Decreased $\beta$-adrenergic Responsiveness in Cornea and Iris-ciliary Body following Topical Timolol or Epinephrine in Albino and Pigmented Rabbits

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The ability of topical timolol and epinephrine to decrease the $\beta$-adrenergic responsiveness of the cornea and iris-ciliary body of albino and pigmented rabbits is reported. The time course of the decrease in responsiveness was determined by comparing in vitro, $\beta$-adrenergic stimulated cyclic AMP synthesis in these tissues from in vivo drug treated and contralateral, untreated eyes. In the cornea, loss of $\beta$-adrenergic responsiveness was maximal within the first hour after a single dose of topical 0.5% timolol and returned to control values within 3–6 hrs. In pigmented iris-ciliary body, however, a greater than 80% decrease of $\beta$-adrenergic responsiveness remained at 6 hrs after topical 0.5% timolol and a 60% decrease remained at 12 hrs. Repeated administration of 0.5% timolol, caused a decrease of $\beta$-adrenergic responsiveness in the iris-ciliary body that persisted for up to 24 hrs. Thus, the long duration of action of timolol observed in pigmented iris-ciliary bodies compared to the much shorter time course observed in nonpigmented tissue in previous studies indicate that pigment may act as a slow releasing depot for this drug. Topical epinephrine was found to decrease $\beta$-adrenergic responsiveness of both the cornea and iris-ciliary body. In albino rabbits, a single topical administration of 2% epinephrine decreased $\beta$-adrenergic responsiveness of the cornea by 42% and of the iris-ciliary body 39% 6 hrs after administration. Pretreatment with topical 1% timolol lessened the effect of epinephrine on the cornea but not on the iris-ciliary body. In pigmented rabbits, a single topical administration of 2% epinephrine desensitized the iris-ciliary body for up to 24 hrs. Repeated topical administration of 2% epinephrine twice-a-day for 7 days in albino rabbits decreased the $\beta$-adrenergic responsiveness of the iris-ciliary body for up to 72 hrs after the last drug treatment. The results of this study indicate that in addition to its high receptor affinity for $\beta$-adrenergic receptors, topical timolol may bind reversibly to ocular pigment, prolonging its bioavailability as a $\beta$-adrenergic antagonist. In addition, topical epinephrine has two time-related effects: initial $\beta$-adrenergic/adenylate cyclase stimulation followed by $\beta$-adrenergic/adenylate cyclase desensitization. We conclude that decreased $\beta$-adrenergic responsiveness, the common pharmacologic effect in the iris-ciliary body following either topical timolol or epinephrine, may be responsible for the decrease in aqueous humor formation observed in glaucoma patients using these medications. Invest Ophthalmol Vis Sci 24:718–724, 1983
Epinephrine is a mixed α- and β-adrenergic agonist that can lower IOP in humans. Glaucoma patients controlled with topical epinephrine routinely administer drops twice-a-day to control their elevated IOP. Two parameters of aqueous humor flow are affected by epinephrine: aqueous humor formation and facility of outflow. Topical administration of epinephrine causes a small transient increase in aqueous humor formation followed by a more protracted decrease and, either initially or after prolonged use, facility of outflow also increases. The decrease in formation and increase in facility are therapeutically advantageous to the glaucoma patients in that they result in a reduction of intraocular pressure.

Following exposure to agonist, several receptors or dependent adenylate-cyclases in a number of tissues have been reported to respond with a receptor-specific loss of sensitivity. The desensitization may involve a decrease in activity of adenylate cyclase enzymes or changes in receptor function. By measuring tissue responsiveness to β-adrenergic stimulation, we have determined the magnitude and the time course of the desensitization in cornea and iris-ciliary body following topical epinephrine.

