β-Adrenergic Receptors in Human Trabecular Meshwork
Identification and Autoradiographic Localization

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β-adrenergic receptors were identified in slide-mounted sections of human trabecular meshwork by in-vitro labeling, light microscopic autoradiography. Autoradiograms were generated after incubation of slide-mounted tissue sections with 125I-cyanopindolol, a selective high-affinity probe for β-adrenergic receptors. Experiments in which an excess of either unlabeled Practolol (β1 selective ligand) or Zinterol (β2 selective ligand) were included suggested that most of the receptors were of the β2 subtype. Data from displacement studies using increasing concentrations of the highly specific β1- and β2-adrenergic receptor antagonists, ICI-89,406 and ICI-118,551, respectively, confirmed the predominance of the β2-adrenergic receptors. Similar results obtained with cultured trabecular endothelial cells suggest that the β-adrenergic receptors may be associated with the endothelial cells of the trabecular meshwork in vivo. These results provide anatomic evidence for the hypothesis that β-adrenergic agents improve outflow by direct action on the trabecular meshwork and provide a rationale for the development of more selective β2-adrenergic agents to increase outflow facility. Invest Ophthalmol Vis Sci 28:772-779, 1987

The pharmacologic complexities of the aqueous inflow and outflow mechanisms are exemplified by epinephrine, an adrenergic agent long used as a mainstay of glaucoma treatment. Experimental observations have supported the concept that epinephrine may act both by decreasing aqueous production1 and by increasing aqueous outflow,2 although its long-term lowering effect on intraocular pressure is thought to result from the latter mechanism.3 Reports have cited both the trabecular meshwork4,5 and the pressure-insensitive uveoscleral outflow pathways6 as target areas for the drug’s effect on outflow facility. Experiments in which the ciliary muscle is retrodisplaced suggest that the epinephrine-induced increase in outflow facility is not mediated by iris or ciliary muscle.7 Conflicting evidence exists as to whether epinephrine’s effect on outflow is an α-adrenergic8 or β-adrenergic9 effect, and may, in fact, differ among species.9

Adrenergic receptors in the anterior segment of the eye of several species have been studied extensively by a variety of techniques. In-vitro ligand binding studies, in which a radiolabeled drug is incubated with a particulate preparation of homogenized tissue, have been valuable in demonstrating β2-adrenergic and β1-adrenergic receptors in rabbit iris-ciliary body preparations,10-12 β2 receptors in rabbit and primate ciliary processes,13 human iris-ciliary body,14 and preparations of nonpigmented bovine ciliary epithelium.15 In-vivo pharmacology has been used to demonstrate a preponderance of β2-adrenergic receptors in the cat anterior segment.16 The evaluation of drug effects on cultured human trabecular cells revealed that they contain high-affinity β2-adrenergic receptors.17,18

The above-mentioned techniques yield valuable information about the presence of various types of receptors and their pharmacologic profiles. Unfortunately, they have not readily yielded information about the precise location of adrenergic receptors in the tissues responsible for aqueous outflow. To answer this question, a morphologic technique is required. Kramer et al,19 for example, used an in-vivo perfusion method to localize catecholamines in the cat uveal tract by fluorescent labeling. Light microscopic autoradiography, however, is even better suited for this task because it allows for anatomic resolution in the micron range and provides a much greater sensitivity in receptor detection than do tissue homogenate techniques.20 Lehto et al21

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used $^3$H-dihydroalprenolol to localize β-adrenergic receptors in the epithelium of the cornea and ciliary processes by autoradiography. In the present study we have employed the technique of light microscopic autoradiography\textsuperscript{22} to identify, characterize, and visualize adrenergic receptors in human trabecular meshwork. Our report provides the first visualization of β2-adrenergic receptors in human trabecular meshwork. These data provide further insight into intrinsic adrenergic influences on intraocular pressure and into the mechanism of action of the widely used, but incompletely understood, adrenergic group of antiglaucoma medications. They also provide a rationale for the development of more selective β2-adrenergic agents to increase outflow facility.

