Influences on the density of $\beta$-adrenergic receptors in the cornea and iris–ciliary body of the rabbit

Arthur H. Neufeld, Kathleen A. Zawistowski, Ellen D. Page, and B. Britt Bromberg

By measurement of the specific binding of $^3$H-dihydroalprenolol, the densities of $\beta$-adrenergic receptors on membranes prepared from homogenized corneas and iris–ciliary bodies of rabbits were studied. Sympathetic denervation, as a result of subconjunctival treatment with 6-hydroxydopamine, causes an increase in the density of $\beta$-adrenergic receptors in membranes prepared from the ipsilateral iris–ciliary body but not the cornea. Topical treatment with epinephrine for 5 days causes a decrease in the density of $\beta$-adrenergic receptors in membranes prepared from cornea and iris–ciliary body, whereas similar treatment with timolol causes an increase in the density of $\beta$-adrenergic receptors. In the cornea, the decrease in receptor density that occurs following in vivo treatment with epinephrine is associated with a decreased ability to synthesize cyclic AMP, whereas the increase in receptor density that occurs following in vivo treatment with timolol is not associated with an altered ability to synthesize cyclic AMP. Our results indicate that the density of $\beta$-adrenergic receptors in the anterior segment of the eye is inversely related to the level of adrenergic stimulation to the tissue but that the ability of a tissue to synthesize cyclic AMP does not necessarily parallel the change in receptor density.

Key words: $\beta$-adrenergic receptor, catecholamines, timolol, dihydroalprenolol, cornea, iris, ciliary body, rabbit, cyclic AMP

Receptors located on the cell membrane provide an interface between an extracellular message and the response of a cell. For example, the $\beta$-adrenergic receptor, upon binding catecholamines such as epinephrine or norepinephrine, activates adenylate clase, which synthesizes cyclic AMP (adenosine 3',5'-monophosphate), the intracellular second messenger that has a profound influence on the physiology of almost every cell.1

In the anterior segment of the eye, several important functions are mediated via this pathway. In the cornea, catecholamines or cyclic AMP stimulates epithelial chloride transport,2–4 inhibits the mitotic rate of the epithelium,5 and suppresses the secretion of collagenase by the keratocytes.6 Cyclic AMP also influences intraocular pressure by increasing the outflow of aqueous humor7,8 and, perhaps, by altering inflow9 and the breakdown of the blood-aqueous barrier as well.10 In addition, evidence from tissues other than those in the eye indicates that the $\beta$-adrenergic–stimulated relaxation of smooth muscle—for example, the iris dilator
Fig. 1. Influences on the density of β-adrenergic receptors in the cornea. All values are the mean ± S.E.M. of the ratio of the density of receptors in the ipsilateral, experimental eye (EXP) to that in the contralateral, control eye (CONT). In vivo treatment of the experimental eye included subconjunctival 6-hydroxydopamine (6-HD), repeated topical epinephrine (EPI), and repeated topical timolol (TIM). Numbers in parentheses are the number of animals treated in each group.

Table I. Density of β-adrenergic receptors

<table>
<thead>
<tr>
<th>Tissue</th>
<th>No. of eyes</th>
<th>Density of β-adrenergic receptors (pmoles 3H-DALP/μg protein)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cornea</td>
<td>9</td>
<td>0.09 ± 0.01†</td>
</tr>
<tr>
<td>Iris-ciliary body</td>
<td>10</td>
<td>0.10 ± 0.01</td>
</tr>
</tbody>
</table>

*Binding of 3H-dihydroalprenolol (3H-DALP) that is specific for the β-adrenergic receptor.
†Mean ± S.E.M.

and vascular smooth muscle—is probably associated with elevation of the cyclic AMP level.11

Thus a key and primary component in the system, which may set the level of the cyclic AMP–mediated response, is the β-adrenergic receptor. This is the first point at which a cell can modulate its physiological response to catecholamine. Studies on cell membranes from diverse tissues indicate that the density of receptors for a hormone (such as insulin12) or a neurotransmitter (such as acetylcholine13) varies inversely with the chronic level of the humoral agent to which the tissue is exposed. In the central nervous system, destruction of nerve terminals and therefore the supply of norepinephrine, with 6-hydroxydopamine, causes an increase in the number of β-adrenergic receptors.14 We have recently demonstrated that adrenergic denervation or decentralization causes an increase in the number of β-adrenergic receptors in the iris-ciliary body of the rabbit.15 In the experiments described here, we determined the extent to which the density of β-adrenergic receptors and the ability to synthesize cyclic AMP in the anterior segment of the eye are influenced by adrenergic compounds that are relevant to the treatment of primary open-angle glaucoma.