Materials and Methods

New Zealand albino (2-3 kg) and pigmented (3-6 kg) rabbits were used. Experimental eyes were treated by either a single topical instillation of 40 μl of drug or 50 μl of drug followed 5 min later by another 50 μl of the same drug; control eyes were either treated with saline or untreated. All drugs were made up in saline immediately prior to use, and concentrations of drugs are expressed as percentage of base in solution.

Rabbits were killed by intravenous injection of sodium pentobarbital, and the anterior segments of both eyes were removed. Corneas and iris-ciliary bodies were carefully dissected, quartered, and placed in 10 ml buffer (100 mM NaCl, 20 mM NaH₂PO₄, 6.9 mM dextrose, 4.5 mM KCl, 0.8 mM MgCl₂, 0.4 mM CaCl₂) containing 0.56 mM indomethacin (2% indomethacin in ethyl alcohol; 1 ml/100 ml buffer).

When indicated, final concentrations of 5 × 10⁻⁴ M isobutylmethylxanthine (IBMX) and 10⁻⁵ M isoproterenol were used. Tissues were preincubated for 20 min in buffer and transferred to fresh buffer containing IBMX. Five minutes later isoproterenol was added, and the incubation continued for 15 min. Incubations were performed at 37°C in unassed buffer. Following incubation, tissues were placed in hot 0.1 N KOH (250 μl) homogenized, cooled, and neutralized with 0.1 N HCl (250 μl). Homogenates were centrifuged (2000 g) for 30 min at 4°C. Amounts of cyclic AMP were determined in the supernatant in duplicate by radioimmunoassay of nonacetylated samples. The pellet was solubilized in 1 M NaOH, and protein concentration was determined by the method of Lowry et al.

In Vivo Antagonism by Timolol

Single treatment: Pigmented rabbits were treated unilaterally with topical timolol (0.5%), and the animals were killed 1, 6, and 12 hrs later. Corneas and iris-ciliary bodies were removed, rapidly preincubated for 20 min, and transferred to fresh buffer containing IBMX. Five minutes later isoproterenol was added and incubation continued for 15 minutes. Cyclic AMP and protein were determined.

Repeated treatments: Albino rabbits were treated unilaterally with topical timolol (0.5%) twice-a-day for 7 days, pigmented rabbits were treated similarly for 4 days. Animals were killed at various times up to 24 hrs after the final topical treatment. The iris-ciliary bodies were removed and challenged in vitro with isoproterenol. Cyclic AMP and protein were determined.

In Vivo Desensitization by Epinephrine

Single treatment: Albino rabbits were treated unilaterally with epinephrine (0.5% or 2%); pigmented rabbits were treated only with 2% epinephrine. Other albino rabbits were pretreated topically with 1% timolol 0.5, or 3 hrs prior to topical instillation of 2% epinephrine. Six hours after treatment with epinephrine the animals were killed. The cornea and the iris-ciliary bodies were removed and challenged in vitro with isoproterenol. Cyclic AMP and protein were determined.

Repeated treatment: Albino rabbits were treated unilaterally twice-a-day with 2% epinephrine for 3.5 days; other albino rabbits were pretreated topically with either 0.5% or 1% timolol prior to topical instillation of 2% epinephrine. Six hours later the animals were killed, the tissues were challenged with isoproterenol and analyzed as described above.

Other albino rabbits were treated unilaterally twice-a-day with 2% epinephrine for 7 days. Twelve, 24 and 72 hrs later, the animals were killed, the iris-ciliary bodies were challenged with isoproterenol and analyzed as described above.