**Materials and Methods**

**Tissue Preparation**

Three human cadaver corneoscleral rims were obtained after corneal buttons had been removed for transplantation and were stored in McCarey-Kaufman medium until ready for use. Radial segments containing sclera, cornea, conjunctiva, trabecular meshwork, and remnants of ciliary body were dissected under a dissecting microscope. In some cases a 6-0 prolene suture was threaded into Schlemm’s canal to serve as a marker for the trabecular meshwork area. The segments of the anterior chamber tissue were frozen without fixation at $-70^\circ$C until ready for further processing.

To assay cultured human trabecular endothelial cells for β-adrenergic receptors, passaged endothelial cells were obtained as described elsewhere.\textsuperscript{23} To confirm the identity of the cultured cells, indirect immunofluorescence assays for fibronectin and laminin were performed on fourth passage human trabecular cells and on cultured human scleral fibrocytes. Cell monolayers were washed with calcium- and magnesium-free, phosphate-buffered saline (PBS) and incubated with one of two primary antibodies at $20^\circ$C for 30 min: antihuman fibronectin (rabbit IgG, 1:10 dilution) or antihuman laminin (sheep IgG, 1:10 dilution). (Antibodies were the gift of Drs. A. Tyl Hewitt and Hilda Kleinman.) The samples were washed in PBS, and then incubated with fluorescein-conjugated secondary antibody: for fibronectin, goat antirabbit IgG (1:20 dilution), and for laminin, rabbit antiseep IgG (1:20 dilution) (Cooper Biomedical; Malvern, PA). Samples were then fixed in 2% paraformaldehyde for 45 min, air dried, and mounted in glycerol-PBS. In control experiments, antiserum that was preincubated for 4 hr at $4^\circ$C with the antigen that it was raised against was substituted for the primary antiserum. A Nikon fluorescence microscope was used to examine and photograph the samples.

Cells were harvested from the culture dish and centrifuged at 1,000 g for 10 min to produce a pellet. The pellet was frozen at $-70^\circ$C until ready for use.

Four radial segments from each of three corneoscleral rims, and the pellet of cultured trabecular cells, were separately embedded in a mixture containing equal parts of homogenized bovine brain and Tissue Tek 2® and sectioned (8 μm) using a microtome (Harri; North Billerica, MA) at $-16^\circ$C. The sections were thaw-mounted onto chrome alum/gelatin subbed microscope slides and stored at $-20^\circ$C until use.

**β-Adrenergic Receptor Labeling**

$^{125}$I-cyanopindolol ($^{125}$ICYP) was used to label β-adrenergic binding sites.\textsuperscript{24} Slide-mounted sections were brought to room temperature and incubated for 70 min in buffer (20 mM Tris HC1/135 mM NaCl; pH, 7.4) containing 50–100 pM $^{125}$ICYP alone, or in the presence of 10 μM DL-propranolol or 100 μM (-)-isoproterenol to determine the nonspecific binding. Coincubations of 100 pM $^{125}$ICYP with 2 μM Zinterol and 2 μM Practolol were carried out in order to compete selectively with $^{125}$ICYP binding to β2 and β1-adrenergic receptors, respectively. These conditions previously had been demonstrated\textsuperscript{25,26} to label β1- and β2-adrenergic receptors selectively in slide-mounted tissue sections.

In another experiment, coincubation of 100 pM $^{125}$ICYP with varying concentrations of ICI-89,406, a selective β1-adrenergic receptor antagonist, or ICI-118,551, a selective β2-adrenergic antagonist, was performed. These ligands previously have been demonstrated to be highly specific for β1- and β2-adrenergic receptors in slide-mounted tissue sections.\textsuperscript{24} After incubation, the tissue sections were washed for three consecutive 20-min periods in buffer at $4^\circ$C, dipped in a deionized water rinse, and dried rapidly under a stream of cold, dry air. All experiments were carried out under subdued lighting conditions.

