-old man who died of a skull fracture, and a 44-year-old woman who died of liposarcoma. The eyes used in this study had no documented ocular diseases. They were frozen in isopentane cooled to −80°C, and stored at −20°C until used. Sections were cut on a cryostat (Cambridge Instruments, Nussloch, Germany) at a thickness of 20 µm, and mounted onto 1.7% gelatin subbed glass slides. For the ligand binding assay, sections were preincubated at room temperature for 60 minutes in 50 mM Tris HCl (pH 7.4) containing 100 mM sodium chloride, 3 mM calcium chloride, and 5% (w/v) bovine serum albumin (BSA). The sections were then incubated in the same buffer containing 1 nM of 125I-iodinated 5-heptenoic acid 7-[3-[3-hydroxy-4-[4-(iodo-131I)phenoxyl]-1-butyl]-7-oxabicyclo[2.2.1]hept-2-yl]-[1S-[1a,2a,(Z),3B(1E,3S*),4a]-C23 H29 O5 (125I-BOP, Cayman Chemical) for 90 minutes at room temperature, and washed at 4°C for 40 minutes in the same buffer with 1% BSA.

Autoradiography was performed by exposing the sections to tritium sensitive film (Hyper film–H: Amersham) for 7–10 days in the dark. Non-specific binding was determined on sections incubated under the same conditions as described above, but which contained in addition either 1–10 µM non-radioactive 1-BOP (TxA₂ agonist: Cayman Chemical), or U-46619 (TxA₂ agonist: Cayman Chemical), or SQ 27427.
Chen, Prasad, Cynader (TxA2 antagonist: kindly provided by Allergan Pharmaceuticals).

IN SITU HYBRIDISATION
The donor eyes from a 47-year-old man who died of tongue cancer and a 52-year-old woman who died of breast cancer were obtained 4-6 hours after the patients died. As soon as they were obtained, the eyes were put into diethyl pyrocarbonate (DEPC) treated tubes and frozen using 2-methylbutane at −80°C. They were then kept in a −80°C freezer until sectioning. For sectioning, the 16 μm thick sections were cut in an anterior posterior horizontal plane on a cryostat and mounted onto double subbed glass slides coated with 0.5% gelatin and 0.02% polylysine (Sigma). After air drying, sections were fixed for 20 minutes at room temperature with 4% paraformaldehyde, then rinsed in PBS and postfixed in 4% paraformaldehyde for 5 minutes, then rinsed in PBS again. The sections were dehydrated through a series of ascending concentrations of fresh ethanol (50% EtOH, 70% EtOH, 95% EtOH, 100% EtOH) for 5 minutes each, and finally sections were air dried up to 2 hours.

A complementary DNA probe encoding the TxA2 receptor (kindly supplied by Masakazu Hirata, Department of Pharmacology, Kyoto University Faculty of Medicine, Japan) from human placenta was used for hybridisation. In order to remove the vector DNA, the cDNA insert was gel purified from a 1% low melting

![Image](http://bjo.bmj.com/)

Figure 1  Autoradiographic views of ³²P-BOP binding sites in eye sections. Sections were incubated with 1 nM ³²P-BOP alone (A) or in the presence of 1 μM I-BOP (B), or with 1 μM unlabelled SQ27472 (C) to determine non-specific binding. In (A) increased optical density represents a greater number of receptors. The arrows illustrate dense concentrations of binding sites within the corneal epithelium, ciliary processes, posterior ciliary artery, and neural retina. Other zones of apparent concentration within the lens and choroid were not displaceable (B and C) using unlabelled competitors (BOP: (B) 1 μM unlabelled SQ27472: (C)). (D) Shows haematoxylin and eosin staining of the section whose autoradiogram is shown in (A). (CE, cornea epithelium; ISM, iris sphincter muscle; CP, ciliary processes; CM, ciliary muscle; L, lens; CH, choroid; NR, neurosensory retina; PCA, posterior ciliary artery.)
point agarose gel by restriction digestion with the enzymes that flank the cDNA insert. This insert was then labelled with [α-32P] dATP (NEB) by using the random primer labelling method of Feinberg and Vogelstein and unincorporated nucleotides were removed using push column purification (Stratagene).