Methods and materials

3H-Dihydroalprenolol (48.6 Ci/mmol) and the cyclic AMP radioimmunoassay kit were purchased from New England Nuclear; l-epinephrine, d-bitartrate, 6-hydroxydopamine, and propranolol were obtained from Sigma. 3-Isobutyl-1-methylxanthine was from Aldrich. Timolol HCl was kindly provided by Merck, Sharp and Dohme. All compounds used were reagent grade.

Treatment of animals. Three groups of male albino New Zealand rabbits, weighing 2 to 3 kg, were treated in the following manner.

6-Hydroxydopamine was injected subconjunctivally in two places in one eye of each animal. Each injection was 50 μl of 1% 6-hydroxydopamine, freshly made up in 2% sodium bisulfite in isotonic saline. The opposite eye received the vehicle only. One week later, the treatment was repeated. One to 2 weeks after the second set of injections, the animals were sacrificed, and the eyes were enucleated.

Two percent l-epinephrine, d-bitartrate in isotonic saline was applied topically to one eye of each rabbit twice a day for 5 days. At the end of this treatment, the animals were sacrificed, and the eyes were enucleated. In the experiments in which topical epinephrine was given to rabbits that had been pretreated with 6-hydroxydopamine, administration of epinephrine was started during the second week after the last 6-hydroxydopamine injection.

One percent timolol HCl in isotonic saline was applied topically to one eye of rabbits twice a day for 4 days. Upon completion of this treatment, some animals were sacrificed, and their eyes were enucleated on the last day of treatment; others...
were not treated for 2.5 days more and then were sacrificed, and the eyes were enucleated.

**Preparation of membranes.** Full-thickness corneas and iris–ciliary bodies were dissected from freshly enucleated eyes. Four corneas per assay were homogenized in a minimal volume of cold 0.25M sucrose, 1 mM MgCl₂, 5 mM Tris-HCl, pH 7.4, in a Polytron tissue homogenizer (Brinkmann Instruments). Four iris–ciliary bodies per assay were similarly homogenized. Further preparation of membranes from both tissues was as described previously.¹⁵

**Assay for β-adrenergic receptors.** β-Adrenergic receptors on the prepared membranes were assayed by the binding/displacement technique using ³H-dihydroalprenolol as originally described by Mukerjee et al.¹⁶ and modified by our laboratory.¹⁵ Specific binding is defined as the amount of ³H-dihydroalprenolol at 1.5 × 10⁻⁸M that can be displaced by propranolol at 5 × 10⁻⁵M. Proof of the specificity of the binding of ³H-dihydroalprenolol in this assay has appeared previously.¹⁵,¹⁷

**Assay for cyclic AMP.** The ability of a tissue to synthesize cyclic AMP in response to epinephrine was determined in vitro. As previously described,¹⁸ freshly excised tissues were preincubated and then incubated in media containing 5 × 10⁻⁴M 3-isobutyl-1-methylxanthine in the presence and absence of 10⁻⁵M epinephrine for 15 min. Following incubation, tissues were immediately homogenized in boiling 0.1N KOH, neutralized, and cooled. Cyclic AMP was determined on the supernatant by radioimmunoassay,¹⁹ and protein was determined on the pellet by the method of Lowry et al.,²⁰ with bovine serum albumin as the standard.

**Results**

**β-Adrenergic receptors.** Table I lists the density of β-adrenergic receptors, determined by the maximal amount of specific ³H-dihydroalprenolol binding per milligram of membranous protein, for the cornea and for the iris–ciliary body of rabbit. Preliminary observations indicated that the affinity of the receptors does not change with derervation or repeated epinephrine treatment; therefore no data are presented on dissociation constants.

**In the cornea.** Adrenergic denervation of the eye, either by superior cervical ganglionectomy or by subconjunctival treatment with 6-hydroxydopamine (Fig. 1), had no ap-

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Fig. 2. Influences on the density of β-adrenergic receptors in the iris-ciliary body. All values are the mean ± S.E.M. of the ratio of the density of receptors in the ipsilateral, experimental eye (EXP) to that in the contralateral, control eye (CONT). In vivo treatment of the experimental eye included subconjunctival 6-hydroxydopamine (6-HD), repeated topical epinephrine to eyes pretreated with 6-hydroxydopamine (6-HD + EPI), and repeated topical timolol (TIM). Numbers in parentheses are the number of animals treated in each group.

parent influence on the density of β-adrenergic receptors in the cornea. However, it should be pointed out that the assay employed is not sensitive enough to detect a change of less than 20% in the cornea.

