Effects of l- and d-Timolol on Cyclic AMP Synthesis and Intraocular Pressure in Water-loaded, Albino and Pigmented Rabbits

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Topical 2% l- or d-timolol reduced the elevation of intraocular pressure induced by water-loading in conscious rabbits. This drug effect appeared on the peak elevation (in pigmented eyes) and on the down-phase (in albino and pigmented eyes) of elevated intraocular pressure. The contralateral eye and the treated eye responded similarly. In urethane anesthetized, water-loaded rabbits, a greater inhibitory effect of l-timolol was observed in pigmented eyes than in albino eyes. Two per cent l-timolol caused alterations of heart rate and arterial blood pressure in water-loaded anesthetized rabbits, but time courses of these alterations did not correlate with the inhibitory effect on the elevation of intraocular pressure. The β-adrenergic antagonistic activity of l-timolol and d-timolol were compared by their ability to inhibit l-isoproterenol-stimulated cyclic AMP synthesis in the rabbit iris-ciliary body preparation in vitro. The IC₅₀s for l- and d-timolol differ by about 1.5 log units. In our studies, d-timolol has little of the intraocular pressure lowering and the β-adrenergic antagonistic activity of l-timolol. Thus, the conscious, water-loaded, pigmented rabbit can be used as a model for studying the effects of β-adrenergic antagonists on intraocular pressure. Invest Ophthalmol Vis Sci 24:1276-1282, 1983

Used as a primary antiglaucoma agent l-timolol is a potent β-adrenergic antagonist. The mechanism of its ocular hypotensive effect is inhibition of the formation of aqueous humor. However, the link between the β-adrenergic antagonism and the inhibition of aqueous humor has not been fully established.1

One of the major difficulties in establishing the mechanism of ocular action of l-timolol is the lack of an experimental animal model. In normotensive albino rabbits, topical l-timolol causes little, if any, significant decrease of intraocular pressure (IOP).2 In water-loaded albino rabbits, the elevation of IOP is inhibited to some extent by bilateral topical treatment of l-timolol 40 min before water-loading.3 However, if unilateral l-timolol is given during the water-loading, there is no detectable alteration of the elevation of IOP in the treated eye compared with the contralateral eye.4

A recent study from our laboratory has shown that pigmented tissue plays a critical role in prolonging the action of l-timolol in the anterior segment of the eye.5 We have therefore devised an experimental model for studying drug action using the water-loaded pigmented rabbit.

When topical l-timolol is absorbed into the circulation, some of the side effects manifested are due to β-adrenergic antagonism.6 D-timolol, the stereoisomer of l-timolol, presumably has less β-adrenergic antagonistic activity.7 If d-timolol lowers IOP, this form of the drug may have less potential to cause side effects. We have therefore compared the potency of l-timolol and d-timolol on β-adrenergic antagonistic activity and the ocular hypotensive effect in the water-loaded, pigmented rabbit model.

Materials and Methods

L-timolol maleate and d-timolol were generously supplied by Merck, Sharp and Dohme Research Laboratory, West Point, PA. These stereoisomers were freshly prepared and concentrations given refer to the base weight. Components for the radioimmunoassay of cyclic AMP (cAMP) were from Collaborative Research Inc., Waltham, MA. Indomethacin, 3-isobutyl-1-methylxanthine (IBMX), l-isoproterenol d-bitartrate, and urethane were from Sigma Chemical Co.
The Water-Loading Model in Albino and Pigmented Rabbits

Two types of rabbits, New Zealand albino rabbits and mixed-breed pigmented rabbits, with starting weights of 2.5 to 3.5 kg were used. Elevated IOP was induced in conscious rabbits by rapidly delivering 60 ml/kg of room temperature distilled water by orogastric intubation. The rabbits were deprived of food for 24 hrs prior to use.

Intraocular pressure was measured with a modified Digilab pneumatometer calibrated for rabbit eyes, and 0.5% proparacaine was used as a local anesthetic. Rabbits were acclimated to pneumatomometry by making several unrecorded measurements prior to beginning each experiment.