Percent decrease of β-adrenergic responsiveness was determined for each animal individually as follows:

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\% \text{ decrease of } \beta \text{-adrenergic responsiveness} = \frac{[\text{cyclic AMP}]_{\text{MAX}} - [\text{cyclic AMP}]_t}{[\text{cyclic AMP}]_{\text{MAX}} - [\text{cyclic AMP}]_0}
\]
DECREASE OF $\beta$-ADRENERGIC RESPONSIVENESS

Fig. 1. Decrease of in vitro isoproterenol-stimulated cyclic AMP synthesis in pigmented rabbit iris-ciliary body (O) and cornea (•) following topical administration of 0.5% timolol (100 μl). Each point (n = 6 animals) is mean ± SEM.

where $[\text{cyclic AMP}]_{\text{max}}$ is a concentration of cyclic AMP following in vitro isoproterenol stimulation of contralateral control tissues, $[\text{cyclic AMP}]$, is the concentration of cyclic AMP following in vitro isoproterenol stimulation of topical drug-treated tissue, and $[\text{cyclic AMP}]_{\text{b}}$ is the basal concentration of cyclic AMP defined as that concentration following in vitro incubation without isoproterenol.

l-epinephrine, d-bitartrate, l-isoproterenol, d-bitartrate, 3-isobutyl, 1-methylxanthine, and indo-methacin were from Sigma Chemical Co.; timolol maleate was generously supplied by Merck Sharp & Dohme Research Laboratories. The components for the radioimmunoassay of cyclic AMP were from Collaborative Research.

Results

In Vitro Isoproterenol-stimulated Cyclic AMP Synthesis

For albino rabbits, the respective basal and stimulated values of cyclic AMP concentration following in vitro incubation with $10^{-5}$ M isoproterenol were for control corneas $17 \pm 1$ (39)* and $188 \pm 7$ (39) pmoles/mg protein/15 min and for control iris-ciliary bodies $78 \pm 5$ (63) and $508 \pm 23$ (63) pmoles/mg protein/15 min. For pigmented rabbits, the respective basal and stimulated values were for control corneas $12 \pm 1$ (39) and $121 \pm 6$ (39) pmoles/mg protein/15 min and for control iris-ciliary bodies $32 \pm 3$ (55) and $121 \pm 11$ (55) pmoles/mg protein/15 min.

In Vivo Antagonism by Timolol

Single treatment-pigmented animals: The time course of the decrease of $\beta$-adrenergic responsiveness by timolol in the pigmented iris-ciliary body was quite prolonged (Fig. 1). For 12 hrs after topical administration of 0.5% timolol, $\beta$-adrenergic responsiveness of the iris-ciliary body to in vitro stimulation with isoproterenol remained depressed by 60–90%. At 12 hrs there is no evidence of recovery of $\beta$-adrenergic responsiveness. The time course of inhibition by timolol of corneas from pigmented animals was substantially different than that for the iris-ciliary bodies. $\beta$-adrenergic responsiveness of the cornea decreased by 60–70% within the first hour after topical timolol, a 20% decrease remained at 6 hrs, and treated and control values were equal by 12 hrs (Fig. 1).

Repeated treatment-albino animals: Repeated treatment of albino rabbits with timolol did not prolong the inhibition in the iris-ciliary body. Following twice-daily topical administration of 0.5% timolol for 7 days, the $\beta$-adrenergic responsiveness of the iris-ciliary body returned to control values 3 hrs after the last drug treatment (Fig. 2).

Repeated treatment-pigmented animals: Repeated treatment of pigmented rabbits with timolol caused prolonged inhibition in the iris-ciliary body. Following twice-daily topical administration of 0.5% timolol for 4 days, the $\beta$-adrenergic responsiveness of the iris-ciliary body remained decreased for at least 24 hrs (Fig. 2).

In Vivo Desensitization by Epinephrine

Single treatment-albino animals: Within 6 hrs after topical epinephrine, $\beta$-adrenergic responsiveness of the cornea decreased by approximately 40% (Fig. 3A). This loss of responsiveness was apparently maximal because it was not dependent upon the dose of epinephrine between 0.5% and 2%. Timolol, given top-
ically, 0.5 or 3 hrs before epinephrine prevented the loss of responsiveness due to topical epinephrine.