As indicated in Fig. 1, membranes prepared from corneas of eyes treated in vivo with epinephrine showed a marked decrease of approximately 40% in the density of β-adrenergic receptors (p < 0.01 vs. 6-hydroxydopamine–treated animals). Also shown in Fig. 1 is the influence of timolol. Membranes prepared from corneas approximately 2.5 days after in vivo treatment of the eyes with timolol had ended had an increase of approximately 40% in the density of β-adrenergic receptors (p < 0.01 vs. 6-hydroxydopamine–treated animals). When membranes were prepared from corneas of timolol-treated eyes on the day that the drug treatment was terminated, a decreased num-

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The number of β-adrenergic receptors was measured (data not shown).

In the iris–ciliary body. Adrenergic de-
ervation of the eye, either by superior cervi-
ganglionection15 or by subconjunctival treatment with 6-hydroxydopamine (Fig. 2),
caused a small, perhaps 20%, increase in the
density of β-adrenergic receptors in the
iris–ciliary body (p < 0.05).

In vivo treatment with topical epinephrine
did not significantly decrease the density of
β-adrenergic receptors in the iris–ciliary
body of normal eyes (data not shown). How-
ever, as indicated in Fig. 2, when eyes were
first denervated by pretreatment with 6-hy-
droxydopamine, topical treatment in vivo
with epinephrine caused a significant reduc-
tion in the density of β-adrenergic receptors
in the iris–ciliary body when compared to the
denervated tissue (p < 0.05).

Fig. 2 also shows the influence of timolol
on the density of β-adrenergic receptors in
the iris–ciliary body. Membranes prepared
from this tissue approximately 2.5 days after
in vivo treatment of the eyes with timolol had
ended had a density of β-adrenergic recep-
tors that was similar to that of denervated
iris–ciliary body. When membranes were
prepared from iris–ciliary bodies of timolol-
treated eyes on the day that the drug treat-
ment was terminated, no significant change
in the density of β-adrenergic receptors in
the treated eyes was measurable (data not
shown).

Cyclic AMP in the cornea. Because the
cornea demonstrated the more marked
changes in the density of β-adrenergic recep-
tors in response to drug treatment, we
determined the ability of the cornea to syn-
thesize cyclic AMP following pharma-
ological manipulation of the density of the
receptors. Following the described in vivo
procedures, either repeated epinephrine
treatment or repeated timolol treatment plus
the 2.5 day hiatus, full-thickness corneas
were incubated in vitro in the presence and
absence of epinephrine. As shown in Fig. 3,
corneas from the eyes repeatedly treated in
vivo with epinephrine had a significantly de-
creased ability to make cyclic AMP in re-
sponse to epinephrine added to the incuba-
tion medium. However, as shown in Fig. 4, corneas from eyes repeatedly treated in vivo with timolol did not have an altered ability to make cyclic AMP in response to epinephrine added to the incubation medium, in spite of the increased density of receptors demonstrated above. Baseline levels of cyclic AMP, i.e., the level in the absence of exogenous epinephrine stimulation during the in vitro treatment, were similar under all conditions.

**Discussion**

Our findings demonstrate that the density of β-adrenergic receptors in a tissue is inversely related to the level of adrenergic stimulation to which the tissue is responding. This phenomenon is clearly seen in the cornea, when treatment with a β-adrenergic antagonist is compared to treatment with a β-adrenergic agonist, and in the iris-ciliary body, when denervation or treatment with a β-adrenergic antagonist is compared to treatment with an adrenergic agonist in a denervated eye.

Topical treatment with epinephrine exposes the cornea to a high pharmacological dose of this compound and the iris-ciliary body to a more attenuated dose. In the cornea, this treatment causes a marked decrease in the density of β-adrenergic receptors; however, a concurrent decrease does not occur in the iris-ciliary body. Nevertheless, the iris-ciliary body has the potential to exhibit the same phenomenon as the cornea. Prior treatment with 6-hydroxydopamine, which destroys the neuronal sites for uptake and inactivation of exogenous drug, leads to a decrease in the density of β-adrenergic receptors in this tissue when the denervated eye is treated with topical epinephrine. Treatment with topical epinephrine for longer periods of time than in our investigation or potentiation by the additional treatment with an inhibitor of neuronal uptake would perhaps also cause a decrease in the density of β-adrenergic receptors in the iris-ciliary body.

