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Effect of Topical Timolol on Tissue Circulation in Optic Nerve Head

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Abstract: The effects of topical 0.5% timolol on tissue circulation in the albino rabbit optic nerve head (ONH) were investigated using a laser speckle tissue circulation analyzer. In the first experiment, the normalized blur (NB) value, a quantitative index of tissue blood flow velocity in the ONH, intraocular pressure (IOP), blood pressure, and pulse rate were measured under general anesthesia before, and 30, 60, 90, and 120 minutes after a 20 μL instillation of timolol in one eye and the vehicle in the other eye in a masked, randomized manner. In the second experiment, one eye of a rabbit received timolol twice daily for 20 days and the fellow eye received the vehicle in a masked, randomized manner. Every 5 days IOP was measured and the NB in the ONH and IOP were measured before treatment and 2 hours after the last instillation on the 20th day. After a single instillation of timolol, PR showed a maximum reduction of 12% and IOP in the timolol-treated eyes showed a maximum decrease in 25%. NB in the ONH and BP did not show any significant change during the experiment. After a 20-day treatment with timolol, IOP showed a maximum decrease of 25% in the timolol-treated eyes and 16% in the vehicle-treated eyes. The NB in the timolol-treated eyes increased significantly by 16% ($P < 0.01$), whereas that in the vehicle-treated eyes showed no significant change. It was suggested that long-term topical timolol with a normal drug regimen caused a significant increase in the peripheral blood velocity in the ONH only in the timolol-treated eyes, at least partly, by local penetration of the drug. Ocular penetration of topically applied timolol is thought to be similar between rabbit and human eyes. Therefore, the present results may have clinical implications. **Jpn J Ophthalmol 1997;41:297–304** © 1997 Japanese Ophthalmological Society

Key Words: Laser speckle phenomenon, optic nerve head, rabbit eye, timolol, tissue circulation.

Introduction

Although intraocular pressure (IOP) has been consistently found to be one of the most important risk factors in the development of open angle glaucoma, various types of evidence indicate that compromise of the tissue circulation in the optic nerve head (ONH) may also play a causal role in glaucomatous injury in the ONH[1–3] Therefore, the possible effects of antiglaucoma agents on ONH tissue circulation, if they exist, are of great clinical importance.

Timolol, a nonselective beta-adrenergic antagonist, has probably been the most widely used antiglaucoma agent. Beta-receptors generally mediate relaxation of vascular smooth muscle and it has been known that systemic beta-antagonists induce vasoconstriction in various tissues.[4,5] Beta-receptors, the majority of which are the beta-2 subtype, have been identified in ONH tissue.[6,7] Because it is very likely that they are vascular beta-2 receptors, if a pharmacologically effective quantity of timolol reaches the ONH through systemic absorption or by direct drug infiltration, as suggested for phenylephrine,[8] the ONH tissue blood flow may be significantly affected by topical timolol.

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There have been many reports of the effect of timolol on the blood flow in the retina,[9–20] choroid,[9–11,15,20,21] or ophthalmic artery.[22,23] However, relatively few reports are available on its effect on the circulation in ONH tissue. According to Jay et al.,[15] the optic nerve blood flow determined with the radioactive microsphere method was not affected in phakic rabbit eyes by topical timolol applied at a rate of 8 drops every 7.5 minutes. In aphakic rabbit eyes wherein more of a topically applied drug was allowed to pass posteriorly, however, timolol given as described above significantly increased the optic nerve blood flow. Later studies using a normal drug regimen failed, however, to confirm the increased blood flow effect of timolol in the optic nerve. Green and Hatchett[10] and Green and Schermerhorn[11] instilled timolol thrice daily for 3–6 weeks in phakic or aphakic rabbits and could not find any significant change in the optic nerve blood flow determined with the radioactive microsphere method. Similarly, Yoshida et al.[20] reported that a single instillation of timolol in human eyes showed no significant effect on the ONH capillary blood flow velocity determined using laser Doppler velocimetry. In the above cited rabbit studies where 15 μm of radioactive microspheres were used, the number of spheres trapped in the optic nerve would not be sufficient to allow detection of a small change in the ONH blood flow.[24,25] Laser Doppler velocimetry used by Yoshida et al.[20] should be much more sensitive in detecting changes in blood flow velocity in the ONH,[26] but their results did not describe the effects of long-term timolol treatment, where concentrations in ONH tissue might accumulate to a much higher level than that after a single instillation.