The prehybridisation buffer consisted of 50% formamide, 25% PIPES (piperazine-N,N'-bis-[2-ethanesulphonic acid]), 5×Denhardt's solution (1×Denhardt's solution is 0.02% BSA, 0.02% Ficoll, and 0.02% polyvinyl pyrrolidone), 1% sodium dodecyl sulphate (SDS), salmon sperm DNA (250 μg/ml), and tRNA (250 μg/ml). Prehybridisation was performed at 37°C for 1 hour with 200 μl of prehybridisation buffer for each eye section. Hybridisation was carried out in the same buffer with the addition of 10% dextran sulphate. For each eye section, 200 μl of buffer containing 5000 cpm/μl of labelled probe was used. Incubation was performed in a humid chamber at 37°C for 18 hours. Following hybridisation, sections were washed in 4×SSC (1×SSC: sodium chloride 150 mM; sodium citrate 15 mM) for 10 minutes at room temperature, 4×SSC for 30 minutes at 37°C and then 2×SSC, 1×SSC, 0.5×SSC for 45 minutes each at 37°C. The sections were then washed in 0.1×SSC for 10 minutes at room temperature, air dried and apposed to x ray film (Hyperfilm: Amersham) for 7 days in the dark.

For a negative control, adjacent sections mounted on separate slides were treated with RNase A (20–40 μg/ml) for 60 minutes at 37°C before hybridisation as described above.

Quantitative analysis of the distribution of performed using a computerised densitometry system (NIH Image: Macintosh). For all three eyes, the images of the sections were captured and the mean autoradiographic densities in different zones representing the ciliary processes, corneal epithelium, posterior ciliary arteries, and retina were measured respectively using the densitometric program and calibrated against the density of co-exposed 32P-plastic standards (125I Micro-scales, Amersham). This enabled us to compare directly the density of images from different films and also to convert grey scales into values of radioactivity per milligram tissue.

**Table 1 Binding densities of 125I-BOP in eye tissues (fmol/mg tissue)**

<table>
<thead>
<tr>
<th>Eye tissues</th>
<th>M (SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ciliary processes</td>
<td>0.12 (0.03)</td>
</tr>
<tr>
<td>Corneal epithelium</td>
<td>0.06 (0.02)</td>
</tr>
<tr>
<td>Posterior ciliary arteries</td>
<td>0.07 (0.02)</td>
</tr>
<tr>
<td>Retina</td>
<td>0.03 (0.01)</td>
</tr>
</tbody>
</table>

**Results**

Representative autoradiograms illustrating the distribution of 125I-BOP binding sites in the human eye are shown in Figure 1. Quantitative densitometric analyses showed that the highest densities of specific binding sites were located in the corneal epithelium, in the ciliary processes, in blood vessels surrounding the optic nerve, and in the retina (Table 1, Fig 1A). The density of binding was reduced dramatically in the presence of 1 μM non-radioactive BOP (Fig 1B), SQ27427 (Fig 1C), and U46619 (data not shown), indicating that specific labelling occurred in these structures. Although both the lens

**Figure 2A**

Autoradiographic visualisation of TxA2 receptor specific mRNA in the eye. Note the concentration of mRNA in the corneal epithelium, ciliary processes, and retina. Sections were hybridised with 5000 cpm/μl of 32P-labelled TxA2 receptor cDNA probes (A). The labelling disappeared after pretreatment with RNase (B).
and choroid showed moderate levels of signal, these were not displaceable and not significantly higher than those of the corresponding non-specific binding levels shown in Figure 1C.

In situ hybridisation investigations of the localisation of mRNAs coding for TxA₂ receptors were broadly consistent with the results obtained from the ligand binding assays (Fig 2A). Within the anterior segment, the highest density of hybridisation sites were found in the corneal epithelium and in the ciliary processes. Although hybridisation to blood vessels was not detected in the posterior segment of the eye, a high density of reaction product was seen in the retina.

Figure 3 illustrates the results of liquid emulsion autoradiography using ¹²⁵I-BOP as a ligand. The results at low power (not shown) were the same as those illustrated in Figure 1. The high magnification views of Figure 3 show that the ciliary process binding sites identified at low power in Figure 1 were concentrated on non-pigmented epithelial cells within the processes rather than on pigmented cells (Fig 3A, B). For the blood vessels at the optic nerve head, emulsion autoradiography showed that the binding was concentrated on the endothelial cells of the ciliary artery rather than on nearby smooth muscle cells (Fig 3C, D), and within the retina a high density of binding sites were seen only within the photoreceptor layer (Fig 3E, F).

**Discussion**

The localisation of TxA₂ receptors has been examined in human eye sections using film autoradiography, liquid emulsion autoradiography, and in situ hybridisation techniques. The results from the three different methods are similar, consistent, and complementary to each other.