The Effect of Timolol Stereoisomers in the Water-Loading Model

Various concentrations of timolol stereoisomers in two 50 μl drops were applied unilaterally to rabbit eyes. IOP was measured in both eyes before and after medication. Either 40 or 90 min later, each rabbit was water-loaded, and IOP was measured every 15 min in both eyes. Each individual rabbit was used repeatedly for the control experiment and for experiments using different timolol stereoisomers. Repeated experiments were performed over the same diurnal period. The order of experiments, control or different medications, was random. At least one week of recuperation was allowed between experiments on the same rabbit.

To investigate the involvement of parameters of systemic circulation in the water-loading model, groups of albino and pigmented rabbits were anesthetized with intravenous 25% urethane (1.5 gm/kg). During the experimental period, additional urethane was administered when necessary to maintain an adequate level of anesthesia. The femoral artery was cannulated and then blood pressure and pulse rate were monitored using a Beckman polygraph. IOP was measured as above except no local anesthetic was given. The protocol for water-loading and drug delivery was the same as that used in conscious rabbits. Repeated experiments on the same rabbit were not performed because of the high fatality rate following urethane anesthesia.

β-Adrenergic Antagonistic Activity of Timolol Stereoisomers

The β-adrenergic antagonistic activity was determined by measuring the inhibition of β-isoproterenol-stimulated cAMP synthesis by rabbit iris-ciliary body preparations in the presence of timolol stereoisomers.

New Zealand albino rabbits (2.5 to 4 kg) were killed by an intravenous overdose of sodium pentobarbital. The anterior segment of the eye was removed, and the iris-ciliary body was dissected, divided into eight pieces, and preincubated in buffer for 1 hr at 37°C. Iris-ciliary body preparations were then transferred to fresh buffer containing 0.5 mM IBMX and various concentrations of l- or d-timolol. Five minutes later β-isoproterenol was added to give a final concentration of 10 μM and the incubation continued for 15 min. At the end of the incubation, cAMP was extracted by homogenizing the tissue in hot 0.1 N KOH and, after cooling, neutralized with 0.1 N HCl. The concentration of cAMP (picomole/mg protein) was measured by radioimmunoassay and the protein concentration was measured by the method of Lowry et al.

Percent inhibition of cAMP synthesis by iris-ciliary body tissue from each eye was calculated as follows:

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\text{% inhibition} = \left( \frac{\text{[cAMP]}_{\text{max}} - \text{[cAMP]}_i}{\text{[cAMP]}_{\text{max}}} \right) \times 100
\]

where [cAMP] max is the concentration of cAMP following β-isoproterenol stimulation, [cAMP] i is the concentration of cAMP following β-isoproterenol stimulation in the presence of timolol stereoisomers, and [cAMP] b is the basal cAMP concentration in the absence of stimulation. Each measurement was performed in duplicate. To minimize the deviation of responses among individual eyes, percent inhibition of cAMP synthesis in the presence of equal concentrations of l- and d-timolol were determined on preparations from the same individual eye.

Results

Effect of Timolol Stereoisomers on Elevated IOP in Water-Loaded Rabbits

In albino rabbits, effects of timolol stereoisomers were observed when treatments preceded water-loading by 40 or 90 min.

Figure 1 demonstrates the changes in IOP following water-loading in the same albino rabbits either untreated or treated with 2% l- or d-timolol 40 min earlier. In the untreated controls IOP remained elevated for 2 hrs, and the peak elevation occurred approximately 45 min after water-loading. When rabbits were pre-treated with 2% l- or d-timolol, no reduction in the peak elevation of IOP occurred, but there was a consistent relative reduction in the down-phase of the IOP profile. That is, IOP returned to normal values sooner when rabbits were treated with the drug. No difference in response between the treatment with 2% l-timolol or 2% d-timolol was noted and no significant difference in IOP was found between treated eyes and contralateral, untreated eyes.
When the interval between the treatment and water-loading was extended to 90 min, the changes in the elevation of IOP (Fig. 2) were similar to the patterns with a 40 min separation interval (Fig. 1). Two percent timolol stereoisomers reduced the down-phase of the elevated IOP profile but did not reduce the peak elevation of IOP. However, compared to the starting IOP, IOP at the time of water-loading was lower when rabbits were treated 90 min before water-loading. This reduction of IOP was, at least in part, due to acclimation since placebo treatment also caused the reduction of IOP in this experimental environment (unpublished data).