**Autoradiography**

The autoradiograms were generated by apposition of glass slides containing sections of anterior segment to Kodak NTB-3 (Rochester, NY) emulsion-coated glass coverslips.\textsuperscript{22} In this technique, the coverslip is glued at one end to the slide containing the tissue section, and is otherwise held against the tissue with number 20 binder clips. After approximately 7 days, the binder clips are removed, and the coverslip is bent away from the slide. Because the coverslip and the slide remained glued at one end, the emulsion on the coverslip can be developed, and the tissue on the glass slide separately stained with toluidine blue, while keeping the autoradiogram in register with the stained tissue sec-
Fig. 1. Autoradiographic localization of 125I-cyanopindolol binding sites in human anterior segment. (A) Bright-field photomicrograph showing the histology of the conjunctival epithelium (arrow), sclera (S), and trabecular meshwork (double arrowheads). (B) Dark-field photomicrograph showing the total autoradiographic grain distribution over the same anterior segment tissue section. In dark-field illumination, the cluster of autoradiographic grains appear as white areas, and the tissue is not visible. Thus, the brightest areas have the highest concentration of binding sites. The highest concentration of specific 125I-cyanopindolol binding sites are found in the conjunctival and corneal epithelium and the trabecular meshwork, with no specific binding present in the sclera. (C) Dark-field photomicrograph showing the absence of specific binding in an adjacent serial section when 10 nM DL-propranolol was included in the incubation buffer. (Bar = 500 μm.)

Results

Histology

Standard light microscopy of hematoxylin–eosin-stained frozen sections was performed first to confirm that the tissue sectioned included readily identifiable portions of the anterior chamber angle. In some cases a 6-0 proline suture had been passed into Schlemm's canal to aid in identification of the trabecular meshwork region. Although the suture was lost in the staining process, it clearly marked Schlemm's canal by dilating its lumen.

Identification of β-Adrenergic Receptors

Low-power observation under dark-field illumination of the autoradiogram of the entire tissue section revealed a high level of specific binding sites for 125I-CYP in the epithelium of the cornea and conjunctiva and in the trabecular meshwork (Figs. 1A, B). Incubation with an excess of unlabeled DL-propranolol (10 μM) left behind only the silver grains representing nonspecific binding, which was very low in all cases (Fig. 1C). Virtually no binding sites were found in the sclera.

β-Adrenergic Receptor Subtypes

β-adrenergic receptor subtypes were explored in two separate sets of experiments. In the first experiments the β₁-adrenergic receptor selective agent, Practolol, and the β₂ selective agent, Zinterol, were employed to probe for β₁ and β₂ selective binding sites. When these ligands are included in an incubation along with 125I-CYP, the amount the grain count is reduced is related to the concentration of β₁- and β₂-adrenergic binding sites, respectively. The histology of the anterior segment tissues corresponding to the autoradiograms is shown in Figure 2A. A comparison of Figures 2B, D demonstrates the marked reduction of grain counts when Zinterol is included in the incubation medium. This
Fig. 2. Autoradiographic characterization of β-adrenergic receptors in the human trabecular meshwork. All sections were incubated with 50 pM 125I-cyanopindolol ([125I-CYP) in the absence (B) or presence of 2 μM Practolol (C), 2 μM Zinterol (D), or 10 μM DL-propranolol (E). (A) Bright-field photomicrograph showing the histology of the trabecular meshwork. (B-E) Dark-field photomicrographs showing the distribution of 125I-CYP binding sites in adjacent sections incubated under the conditions described previously. Note that the selective β-adrenergic receptor antagonist, Practolol, does not displace any of the 125I-CYP binding (C), whereas an identical concentration of the β2-adrenergic receptor agonist, Zinterol (D), effectively reduces 125I-CYP binding to almost blank (E), concentrations. (Bar = 25 μm.)

Reduction in grain counts approaches the reduction observed when DL-propranolol, a nonsselective β-adrenergic receptor agent, was included in the incubation medium (Fig. 2E). When Practolol is included, on the other hand, no change in the number of grain counts is observed (Figs. 2B, C; Table 1). These findings suggest a preponderance of β2-over β1-adrenergic receptors in the trabecular meshwork.