We have recently developed an apparatus equipped with a diode laser for noncontact, two-dimensional estimation of ocular fundus tissue circulation utilizing the laser speckle phenomenon[27] with which a quantitative index of the tissue blood flow velocity in an area of 0.42 × 0.42 mm of the ONH, normalized blur (NB) value, was measured with reproducibility of approximately 10%. Further, Araie and Muta[28] have already reported that, using this apparatus, twice daily 20-day instillation of 0.5% betaxolol caused a significant increase in ipsilateral ONH tissue blood velocity in albino rabbits. Using the same apparatus, we studied the effects of a single or twice daily 20-day instillation of timolol on ONH tissue circulation in rabbit eyes. The NB in the ONH also was found to show a good correlation with the blood flow rate in the ONH determined using the hydrogen gas clearance method of albino rabbits.[29]

Materials and Methods

Laser Speckle Tissue Circulation Analyzer

The ONH tissue circulation was evaluated with a laser speckle tissue circulation analyzer, details of which have been described elsewhere.[27] The apparatus consists of a fundus camera (TRC-WT3®, Topcon, Tokyo) equipped with a diode laser (wavelength 808 nm) and an image sensor (100 × 100 pixels, BASIS type, Canon, Tokyo). A halogen lamp illuminated the fundus where the laser beam was focused. The scattered laser light was imaged onto the image sensor, which corresponds to a field of 0.62 × 0.62 mm in the rabbit ONH, where a speckle pattern appeared. The difference between the average of the speckle intensity (I_{mean}) and the speckle intensity for successive scannings of the image speckles at the pixels on the sensor plane was calculated, and the ratio of I_{mean} to this difference was defined as NB. Normalized blur is nearly equivalent to the reciprocal of speckle contrast described by Briers and Fercher[30] and Fercher and Briers[31] and is thought to be indicative of tissue blood velocity. The results are displayed in a color graphic showing the two-dimensional variation of the NB level over the field of interest. The average of NB levels in the measured field in the ONH was expressed as NB_{av}. The coefficient of reproducibility of a 5-minute or 24-hour interval in vivo measurement of NB_{av} in 70 × 70 pixels, which corresponds to 0.42 × 0.42 mm in the rabbit ONH, was approximately 10%.[27]

Drug

Timolol maleate 0.5% ophthalmic solution was purchased from Banyu Pharmaceutical Company (Tokyo). The vehicle was kindly supplied by the same company.

NB_{av} Measurement in ONH

Albino rabbits weighing 2.5–3.0 kg were used and handled in accordance with the ARVO Resolution on the Use of Animals in Research. The animals were entrained to a schedule of alternating 12-hour periods of light and dark (light on at 4:00 AM) for at least 3 weeks prior to use. After dilating the pupil with one drop of Mydrin M® (0.4% tropicamide, Santen Pharmaceutical, Osaka), the image speckles from the largest square field in the ONH free of visible surface vessels were recorded to measure the NB_{av} value in the ONH tissue ($NB_{av(ONH)}$) (Figure 1). Color fundus photographs were taken to record the site of NB measurement referring to visible surface vessels near the measurement field as markers

to identify tissue sites. The animals were sacrificed at the end of the single instillation experiment, but not at the end of the 20-day instillation experiment.

Experimental Protocol

Effect of single instillation. General anesthesia was induced by intravenous injection of 30 mg/kg pentobarbital sodium and maintained carefully with additional small doses. The femoral artery was cannulated with a polyethylene catheter and connected to a pressure transducer (DTX®, Spectramed, CA, USA) for measurements of the femoral arterial blood pressure and pulse rate. The mean femoral arterial blood pressure (BP_m) was calculated according to the formula:

$$BP_m = BP_d + 1/3(BP_s - BP_d)$$

where BP_d and BP_s are diastolic and systolic femoral arterial blood pressure. After dilating the pupil with one drop of Mydrin M®, the $NB_{av(ONH)}$ was measured as described above. The average of six measurements obtained at intervals of 1 minute was adopted as the initial value.