The effect of 2% timolol stereoisomers on elevated IOP in water-loaded pigmented rabbits is shown in Figure 3. Pretreatment with 2% l-timolol 90 min before water-loading decreased both the peak elevation of IOP and the down-phase of elevated IOP. The average maximal IOP in untreated eyes was 36 mmHg and in 2% l-timolol treated eyes was 29 mmHg. The difference between these two values is statistically significant ($P < 0.01$). Two percent d-timolol was less effective than...
2% L-timolol at reducing the peak of the IOP profile; the averaged maximal IOP was 32 mmHg after treatment with d-timolol. This value is statistically different from the control \( (P < 0.05) \). In either unilateral treatment with L-timolol or d-timolol, there was no statistical difference between treated eyes and contralateral eyes.

When 0.2% timolol stereoisomers were given topically to the same pigmented rabbits 90 min before water-loading, there were no significant effects on either peak elevation or the down-phase of the IOP profile (Fig. 4).

Effects of L-Timolol on Cardiovascular Parameters in Water-Loaded Anesthetized Rabbits

Because 2% L-timolol caused the maximal effect on elevated IOP induced by water-loading, this drug was tested for its ability to alter systemic fluid dynamics using either albino (Fig. 5) or pigmented rabbits (Fig. 6). We compared the time courses of alterations in cardiovascular parameters with the time course of the inhibitory effect on IOP following L-timolol treatment to clarify whether the effect of L-timolol on IOP in the water-loaded rabbit was a subsequent response to an alteration of systemic fluid dynamics caused by the drug.

Water-loading caused an elevation of IOP in urethane anesthetized rabbits that persisted for at least 2 hrs. The peak elevation appeared approximately 15 min after water-loading in control experiments. A greater inhibitory effect of 2% L-timolol on the elevation of IOP was observed in pigmented rabbits than in albino rabbits.
The effect of 2% l-timolol on heart rate and arterial blood pressure was similar in albino and pigmented rabbits. Both water-loading and treatment with 2% l-timolol slowed the heart rate, but the time course of bradycardia did not correlate with the time course of alteration of IOP. During the 90-min period before water-loading, l-timolol gradually decreased the arterial blood pressure. Water-loading caused a transient increase in arterial blood pressure that lasted only 10 min and was partially blocked by the pretreatment with 2% l-timolol. The time course of changes in arterial blood pressure did not correlate with the time course of the effect of l-timolol on elevated IOP. Therefore, treatment with 2% l-timolol caused alterations of cardiovascular parameters in anesthetized rabbits, but these alterations were not correlated with the inhibitory effect of l-timolol on IOP in the water-loading model.

**Comparison of \( \beta \)-Adrenergic Antagonistic Activity of Timolol Stereoisomers**

Figure 7 demonstrates the ability of l- and d-timolol to inhibit in vitro cAMP synthesis in rabbit iris-ciliary
body stimulated with 10 \(\mu\)M \(\alpha\)-isoproterenol. Dose-response curves in the figure are the best-fit graphs using the equation in Appendix A of the method of Brown et al.\textsuperscript{13} The concentrations of \(L\) and \(D\)-timolol required to inhibit 50\% of the cAMP synthesis (\(I_{50}\)) differ about 1.5 log units. Thus \(D\)-timolol has approximately 3\% of the \(\beta\)-adrenergic antagonistic activity of \(L\)-timolol in rabbit iris-ciliary body. At concentrations higher than 10 \(\mu\)M, both stereoisomers caused essentially complete inhibition of \(\beta\)-adrenergic stimulation.

**Discussion**

Our study indicates that the water-loaded pigmented rabbit is a good model for studying the action of \(L\)-timolol whereas the water-loaded albino rabbit is not. Our laboratory has recently demonstrated that \(\beta\)-adrenergic antagonism persists longer in iris-ciliary body from pigmented rabbits than from albino rabbits.\textsuperscript{5} Apparently, pigmented tissue in the anterior segment of the eye provides a depot for timolol stereoisomers, which are subsequently released to bind to nearby \(\beta\)-adrenergic receptors. By this mechanism, an inhibitory effect of \(\beta\)-adrenergic antagonism on the formation of aqueous humor may persist for many hours.