To further confirm the above observation, a second experiment using the highly selective compounds ICI-118,551 and ICI-89,406 for β2- and β1-adrenergic receptors, respectively, was performed. Data from these competition experiments show that the selective β2-adrenergic receptor antagonist ICI-118,551 was 10- to 1000-fold more potent in reducing the specific grain count than the selective β1-adrenergic receptor antagonist ICI-89,406 (Table 1). The nonspecific binding was determined using isoproterenol, which has been shown to yield higher levels of nonspecific binding than propranolol.24

Table 1. Pharmacologic specificity of 125I-cyanopindolol ([125I-CYP) binding in human trabecular meshwork

<table>
<thead>
<tr>
<th>Study</th>
<th>Total (50 pM 125I-CYP)</th>
<th>Practolol (2 μM)</th>
<th>Zinterol (2 μM)</th>
<th>DL-propranolol (10 μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Study 1</td>
<td>218 ± 19</td>
<td>235 ± 12</td>
<td>27 ± 2</td>
<td>8 ± 3</td>
</tr>
<tr>
<td>Study 2</td>
<td>382 ± 37</td>
<td>323 ± 42</td>
<td>299 ± 34</td>
<td>224 ± 29</td>
</tr>
<tr>
<td></td>
<td>211 ± 24</td>
<td>201 ± 24</td>
<td>179 ± 13</td>
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</tbody>
</table>

Data represent silver grains per 500 μm². The mean counts were determined in a 570-μm² area from four representative sections of trabecular meshwork from each of three conncociliary rims (study 1) or from one fresh eye bank eye (study 2). In study 1, incubations were carried out in a medium containing 50 pM 125I-CYP with the addition of 2 μM Practolol or 2 μM Zinterol to displace a fraction of 125I-CYP that binds to β2- and β1-adrenergic receptors, respectively, and with the addition of 10 μM DL-propranolol to displace 125I-CYP binding from both β1- and β2-adrenergic receptors. In study 2, incubations were carried out in a medium containing 100 pM 125I-CYP with the addition of 100 nM and 1 μM ICI-89,401 or ICI-118,551 to displace a fraction of 125I-CYP that binds to β1- and β2-adrenergic receptors, respectively, and with the addition of 100 μM (-) isoproterenol to displace 125I-CYP binding from both β1- and β2-adrenergic receptors.

β-Adrenergic Receptors in Cultured Trabecular Endothelial Cells

Cultured cells were demonstrated to be trabecular endothelial cells by the following criteria: (1) light mi-
The microscopic appearance of a monolayer of closely packed, broad, flat cells with multiple cell processes (Fig. 3A); (2) immunofluorescent studies revealing fibronectin staining of cell margins (Fig. 3B) and association of laminin with the cells (Fig. 3C); and (3) electron microscopic ultrastructure. These criteria have been used previously by other authors to define cultured trabecular endothelial cells. Fibroblasts grown from adjacent sclera showed different morphologic characteristics (Fig. 3D) and did not stain for laminin.

Autoradiography of sections of centrifuged pellets of cultured trabecular endothelial cells revealed the presence of β-adrenergic receptors, predominantly of the β2 subtype, associated with the cells (Fig. 4).

**Discussion**

This study demonstrates the presence of β-adrenergic receptors, predominantly of the β2 subtype, in specimens of human trabecular meshwork. Although β-adrenergic receptors have previously been localized in rabbit iris and ciliary body tissue homogenates, they have not been definitively localized in the trabecular meshwork of any species.

Demonstration of the receptors was made possible with light microscopic autoradiography of mounted tissue sections. This technique uses dry (as opposed to wet) emulsion-coated coverslips, which greatly reduces the amount of artifact induced by diffusion of ligand.
In addition, the use of in-vitro, rather than in-vivo, techniques is distinctly advantageous because of the reduction of nonspecific binding by washing, because of its capability to examine different receptors or receptor subtypes in adjacent sections, and because it can be used on postmortem tissue, including that of humans. The use of a high specific activity iodinated ligand for the β-adrenergic receptor studies conveniently reduced the autoradiogram exposure time to 7–10 days.