The cornea has a decreased ability to make cyclic AMP in response to epinephrine in vitro, once the receptor density has decreased following in vivo epinephrine treatment. This probably means that the kinetics for association between the stimulated β-adrenergic receptor and adenylate cyclase are less favorable because there are less receptors available in the membrane. Thus the tissue makes less cyclic AMP when stimulated with epinephrine, and perhaps the physiological or pharmacological response to epinephrine is also diminished.

Our findings of decreased binding of 3H-dihydroalprenolol to membranes prepared from eyes immediately after completion of timolol treatment are consistent with the findings reported for the dopamine receptor after haloperidol treatment. If these membrane preparations are prepared and assayed immediately, sufficient antagonist is probably still present at the receptor sites to interfere significantly with the assay. When a few days are allowed for the timolol to clear from the tissue, the increased number of β-adrenergic receptors becomes apparent. It is likely therefore that an increased number of β-adrenergic receptors (although inhibited) already exists on the day that the timolol is withdrawn.

By several days after the end of treatment with the potent β-adrenergic antagonist timolol, the density of β-adrenergic receptors in the cornea and iris-ciliary body has increased. Thus the cellular response to the presence of the antagonist and therefore the inability to perceive β-adrenergic stimulation is identical to that seen when the neurotransmitter is removed by treatment with 6-hydroxydopamine, at least for the iris-ciliary body. In effect, we produced the equivalent of denervation supersensitivity of the β-adrenergic receptors by administering a β-adrenergic antagonist. A similar finding has been reported for the dopamine receptors of the central nervous system after treatment with haloperidol, and Amer has suggested that such antagonist-induced changes in the cyclic AMP system are important in the mechanism of action of β-adrenergic antagonists for the treatment of systemic hypertension.

However, the results of our determinations of the ability of the corneas to synthesize cyclic AMP following repeated timolol

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treatment do not support Amer's hypothesis. The increased numbers of receptors in the membrane following timolol treatment apparently do not alter the kinetics of association between β-adrenergic receptors and adenylyl cyclase, probably because the amount of adenylyl cyclase is limiting. Thus the tissue does not make more cyclic AMP even though it has more β-adrenergic receptors. Therefore the increased number of receptors must be spare receptors, and perhaps, once timolol is withdrawn, the physiological response to epinephrine, mediated via cyclic AMP, will be unaltered.

The procedures that we have used to influence the density of β-adrenergic receptors are relevant to the medical therapy of primary, open-angle glaucoma. In patients with this disease, local treatment with 6-hydroxydopamine causes an enhanced sensitivity to topically applied epinephrine. Certainly the major effect is due to loss of the adrenergic terminals, which allows more epinephrine to be available. Any increased density of β-adrenergic receptors will probably soon be reversed with continued therapy with epinephrine; therefore changes in sensitivity occurring through the cyclic AMP pathway will not play a major role.

Topical epinephrine is widely used to reduce the intraocular pressure associated with primary, open-angle glaucoma. This drug, however, often becomes clinically less effective with prolonged use. Indeed, in rabbits, the ability of epinephrine to decrease intraocular pressure and increase cyclic AMP levels in the aqueous humor diminishes after 5 days of topical administration. Thus the loss of effectiveness of epinephrine may be due, at least in part, to a drug-induced decrease in the density of its own receptors, as we have shown here. In addition, when giving a drug with an action deep within the eye, one often discounts any effect on the cornea, particularly when no gross alterations are apparent. Our results indicate that topical epinephrine does influence the level of β-adrenergic receptors in the cornea in a manner that may compromise the ability of this tissue to respond to stress. Thus, to the extent that cyclic AMP regulates Cl⁻ transport, 4 mitotic rate, 5 and secretion of collagenase from cells in the cornea, the potential for stimulation of these responses via β-adrenergic input may be decreased in eyes treated with topical epinephrine.

Treatment with timolol 25-26 apparently does not provide the opposite possibility. Certainly, chronic use of timolol will cause the inhibition of important β-adrenergic pathways in the cornea. However, when topical therapy with timolol is withdrawn, tissues in the anterior segment apparently do not synthesize increased amounts of cyclic AMP, although there are more β-adrenergic receptors present. Thus a change in the density of β-adrenergic receptors is not always associated with a similar change in the next step of the pathway.

REFERENCES
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