Galin et al suggested that reduction of the osmolarity of the blood is involved in the mechanism of elevating IOP following water-loading.\textsuperscript{14} Our study demonstrates that alterations in heart rate and arterial blood pressure after water-loading are not critical in elevating IOP nor in the action of timolol. In both albino and pigmented rabbits, changes in these cardiovascular parameters do not correlate with either the time course of the elevation of IOP or the relative reduction of IOP by \(L\)-timolol. We have found no evidence that the action of timolol stereoisomers on elevated IOP in pigmented rabbits is due to a systemic cardiovascular effect. We note that \(L\)-timolol alters cardiovascular parameters in both albino rabbits, in which a relatively small effect on elevated IOP occurs, and in pigmented rabbits, in which a relatively large effect occurs. The one obvious difference is the pigment in the eye. We postulate that there is a local site of action of timolol on elevated IOP in the treated eye of the water-loaded pigmented rabbit.

When either timolol stereoisomer was given to conscious rabbits, the contralateral eye and the treated eye responded similarly. A smaller contralateral effect occurs in humans\textsuperscript{15} and in cats.\textsuperscript{16} Perhaps there was sufficient timolol stereoisomers absorbed into the circulation to act locally in the contralateral eye. After unilateral application of 2\% \(L\)-timolol to rabbit eyes, apparently sufficient drug was absorbed systemically to cause cardiovascular responses. Alternatively, bilateral control of IOP via the central nervous system may be influenced by timolol, but this mechanism has not been investigated. Obviously, a better understanding of the contralateral action of timolol stereoisomers is needed.

The ciliary processes are the major site of action of \(L\)-timolol to decrease the formation of aqueous humor. We have determined that \(D\)-timolol has approximately 3\% of the \(\beta\)-adrenergic antagonistic activity of \(L\)-timolol and is significantly less potent in reducing the rise in IOP induced by water-loading. Therefore, although there may be few clinical side effects with topical \(D\)-timolol, this drug does not appear to offer the potency of \(L\)-timolol for lowering IOP.

The dose-response relationship between topical timolol stereoisomers and the effect on elevated IOP in water-loaded pigmented rabbits has not been completely established in this study. Although pretreatment with 2\% of either timolol stereoisomer caused an inhibition of the elevation of IOP, when the concentration was reduced to 0.2\% no detectable inhibition with either stereoisomer was observed. As determined in this study, \(D\)-timolol has 3\% of the \(\beta\)-adrenergic antagonistic activity of \(L\)-timolol. Therefore, 0.2\% \(L\)-timolol should have caused more \(\beta\)-adrenergic antagonism than 2\% \(D\)-timolol and would have been expected to lower IOP in the water-loading model. Our observation that 0.2\% \(L\)-timolol was insufficient indicates that the relative bioavailability of timolol stereoisomers, via absorption and deposition in the ocular pigmented tissue, may not parallel in vitro \(\beta\)-adrenergic antagonism.
The d-timolol that we used may have contained a trace of l-timolol. However, this amount of l-timolol would not account for the ability of d-timolol to decrease the elevated IOP in the water-loading model. Thus, the d-timolol used in this study has intrinsic $\beta$-adrenergic antagonistic activity. In the cat, perfusion of a high concentration of d-timolol intracameral may have produced a maximal inhibitory effect on the formation of aqueous humor by acting at $\beta$-adrenergic receptors.17

Our observation that topical d-timolol caused less effect on IOP than l-timolol suggests that this phenomenon is stereospecific. The finding of stereospecificity favors a receptor mediated mechanism of action and suggests that $\beta$-adrenergic antagonism may be the relevant mechanism for the action of timolol in the ciliary processes.

Key words: l-timolol, d-timolol, cyclic AMP, $\beta$-adrenergic antagonism, water-loading, intraocular pressure, rabbit

References