In the autoradiographic binding studies, the

---

**Figure 3** Microautoradiograms of ¹²⁵I-BOP binding in anterior segment. Bright field (A) and dark field (B) views of the same section after incubation with 1 nM ¹²⁵I-BOP alone. Silver grains were located mainly on non-pigmented epithelial cells within the ciliary processes. In the posterior segment bright field (C) and dark field (D) autoradiograms show silver grains decorating the endothelial cells (E, F) of the ciliary artery. Note the absence of silver grains on the immediately adjacent smooth muscle cells (C). In the retina only the photoreceptor layer is labelled (F) and the ganglion cell layer, bipolar cell layer, and inner or outer plexiform layers illustrated in (E) are essentially unlabelled. (NPE, non-pigmented epithelium; PE, pigmented epithelium; EC, endothelial cells of artery; SMC, smooth muscle cells of artery; GCL, ganglion cell layer; BCL, bipolar cell layer; IPL, inner plexiform layer; OPL, outer plexiform layer; PL, photoreceptor layer.)
localisation of thromboxane A2 receptors and the corresponding mRNAs in human eye tissue

binding sites representing the TxA2 receptor were primarily localised to the corneal epithelium, to the ciliary processes of the ciliary body, to the retina, and to the vessels surrounding the optic nerve. These binding sites could be displaced using unlabelled I-BOP, U-46619 and SQ27427 attesting to the specificity of the binding in these structures. The film autoradiographic method suffers from limited spatial resolution and a relatively high noise/signal ratio. Its spatial resolution is limited to about 100 μm. The liquid emulsion method gives more detailed information about the location of binding sites which enable a more precise assessment of possible function of the TxA2 sites.

The localisation of the TxA2 receptor in human eye was examined not only at the protein level but also at the mRNA level. The distribution of mRNA encoding the TxA2 receptor, revealed by in situ hybridisation, showed a good correlation with that of the corresponding receptor distribution within the cornea, ciliary processes, and retina. The correlation was not exact, however. TxA2 receptor mRNA was not found concentrated within the posterior ciliary artery, on which the 125I-BOP showed a high density of binding, but the mRNA was highly concentrated within corneal epithelium cells which show comparable levels of TxA2 binding. These differences may be due to poor penetration of the various ligands in different ocular tissues, individual variation, or some unexplained variation in the localisation of receptors and their mRNAs. One possibility is that receptor turnover in the ciliary arteries is very slow, so there would be only a low concentration of mRNA at any given time, while receptors would be present in higher numbers. Conversely, receptor turnover in the corneal epithelial cells may be unusually rapid, so there may be relatively low numbers of receptors present at any given time, but the tissue might still show a high concentration of mRNA. The blood supply of the eye is derived from two separate systems, the retinal vessels and the uveal vessels. The posterior ciliary arteries blood to the uveal vessels and provide the blood supply to the choroid and retina. During the past decade, anterior ischaemic optic neuropathy has been recognised as a commonly seen clinical entity. Optic disc cupping and visual field defects are the cardinal features of glaucoma and low tension glaucoma. There is evidence that the optic nerve head changes are due to vascular disturbances in the anterior part of the optic nerve, and that the posterior ciliary arteries are the main source of blood supply to the posterior part of optic nerve. Thus, the present results, showing that TxA2 receptors are highly concentrated in the wall of the posterior ciliary arteries, suggest that this receptor system might be involved in the pathophysiological mechanism of glaucomatous optic neuropathy.

At a microscopic level, the binding sites were found in endothelial layers but under high power, the adjacent smooth muscle cells lining the posterior ciliary arteries were found not to contain many receptors. It has been established that many eicosanoids have powerful effects on the vasculature, causing vasodilatation or vasoconstriction was well as increased permeability in the eye. Most of them are potential vasodilators, whereas TxA2 causes vasoconstriction in practically all vascular beds. It has been shown that the level of TxA2 in plasma was increased in patients with essential hypertension as well as biosynthesis of TxA2 in spontaneously hypertensive rats. The selective TxA2 receptor antagonist BM13177 also can induce a strong decrease of arterial blood pressure with a maximum reduction of 21-6% in the third minute after intravenous injection. In the past few years, the study of TxA2 in vascular tissues has been based mainly on vascular smooth muscle constriction assays. There have been few direct binding studies examining the TxA2 receptor localisation on endothelial cells. Direct functional studies have been reported recently by Sung et al. They demonstrated the existence of TxA2 receptors in hypertensive membrane preparations of bovine pulmonary artery endothelial cells using a ligand binding assay with a potent TxA2 specific antagonist, 25I-PTA-OH. They found that U-46619 suppressed prostacyclin-PGI2 (the most potent vasodilator and platelet inhibitor) production in cultured endothelial cells. Furthermore, the suppression of PGI2 by U-46619 could be blocked by the TxA2 specific antagonists, I-PTA-OH or 25I-BOP. Thus, it has been hypothesised that the vasoconstriction effect of TxA2 may be caused by suppressing the release of vasodilators from vascular endothelial cells, resulting in an enhanced vasoconstriction of blood vessels. Our results indicating TxA2 receptor binding in endothelial cells of posterior ciliary artery support this hypothesis and may also help us to understand the pathological mechanism of glaucomatous optic neuropathy. Further studies in glaucomatous eyes with a focus on the optic nerve head and associated blood vessels are required to address the question of whether there are any changes in the TxA2 receptor binding sites in glaucomatous eyes.