The subtype of β-adrenergic receptor represented was determined in two sets of competition experiments. In the first, the tissue sections were coincubated with $^{125}$I-CYP, a ligand that binds with equal affinity to both $\beta_1$- and $\beta_2$-adrenergic receptors, and an excess of unlabeled Zinterol, a $\beta_2$-adrenergic agonist, or Practolol, a $\beta_1$-adrenergic antagonist. The finding that almost all of the radiolabeling was abolished by Zinterol and almost none by Practolol suggested that most of the $\beta_1$-adrenergic receptors were of the $\beta_2$ subtype. Because these experiments were hampered by the fact that only one concentration of each ligand was employed and because the selectivity of these ligands may not have been optimal, additional coincubation experiments were performed, using the most selective $\beta_1$- and $\beta_2$-adrenergic receptor agents available: ICI-89,406 and ICI-118,551, respectively. These highly specific ligands were used in varying concentrations to generate dose-
Fig. 4. Autoradiographic characterization of β-adrenergic receptors in slide-mounted sections of cultured trabecular endothelial cells. (A–D) Bright-field photomicrographs of adjacent tissue sections incubated with 50 pM 125I-cyanopindolol (125I-CYP) in the absence (A) or presence of 2 µM Practolol (B), 2 µM Zinterol (C), or 10 µM DL-Propranolol (D). In these bright-field photomicrographs the focus is on the autoradiographic grains, and the darkly stained endothelial cell nuclei are also visible. Note the high concentration of grains (A) (total) and the inability of the selective β1-adrenergic receptor antagonist, Practolol (B), to displace 125I-CYP binding. In contrast, the β2-adrenergic receptor agonist Zinterol (C) effectively decreases 125I-CYP binding almost to blank (D) concentrations. (Bar = 10 μm.)

response data. ICI-118,551 appears to be more potent at displacing 125I-CYP-specific grain counts than ICI-89,406 by at least one—and perhaps three—orders of magnitude, again implying a preponderance of β2-adrenergic receptors.

Experiments with cultured trabecular endothelial cells confirmed the presence of β-adrenergic receptors, predominantly of the β2 subtype, in the trabecular meshwork. Although the relationship between the binding characteristics of the cultured cells and trabecular endothelial cells in vivo is unknown, these experiments suggest that at least a portion of the β2-adrenergic receptors in the trabecular meshwork is associated with the endothelial cells. The association of β2-adrenergic receptors with endothelial cells has been demonstrated previously in human umbilical cord vein, feline and rat cerebral microvessels, and cultured bovine aortic endothelial cells. Homogenates of both fresh and cultured bovine corneal endothelium contain β2-adrenergic receptors. One can speculate that some function of the trabecular endothelial cells is influenced by the binding of adrenergic agents to these receptors. Supporting this concept is the demonstration by Polansky et al. that cultured trabecular endothelial cells can respond to catecholamines with a rise in cyclic adenosine monophosphate.

Limitations in our technique require that certain caution be used in interpreting the data. First, it must be noted that the data is semiquantitative, in part because a series of density standards for autoradiography was not employed. These standards are necessary to define the nonlinear relationship between grain counts and radioactivity. One cannot assign precise relative ratios of the proportion β2 to β1-adrenergic receptors, although β2 receptors appear to be the vastly predominant subtype. Secondly, the relatively few concentrations of β-adrenergic agents used in the dose response data compares unfavorably with the many points usually displayed in dose–response curves from experiments employing tissue homogenates. Thus we were not able to determine the relative affinities of the receptors. One of the drawbacks of autoradiography, especially on coverslips, is the technical difficulty of obtaining large numbers of data points. Nevertheless, our data does provide convincing semiquantitative evidence, not available at this time with other techniques.
for the presence of β-adrenergic receptors, predominantly of the β2 subtype, in the intact trabecular meshwork.

The data presented in this article clearly demonstrate the presence of β2-adrenergic receptors in the human trabecular meshwork. Localization of adrenergic receptors is of potential importance in designing new drugs for the treatment of glaucoma. It would be of interest to determine if receptor concentration is altered in glaucoma or with aging. Preferential localization of receptors to regions of the trabecular meshwork, such as along Schlemm’s canal or in the juxtacanalicular meshwork also would be interesting. Through further experiments, light microscopic autoradiography of receptors may be able to provide answers to these questions.

**Key words:** autoradiography, β-adrenergic receptors, cell culture, human, trabecular meshwork

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**References**