Characterization of β-Adrenergic Receptors in Cultured Human Trabecular Cells and in Human Trabecular Meshwork

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The characterization of β-adrenergic receptors on cultured human trabecular cells and trabecular meshwork from human autopsy eyes was carried out by radioligand binding utilizing (125I)-iodopindolol. In cultured cells, the observed binding of (125I)-iodopindolol was of high affinity (Kd = 43 pM) and saturable. Scatchard plots were linear and revealed a Bmax of 33 ± 7 fmol/mg of protein. Competition studies with a series of agonists and antagonists revealed that human trabecular cells contain a single class of β-adrenergic receptors of the β2 subtype. Similarly, the IC50 of ICI 89,406 (176 nM) in human trabecular meshwork from autopsy eyes supports the presence of β2-adrenergic receptors in this tissue. Invest Ophthalmol Vis Sci 30:51-57, 1989

The trabecular meshwork is the primary structure through which aqueous humor must flow during its egress from the anterior chamber of the eye. It is composed of branching, interlacing collagenous beams, each enclosed by a layer of endothelial cells. The trabecular meshwork is one of several structures in the anterior chamber angle through which filtration may occur. These include the juxtacanalicular tissue, the canal of Schlemm, and the efferent venous collector channels. Impaired drainage of aqueous humor through these structures is thought to underlie the primary pathophysiologic abnormality in open-angle glaucoma, namely, diminished outflow facility of aqueous humor from the anterior chamber.1-2 Only limited success has been achieved in finding histologic or morphologic changes in the trabecular meshwork or its adjacent structures that would account for the alterations in outflow of aqueous humor that accompany glaucoma. The possibility that changes in the mucopolysaccharide composition of the trabecular meshwork, or in the mechanism by which endothelial pinocytotic vesicles apparently transport materials (including aqueous humor) into the canal of Schlemm, has been studied in an effort to explain the reduced outflow of aqueous humor, hence elevated intraocular pressure, in glaucoma.

The trabecular meshwork/filtration apparatus is also thought to be the site at which pharmacologic agents act to improve the outflow facility of the eye. Epinephrine, used for the treatment of glaucoma since 1894,4 is thought to improve aqueous outflow although the precise mechanism that underlies this effect is not known.5-7 Specifically, it remains unclear whether this effect is mediated primarily by α-adrenergic5-8-12 or β-adrenergic receptors.13-14 These discrepancies may be partially due to species variability. It has been suggested that in rabbits, both α- and β-adrenergic agonists increase outflow facility, whereas in humans and monkeys there is no apparent effect from stimulation of α-adrenergic receptors although an increase does occur after stimulation of β-adrenergic receptors.6,7,14

Further understanding of the pharmacologic properties and characteristics of the trabecular meshwork has come from results of the in vitro study of human trabecular cells.15-17 Trabecular cells obtained from humans and grown in primary culture have been used to study changes in intracellular cAMP production in response to catecholamines.15 Furthermore, possible morphologic alterations have been observed in these cells in response to prolonged administration of epinephrine.18 These functional effects are thought to be initiated following occupancy of β-adrenergic receptors by epinephrine on the trabecular cells.

We have characterized the pharmacologic properties of the β-adrenergic receptors of human trabecular cells grown in culture, and have compared their prop-

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Fig. 1. Photomicrograph of the trabecular meshwork region of a human eye fixed 24 hr postmortem shows the region from which trabecular tissue was obtained. After iris was removed from its insertion, gentle scraping was performed to obtain the tissue from Schwalbe’s line to the scleral spur (between the black arrows), and medial to the inner wall of the canal of Schlemm (SC) (×50).

erties to those observed in studies of trabecular meshwork tissue that was obtained from human eyes shortly after death. Our findings indicate that the β-adrenergic receptors of human trabecular cells in vitro and in tissue from the trabecular meshwork in vivo are characterized by the presence of β-adrenergic receptors of the β2 subtype.