In a masked manner, one eye of each animal received 20 μL of 0.5% timolol and the fellow eye received the vehicle of 18:00 (timolol group, n = 8). To serve as a control, both eyes received the vehicle of timolol at 18:00 in another group of rabbits that were anesthetized and treated in the same way as in the timolol group (control group, n = 8). During the experiment, $NB_{av(ONH)}$ and IOP in both eyes of the timolol group and one randomly chosen eye of the control group, BP_m and pulse rate were monitored prior to instillation, and at 30, 60, 90, and 120 minutes after instillation. IOP was monitored with a calibrated applanation pneumotonometer. Arterial P_{O_2}, P_{CO_2} and pH were checked before instillation, and 60 and 120 minutes after instillation using the pH/Blood Gas Analyzer, Model 170 (Corning Glass, Corning, NY, USA). Body temperature was monitored with a rectal thermometer.

Figure 1. Measurement field of NB in ONH tissue. Image speckles from inferior field of ONH where no discrete vessels were visible (□ = 0.42 × 0.42 mm).

Effect of 20-day topical treatment. The IOP was measured in both eyes with a calibrated applanation pneumotonometer after instillation of topical anesthesia (0.4% oxbuprocaine hydrochloride) at 20:00 under dim light. Relatively weak general anesthesia was induced by intravenous injection of 15 mg/kg pentobarbital sodium. The pupil was dilated as described above. Fifteen minutes after induction of general anesthesia, the $NB_{av(ONH)}$ in both eyes was measured as described above, and the average of six measurements obtained at intervals of 1 minute was adopted as the initial value. Color fundus photographs were taken to record the site of NB measurement.

From the next day on, one eye of each animal received 20 μL of 0.5% timolol, and the fellow eye received the vehicle of each drug twice daily (6:00 and 18:00) for 20 days in a masked manner (timolol group, n = 24). To serve as a control, both eyes of another group of rabbits, which were treated in the same way as in the timolol group, received the vehicle of timolol twice daily (6:00 and 18:00) for 20 days (control group, n = 15). During the treatment period, the light schedule was the same as described above and the IOP was measured under topical anesthesia in both eyes of the timolol group and one randomly chosen eye of the control group at 20:00 on the 5th, 10th, 15th, and 20th day under dim light. On the 20th day, after measuring the IOP under topical anesthesia, general anesthesia was induced. The $NB_{av(ONH)}$ at the same site of ONH tissue was measured again as described above in both eyes of the timolol group and a chosen eye of the control group. All measurements were carried out by investigators unaware of the treatment given the animals.

Calculations and Statistical Analysis

The results are presented as mean ± standard error of mean. Paired Student's *t*-tests or unpaired *t*-tests were applied to evaluate statistical significance. For multiple comparisons, Bonferroni's correction was used to calculate *P* values. Significance levels of $P < 0.05$ were considered statistically significant.

Results

Effect of Single Instillation

Only those rabbits whose systemic condition parameters, except for pulse rate, showed little change

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Table 1. Values of Systemic Condition Parameters Before and After Single Topical Instillation of Timolol or Vehicle

	Time (Minutes)				
	0	30	60	90	120
Timolol Group					
BP_m (mmHg)	100.2 ± 2.4	96.2 ± 4.5	95.4 ± 5.0	95.0 ± 5.8	96.4 ± 5.7
BT (°C)	38.3 ± 0.2	—	38.2 ± 0.2	—	38.2 ± 0.2
pH	7.45 ± 0.02	—	7.44 ± 0.02	—	7.44 ± 0.02
P_{CO_2}	34.4 ± 3.7	—	32.5 ± 3.8	—	29.7 ± 3.7
P_{O_2}	88.5 ± 2.1	—	90.3 ± 2.6	—	91.4 ± 2.9
Control Group					
BP_m (mmHg)	98.3 ± 2.5	95.9 ± 2.6	97.0 ± 2.5	96.5 ± 2.4	97.6 ± 2.6
BT (°C)	38.4 ± 0.2	—	38.2 ± 0.2	—	38.3 ± 0.2
pH	7.44 ± 0.02	—	7.43 ± 0.02	—	7.44 ± 0.02
P_{CO_2}	34.1 ± 3.6	—	33.5 ± 3.8	—	32.8 ± 3.2
P_{O_2}	89.8 ± 2.0	—	90.6 ± 2.5	—	90.4 ± 2.6

BP_m = mean femoral arterial blood pressure. BT = body temperature. Figures are mean ± standard error of mean (n = 8).