The blood supply of the eye is derived from two separate systems, the retinal vessels and the uveal vessels. The posterior ciliary arteries blood to the uveal vessels and provide the blood supply to the choroid and retina. During the past decade, anterior ischaemic optic neuropathy has been recognised as a commonly seen clinical entity. Optic disc cupping and visual field defects are the cardinal features of glaucoma and low tension glaucoma. There is evidence that the optic nerve head changes are due to vascular disturbances in the anterior part of the optic nerve, and that the posterior ciliary arteries are the main source of blood supply to the posterior part of optic nerve. Thus, the present results, showing that TxA2 receptors are highly concentrated in the wall of the posterior ciliary arteries, suggest that this receptor system might be involved in the pathophysiological mechanism of glaucomatous optic neuropathy.

At a microscopic level, the binding sites were found in endothelial layers but under high power, the adjacent smooth muscle cells lining the posterior ciliary arteries were found not to contain many receptors. It has been established that many eicosanoids have powerful effects on the vasculature, causing vasodilatation or vasoconstriction was well as increased permeability in the eye. Most of them are potential vasodilators, whereas TxA2 causes vasoconstriction in practically all vascular beds. It has been shown that the level of TxA2 in plasma was increased in patients with essential hypertension as well as biosynthesis of TxA2 in spontaneously hypertensive rats. The selective TxA2 receptor antagonist BM13177 also can induce a strong decrease of arterial blood pressure with a maximum reduction of 21-6% in the third minute after intravenous injection. In the past few years, the study of TxA2 in vascular tissues has been based mainly on vascular smooth muscle constriction assays. There have been few direct binding studies examining the TxA2 receptor localisation on endothelial cells. Direct functional studies have been reported recently by Sung et al. They demonstrated the existence of TxA2 receptors in hypertensive membrane preparations of bovine pulmonary artery endothelial cells using a ligand binding assay with a potent TxA2 specific antagonist, 25I-PTA-OH. They found that U-46619 suppressed prostacyclin-PGI2 (the most potent vasodilator and platelet inhibitor) production in cultured endothelial cells. Furthermore, the suppression of PGI2 by U-46619 could be blocked by the TxA2 specific antagonists, I-PTA-OH or 25I-BOP. Thus, it has been hypothesised that the vasoconstriction effect of TxA2 may be caused by suppressing the release of vasodilators from vascular endothelial cells, resulting in an enhanced vasoconstriction of blood vessels. Our results indicating TxA2 receptor binding in endothelial cells of posterior ciliary artery support this hypothesis and may also help us to understand the pathological mechanism of glaucomatous optic neuropathy. Further studies in glaucomatous eyes with a focus on the optic nerve head and associated blood vessels are required to address the question of whether there are any changes in the TxA2 receptor binding sites in glaucomatous eyes.

The blood supply of the eye is derived from two separate systems, the retinal vessels and the uveal vessels. The posterior ciliary arteries blood to the uveal vessels and provide the blood supply to the choroid and retina. During the past decade, anterior ischaemic optic neuropathy has been recognised as a commonly seen clinical entity. Optic disc cupping and visual field defects are the cardinal features of glaucoma and low tension glaucoma. There is evidence that the optic nerve head changes are due to vascular disturbances in the anterior part of the optic nerve, and that the posterior ciliary arteries are the main source of blood supply to the posterior part of optic nerve. Thus, the present results, showing that TxA2 receptors are highly concentrated in the wall of the posterior ciliary arteries, suggest that this receptor system might be involved in the pathophysiological mechanism of glaucomatous optic neuropathy.
TxA₂ acts as an agonist at a specific, G protein coupled surface receptor which is linked to phospholipase C. Activation therefore results in a rise of IP₃ and diacylglycerol and consequent increase in intracellular free calcium concentration. Since both Ca²⁺ and GTP are involved in signal transduction processes of photoreceptors, our results, which show that TxA₂ receptors are concentrated in retinal photoreceptors, imply a possible and rather unexpected role of TxA₂ receptors in the phototransduction process. The exact pathophysiological implications and mechanisms of this process need to be explored further.

Localisation of thromboxane A2 receptors and the corresponding mRNAs in human eye tissue.
Z Chen, S Prasad and M Cynader

*Br J Ophthalmol* 1994 78: 921-926
doi: 10.1136/bjo.78.12.921

Updated information and services can be found at:
http://bjo.bmj.com/content/78/12/921

**Email alerting service**

Receive free email alerts when new articles cite this article. Sign up in the box at the top right corner of the online article.

**Notes**

To request permissions go to:
http://group.bmj.com/group/rights-licensing/permissions

To order reprints go to:
http://journals.bmj.com/cgi/reprintform

To subscribe to BMJ go to:
http://group.bmj.com/subscribe/