Materials and Methods

Tissue Preparation

Freshly enucleated eyes from human donors (ages 56 to 75) were obtained within 24 hr of death due to cardiorespiratory failure and were dissected on ice in tissue buffer (20 mM Hepes buffer, pH 7.5, containing 145 mM NaCl). The pars plana was incised 4 mm posterior to the corneascleral limbus, and the anterior segment was removed intact by surgical separation. After removal of the iris/ciliary body, the corneoscleral remnant, which included the trabecular meshwork, was scraped from the scleral spur to Schwalbe’s line and placed in tissue buffer for further processing. Sections of the corneoscleral/trabecular meshwork remnant were also sent for histopathological analysis to confirm the presence of trabecular meshwork (Fig. 1). Tissues from human eyes were pooled in tissue buffer, homogenized with a Brinkmann (Westbury, NY) Polytron (five times for 1 sec at setting 6-7), and centrifuged at 20,000 g. Membranes were resuspended in tissue buffer and stored frozen at −70°C until they were thawed for binding assays. Tissues from seven eyes were thawed and pooled for an additional wash at 20,000 g and resuspended in a suspension of 25% Percoll (Pharmacia Fine Chemicals, Uppsala, Sweden) in 20 mM Hepes buffer, pH 7.5, containing 145 mM NaCl. The Percoll suspension containing tissue was placed in a Beckman Polyallomer (Palo Alto, CA) centrifuge tube (16 x 76 mm) and centrifuged at 20,000 rpm in a 70.1 Ti rotor to form a self-generated isopycnic gradient. Centrifugation resulted in the separation of plasma membranes from the melanin pigment-containing pellet at the bottom of the tube. The properties of β-adrenergic receptors were then characterized in binding assays that used the radioligand (125I)-iodopindolol (125I-IPIN). (125I)-IPIN was prepared by a modification of the method of Barovsky and
Fig. 2. Human trabecular cells grown postconfluency as described in Methods (×100).

Brooker,20 as described by Wolfe and Harden,21 and used in binding assays to assess the properties of β-adrenergic receptors in human eye tissue as previously described.19

Cell Culture

Human trabecular meshwork cells were obtained from the eyes of a 30-year-old patient with no known ocular disease and grown for 2 to 4 weeks postconfluence. This was approximately 1 week longer than required to obtain optimally stable, morphologically differentiated monolayers.15"17 Fourth-passage cells from frozen stock were plated at 5000 cells/cm² in 100 × 15 mm dishes (Falcon Labware, Oxnard, CA), and grown in Dulbecco's modified Eagle's medium (Gibco Laboratories, Grand Island, NY) containing 2 mM glutamine, 15 μg/ml gentamycin, and 2.5 mg/ml Fungizone. Medium was changed every 2-3 days and was supplemented with 15% fetal calf serum (Sterile Systems Inc., Logan, UT) during the growth phase to confluency in an atmosphere of 10% CO₂-90% air at 37°C. In addition, 250 ng/ml of fibroblast growth factor (FGF) was added every other day until confluence was achieved. At confluence, cells were subsequently maintained in 10% fetal calf serum and grown in the absence of FGF for 10–14 days prior to harvest (Fig. 2). The medium was changed 18–24 hr prior to harvesting at which time cells were washed twice with phosphate-buffered saline and once with 20 mM Hepes buffer, pH 7.4, containing 145 mM NaCl. Cells were removed with a rubber policeman and centrifuged at 1500 g for 10 min. The supernatant was discarded and the pellet was stored at −70°C. Before use, cells were thawed and resuspended in 20 mM Hepes buffer, pH 7.4, containing 145 mM NaCl. Membranes were prepared by homogenizing the cells with a Brinkmann Polytron at setting 5-6 for 5 sec and centrifuging at 20,000 g for 15 min. The supernatant was discarded, and the membranes were resuspended in buffer as above prior to their addition to the binding assays.

Assay of β-Adrenergic Receptors

Properties of β-adrenergic receptors were assessed by adding aliquots of crude membranes to assay tubes containing, in a final volume of 0.25 ml, 12 mM Hepes buffer and 0.5% NaCl, bovine serum albumin (0.0004%) and ascorbic acid (1.1 mM). Assays were carried out in triplicate in the presence of 100 μM GTP. Specific binding was defined as the amount of ([125I]-IPIN bound in the absence of a competing ligand minus the amount bound in the presence of 100 μM (−)-isoproterenol. Equilibrium binding was performed for 20 min at 37°C in disposable polypropylene tubes (Walter Sarstedt, Princeton, NJ). Reactions were terminated by dilution with 10 ml of buffer (10
mM Tris HCl, pH 7.5, containing 154 mM NaCl) at room temperature. Samples were then filtered over Schleicher and Schuell (Keene, NH) glass-fiber filters (No. 30) on a millipore vacuum filter manifold. The filters were washed with an additional 10 ml of buffer at room temperature and dried with suction. Radioactivity was determined in an LKB 1274 or a Beckman 4000 gamma counter. Protein was determined by the method of Bradford using bovine serum albumin as a standard.