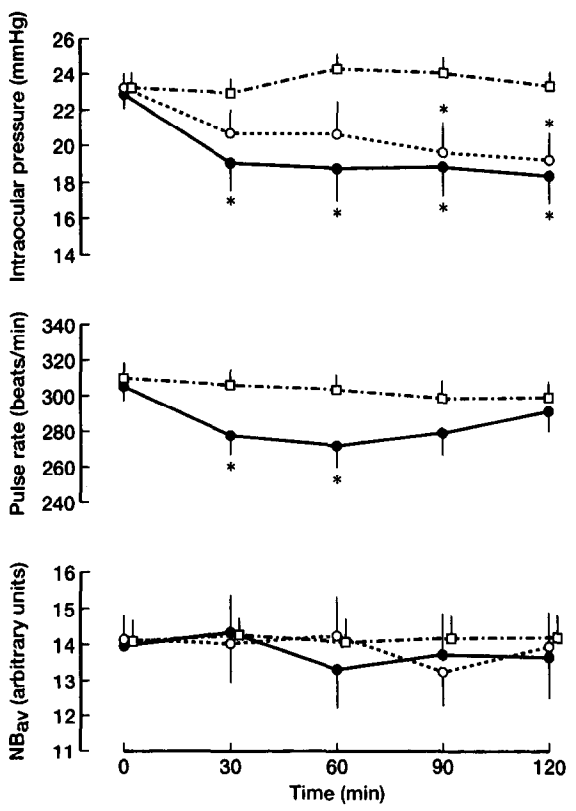

Figure 2. Time course (minutes) of NB_{av} (70 × 70) obtained from ONH tissue, pulse rate, and intraocular pressure after single instillation of timolol (●) or vehicle (○) in timolol group, and vehicle in control group (□). Each plot represents mean value of eight rabbits. Error bars: Standard error of mean.
*$P < 0.05$ by unpaired t-test for difference from control.

during the experiments and were within the normal range for healthy rabbits[32,33] were accepted for the study. Systemic parameters obtained in these rabbits are shown in Table 1.

Figure 2 summarizes the time course of NB_{av}, IOP, and pulse rate after instillation of timolol and the vehicle. The IOP and pulse rate showed no significant change during the experimental period in the control group. The IOP in the timolol-treated eyes was significantly lower at 30, 60, 90, and 120 minutes and that in the vehicle-treated eyes of the timolol group was significantly lower at 90 and 120 minutes, compared with that of the control group ($P < 0.05$ with Bonferroni's correction). The pulse rate in the timolol group was significantly lower at 30 and 60 minutes compared with that of the control group ($P < 0.05$). The NB_{av} showed no significant change during the experimental period in the timolol-treated and vehicle-treated eyes of the timolol group, and in the eyes of the control group.

Effect of 20-Day Topical Treatment

The IOP in the control group showed no significant change during the experimental period. The IOP in the timolol-treated eyes was significantly lower between 5 and 20 days, and that in the vehicle-treated eyes of the timolol group was significantly lower between 10 and 20 days, compared with data of the control group ($P < 0.01$ with Bonferroni's correction) (Figure 3).

Average values of $NB_{av(ONH)}$ before and after 20 days of topical treatment are summarized in Table 2. Baseline values of $NB_{av(ONH)}$ were not significantly different among the timolol- and vehicle-treated

eyes of the timolol group and those in the control group. After treatment, the average change from baseline in $NB_{av(ONH)}$ was not statistically significant in the vehicle-treated eyes of the timolol group and those of the control group. In the timolol-treated eyes, there was a significant increase in $NB_{av(ONH)}$ of 15.7% ($P < 0.01$) from baseline value. In comparison with the vehicle-treated eyes in the timolol group or the eyes in the control group, $NB_{av(ONH)}$ in the timolol-treated eyes after treatment was significantly greater ($P < 0.01$ with Bonferroni's correction). The difference in the $NB_{av(ONH)}$ before and after completion of treatment was significantly larger in the timolol-treated eyes than in the vehicle-treated eyes in the timolol group ($P < 0.05$ with Bonferroni's correction) or in the control group ($P < 0.01$ with Bonferroni's correction).

After completion of 20-day treatment, the bilateral difference of IOP was 2.0 ± 0.5 mmHg; bilateral difference of $NB_{av(ONH)}$ was 1.9 ± 0.3. There was no significant correlation between IOP and $NB_{av(ONH)}$ ($r = 0.050$).