Within 6 hrs after 2% topical epinephrine, β-adrenergic responsiveness of the iris-ciliary body decreased by approximately 40% (Fig. 3B). This loss of responsiveness may not be maximal for this tissue because it is dose dependent in this range; 0.5% topical epinephrine caused significantly less desensitization. Topical timolol, given 0.5 or 3 hrs before epinephrine did not prevent the loss of responsiveness in the iris-ciliary body due to topical epinephrine.

**Single treatment-pigmented animals:** A single topical administration of 2% epinephrine effectively desensitized both cornea and iris-ciliary body to β-adrenergic stimulation (Fig. 4). Within 6 hrs after topical epinephrine, β-adrenergic responsiveness of the cornea decreased by approximately 55% (Fig. 4). The β-adrenergic responsiveness remained decreased for at least 24 hrs. Within 6 hrs after topical epinephrine, β-adrenergic responsiveness of the iris-ciliary body decreased by approximately 33%, significantly less than the decrease in the cornea (Fig. 4). The β-adrenergic responsiveness of the iris-ciliary body returned to within 20% of control values 24 hrs after drug treatment. Thus, topical epinephrine desensitized the cornea to a greater extent and with longer duration than the iris-ciliary body.

**Repeated treatment-albino animals:** Repeated topical 2% epinephrine decreased β-adrenergic responsiveness of the iris-ciliary body for 72 hrs (Fig. 5), but did not cause a greater decrease of β-adrenergic responsiveness in the iris-ciliary body compared to the decrease found after a single administration of epinephrine (unpublished data). Repeated pretreatment with topical timolol prior to epinephrine administration did not diminish the magnitude of the decrease in β-adrenergic responsiveness in the iris-ciliary body (unpublished data).

**Discussion**

This investigation is concerned specifically with unraveling the apparent paradox of how both timolol and epinephrine can affect the ciliary processes and decrease aqueous humor formation. We have studied the mechanisms and duration of pharmacologic effectiveness of these drugs as they may relate to the prolonged hypotensive response. To demonstrate these effects at the cellular level, we have measured the responsiveness of the β-adrenergic pathway of the rabbit iris-ciliary body following topical treatment with timolol or epinephrine. Although neither drug may substantially alter aqueous humor formation in this species, we propose that the receptor mechanisms and duration of decreased β-adrenergic responsiveness are analogous in glaucoma patients receiving these drugs as therapy.

Both timolol and epinephrine interact with β-adrenergic receptors and are effective topical agents for lowering intraocular pressure. Nevertheless, they have considerably different pharmacologic actions. At the β-adrenergic receptor, timolol causes only β-adrenergic antagonism; whereas, epinephrine has two actions, acute stimulation of the β-adrenergic pathway followed by prolonged desensitization of this pathway.
Can the influence of these drugs on aqueous humor formation be correlated with studies on β-adrenergic responsiveness conducted in animals? Earlier studies using albino animals demonstrated that following topical administration of timolol, β-adrenergic responsiveness of iris-ciliary body decreases to a minimum within the first hour and returns to normal soon thereafter. These observations indicate that timolol rapidly washes out of the tissue and that β-adrenergic antagonism in albino rabbits does not have the same time course as ocular hypotension in humans. However, the time course of this event is quite different in pigmented eyes. A significant decrease of isoproterenol-stimulated cyclic AMP synthesis by iris-ciliary body persists for 12 hrs following a single topical administration of 0.5% timolol. In the three nonpigmented tissues that we have studied, ie, the corneas of pigmented or albino rabbits and the iris-ciliary body from albino rabbits, timolol-induced β-adrenergic antagonism is maximal within the first hour and does not persist for more than a few hours. Apparently, the elimination of timolol from pigmented tissues is significantly different from that of nonpigmented tissue; the biological half-life is greater in pigmented tissue.