### Data Analysis

Saturation data were analyzed by the method of Scatchard to provide an estimate of the density of receptors and the affinity of the receptor (K_d) for (I^{125}I)-IPIN. Dose-response curves for the inhibition of the binding of (I^{125}I)-IPIN were analyzed by the computer-aided curve-fitting program NEWFIT-SITES on the NIH-sponsored PROPHET computer system. Using nonlinear regression analysis, the mathematical modeling program MLAB was used to identify the presence of one or two classes of binding sites by comparing the sum of squares of the residuals for one- and two-site models. A two-site model was considered to fit the data significantly better than a one-site model if an F test of the sum of squares of the residuals yielded a significance level of improvement of fit of $P < 0.001$. K_d values for the binding of a competing drug were calculated from the IC_{50} values using the method of Cheng and Prusoff.

### Results

#### Direct Binding of Cultured Human Trabecular Cells

Direct binding of (I^{125}I)-IPIN to membranes from cultured human trabecular cells was carried out with six to eight concentrations of (I^{125}I)-IPIN ranging from 10 to 400 pM. The observed binding of (I^{125}I)-IPIN was of high affinity (K_d = 43 ± 4 pM) and saturable (Fig. 3). Scatchard plots were linear and revealed a B_{max} of 33 ± 7 fmol/mg of protein.

#### Analysis of Indirect Binding

The ability of several agonists and antagonists to compete for binding sites for (I^{125}I)-IPIN was determined. The affinities of drugs were assessed by incubating homogenates of cultured human trabecular cells with 150 pM (I^{125}I)-IPIN in the presence of 16 to 20 concentrations of competing drug. The relative proportions of $\beta_1$- and $\beta_2$-adrenergic receptors were determined by computer-assisted analysis of dose-response curves resulting from the inhibition of the binding of (I^{125}I)-IPIN with 20 concentrations of the $\beta_1$-selective antagonist ICI 89,406 or the $\beta_2$-selective antagonist ICI 118,551. In the cultured trabecular meshwork cells, the inhibition curves were best explained by the presence of a single population of binding sites. ICI 118,551 was approximately 90-fold more potent in displacing (I^{125}I)-IPIN from the $\beta_2$-adrenergic receptors in human trabecular cells than was ICI 89,406 (Fig. 4). The observed order of potency for the inhibition of the binding of (I^{125}I)-IPIN in vitro by agonists was isoproterenol > epinephrine > norepinephrine (Fig. 5, Table 1).

A similar assay for (I^{125}I)-IPIN binding sites was performed in postmortem human trabecular tissue pooled from seven patients. The results obtained were similar to those found in vitro since the inhibition curve was best fit by a model that assumes the presence of one class of binding sites. The IC_{50} value of ICI 89,406 in displacing specifically bound (I^{125}I)-IPIN from postmortem human trabecular tissue was 176 nM and is similar to that obtained in the human trabecular cells grown in tissue culture (Fig. 6). Using the assumption that the affinity of the radioligand (I^{125}I)-IPIN is similar in both human trabecular tissue and cultured cells, we may estimate that the density of receptors in the human trabecular meshwork is approximately 70 fmol/mg.

### Discussion

The ability to propagate human trabecular cells in serial culture facilitates the study of receptor-mediated hormone interactions at the level of the cell membrane that cause subsequent biochemical responses in this tissue. The presence of saturable specific binding of (I^{125}I)-IPIN demonstrates the conservation of membrane-bound $\beta$-adrenergic receptors in broken cell preparations of human trabecular cells grown in tissue culture.

$\beta$-Adrenergic receptors have been classified into two subtypes based on the pharmacologic specificity of adrenergic receptor-mediated responses to selective drugs. Analysis of the subtypes of membrane-bound $\beta$-adrenergic receptors can be carried out by using radioligand binding assays in which selective antagonists compete for specifically bound (I^{125}I)-IPIN. In our experiments, $\beta$-adrenergic receptors on trabecular cells grown in culture had a high affinity...
Fig. 3. Density of β-adrenergic receptors in human trabecular cells. A representative saturation curve for binding of membranes of human trabecular cells grown in culture to (125I)-IPIN is shown. Total binding (●), specific binding (▲), and nonspecific binding (■) are shown in addition to the linear Scatchard transformation (inset).

for the β2-selective antagonist ICI 118,551, and a low affinity for the β1-selective antagonist ICI 89,406. This is consistent with the reported properties of the β2 subtype of the β-adrenergic receptor. The potency of ICI 89,406 at β-adrenergic receptors in human trabecular tissue from postmortem eyes was similar to that which we observed in vitro. Furthermore, in both tissues, only a single homogeneous class of binding sites was found.

Although considerably more efforts are necessary to define the mechanism(s) by which adrenergic drugs facilitate outflow, our findings provide some potentially useful information. Physiological studies in monkeys suggest that outflow facilitated by adrenergic agonists is mediated by β-adrenergic receptors since cyclic AMP was elevated in aqueous humor with agonist stimulation and the response was blocked by propranolol.6,7 Similarly, clinical investigation in humans suggested that timolol was able to block the ability of epinephrine to increase outflow facility.13 The use of the nonselective β-adrenergic antagonists propranolol and timolol, however, does not provide useful information concerning the β-adrenergic receptor subtype that mediates their ef-

Fig. 4. Inhibition of the binding of (125I)-IPIN by selective antagonists in membranes of cultured human trabecular cells. Assays were carried out with 100 pM (125I)-IPIN. The IC50 value and Hill coefficient were 3.5 nM and 0.94 for ICI 118,551, and 270 nM and 0.90 for ICI 89,406. The data are from a representative experiment performed in triplicate.

Fig. 5. Inhibition of the binding of (125I)-IPIN by selective agonists in membranes of cultured human trabecular cells. Assays were carried out with approximately 140 pM (125I)-IPIN. The IC50 values for isoproterenol (0.9 μM), epinephrine (4.7 μM), and norepinephrine (18.7 μM) are the means of triplicate determinations. The Hill coefficients for the agonists were each approximately 0.9. The data are from a representative experiment performed in triplicate.
Antagonists

Agonists

Isoproterenol nonselective 163 ± 20
Epinephrine nonselective 1165 ± 65
Norepinephrine $\beta_1$ 4780 ± 270

Table 1. Apparent equilibrium constants for drugs acting at $\beta$-adrenergic receptors in human trabecular cells

<table>
<thead>
<tr>
<th>Drug</th>
<th>Selectivity</th>
<th>$K_i \pm SEM$ (nM)</th>
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<tr>
<td>Antagonists</td>
<td></td>
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<tr>
<td>ICI 118,551</td>
<td>$\beta_2$</td>
<td>1.0 ± 0.1</td>
</tr>
<tr>
<td>ICI 89,406</td>
<td>$\beta_1$</td>
<td>91.0 ± 0.1</td>
</tr>
<tr>
<td>Agonists</td>
<td></td>
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<tr>
<td>Isoproterenol</td>
<td>nonselective</td>
<td>163 ± 20</td>
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<tr>
<td>Epinephrine</td>
<td>nonselective</td>
<td>1165 ± 65</td>
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<tr>
<td>Norepinephrine</td>
<td>$\beta_1$</td>
<td>4780 ± 270</td>
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Competition experiments for binding sites for (17$I$)-IPIN were performed as described in Materials and Methods. All assays were performed in the presence of 100 $\mu$M GTP and represent the average of at least two experiments performed in triplicate. The order of potency is characteristic of the $\beta_2$ subtype of the $\beta$-adrenergic receptor.

Fig. 6. Inhibition of the binding of (12$I$)-IPIN to $\beta$-adrenergic receptors by ICI 89,406 in human trabecular tissue obtained from postmortem eyes. Assays were carried out with approximately 70 pM of (12$I$)-IPIN. The observed $IC_50$ value of 176 nM represents the results of triplicate determinations from tissue pooled from seven eyes. The Hill coefficient was 1.07, and the data were best fit to a model suggesting the presence of a single class of binding sites.

Key words: trabecular cells, trabecular meshwork, $\beta$-adrenergic receptors, $^{125}$I-hodopindolol
References