Thus, the time course of β-adrenergic antagonism in iris-ciliary body of pigmented rabbits parallels more closely the time course of the reduction in IOP observed in glaucoma patients. By demonstrating the potential for a long duration of action of timolol, these observations support the hypothesis that the major drug action of timolol, responsible for decreasing IOP, is β-adrenergic antagonism in the ciliary processes. The iris-ciliary body pigment apparently acts as a slow releasing depot for this drug.

Following topical administration, the concentration of epinephrine in the aqueous humor reaches a maximum within the first hour and declines thereafter. The α-adrenergic-mediated responses of epinephrine, vasoconstriction, and mydriasis, generally do not persist for longer than 3 hrs, and the duration of these effects gives a first approximation of the bioavailability of epinephrine to receptors in the anterior segment. Because an even lower concentration of epinephrine must reach the β-adrenergic receptors of the ciliary processes, the time course of peak stimulation of cyclic AMP synthesis must be within 3 hrs. This time course of action within the ciliary processes is supported by the appearance and decay of cyclic AMP in the aqueous humor and corresponds to the early stimulation of aqueous humor formation following topical epinephrine.

In addition to transient stimulation of the β-adrenergic receptors, epinephrine also causes desensitization. β-adrenergic receptors in a number of tissues undergo a receptor-specific loss of sensitivity following stimulation with agonist. This desensitization frequently involves a decrease in the number of receptors at the cell membrane and loss of responsiveness of the tissue. In other tissues, β-adrenergic stimulation causes desensitization of adenylate cyclase that likewise causes a loss of responsiveness of the tissue.

Following topical administration, epinephrine causes a loss of β-adrenergic responsiveness in both the cornea and the iris-ciliary body. In the cornea,
the loss of responsiveness is associated with a decrease in number of β-adrenergic receptors.27,28 In the iris-ciliary body, loss of receptors following topical epinephrine has been observed in 6-hydroxydopamine-pretreated eyes.27 In normally innervated iris-ciliary processes, a decrease in activity of the guanyl nucleotide regulatory protein of adenylate cyclase occurs following topical epinephrine.21 Therefore, in the iris-ciliary body, loss of responsiveness following topical epinephrine can occur by at least two mechanisms.

Our present data demonstrate that this phenomenon persists for 12–24 hrs after a single drug administration and for up to 2–3 days following repeated administration. In the pigmented iris-ciliary body, β-adrenergic antagonism due to timolol has a rapid onset, decreases responsiveness by 70–80%, and decays slowly. In this tissue, β-adrenergic desensitization due to epinephrine has a slower onset, decreases responsiveness by 30–40%, and decays within 24 hrs. Thus, both drugs could cause a decrease in aqueous humor formation 3–12 hrs after administration by causing decreased responsiveness of the same physiologic phenomenon. β-adrenergic antagonism by timolol should be more effective than β-adrenergic desensitization by epinephrine because both a more rapid onset of action and a greater decrease in responsiveness are achieved with timolol.

For either of these hypothetical mechanisms, β-adrenergic antagonism or desensitization, to account for a specific β-adrenergic pathway dependent decrease in aqueous humor formation, β-adrenergic tone maintaining normal aqueous humor formation must be postulated.29–31 Tone to the ciliary epithelium has not been conclusively demonstrated although there is a dense network of adrenergic nerves in the stroma of the ciliary processes.32

In summary, both timolol and epinephrine decrease β-adrenergic responsiveness of the iris-ciliary body. The duration of this effect in pigmented tissues parallels the time course of the hypotensive effect which is achieved in many glaucoma patients controlled with these drugs. The greater magnitude of decreased β-adrenergic responsiveness achieved with timolol is consistent with the observation of its greater clinical utility in decreasing aqueous humor formation. The smaller effect of epinephrine in the ciliary processes is consistent with the hypothesis that its major clinical usefulness is due to its ability to increase outflow facility.

Key words: β-adrenergic antagonist, β-adrenergic agonist, β-adrenergic desensitization, timolol, epinephrine, cyclic AMP, aqueous humor formation, ciliary body, cornea

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References