Discussion

According to Koelle et al.,[34] the penetration depth of near infrared laser (wavelength 811 nm) in the cat optic nerve exceeds 1 mm. Thus, in the present apparatus, the effective depth of sampling in the ONH tissue will be greater than 1 mm, and some contribution is expected from the retroscleral region (retrolaminal region in human eyes) to the measured NB, in addition to that from the anterior ONH and scleral region.

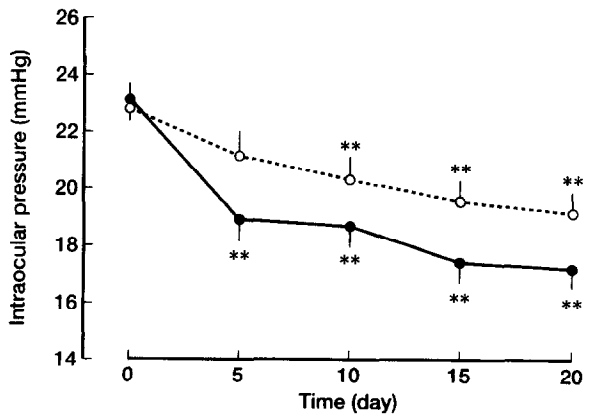

Figure 3. Time course (day) of IOP after topical instillation of timolol (●) or vehicle (○) in timolol group. Each plot represents the mean value of 24 or 15 rabbits. Error bars: standard error of mean.
**$P < 0.01$ by paired t-test for difference from baseline IOP.

$NB_{av(ONH)}$ in the rabbit showed a robust and significant correlation with a change in the ocular perfusion pressure (OPP) after injection of a lethal dose of pentobarbital.[27] Using the same apparatus, Sugiyama et al.[29] measured $NB_{av(ONH)}$ and the ONH tissue blood flow rate determined with the hydrogen gas clearance method in the same rabbit eye before and after inhalation of 10% carbon dioxide (CO_2) or intravenous injection of a small amount (10^{-10} mol/kg) of endothelin-1 (ET-1). Immediately after inhalation of 10% CO_2, $NB_{av(ONH)}$ increased by 31% and the ONH tissue blood flow rate determined with the hydrogen gas clearance method increased by 22%. Fifteen minutes after intravenous injection of 10^{-10} mol/kg ET-1, $NB_{av(ONH)}$ decreased by 22% and the ONH tissue blood flow rate determined with the hydrogen gas clearance method decreased by 19%. Further, a significant correlation ($r = 0.92$, $P < 0.01$) was found between a relative change in NB_{av} and the ONH tissue blood flow rate determined with the hydrogen gas clearance method. These results suggest that $NB_{av(ONH)}$, which is a quantitative index of blood velocity in ONH tissue, also correlates with the ONH tissue blood flow rate.

In the current study, NB_{av} obtained from the ONH tissue showed little change after a single instillation of timolol. On the other hand, after 20 days of twice daily timolol treatment, NB_{av} obtained from ONH tissue in the timolol-treated eyes showed a significant increase of about 15%, while it showed no change in the vehicle-treated contralateral eye. The results of the long-term instillation experiment may be explained by: (1) the beta blocking effect of systemically absorbed timolol; (2) the beta blocking effect of timolol, which penetrated the ONH tissue of

Table 2. NB_{av} (70 × 70) in ONH Tissue Before Treatment and After 20-Day Instillation of Timolol

	n	Before	20 Days	Δ
Timolol	24	14.2 ± 0.3	16.5 ± 0.3[a,b]	2.2 ± 0.3[c]
Vehicle	24	14.1 ± 0.5	14.6 ± 0.4	0.4 ± 0.3
Control	15	14.0 ± 0.4	13.7 ± 0.3	−0.3 ± 0.3

Mean ± standard error of mean.
Timolol: timolol-treated eyes. Vehicle: vehicle-treated contralateral eyes. Control: eyes in control experiment. Δ: difference between values on 20th day and before treatment.
[a]$P < 0.01$ (paired t-test) compared with value before treatment or in vehicle-treated eyes.
[b]$P < 0.01$ (unpaired t-test with Bonferroni's correction) compared with control.
[c]$P < 0.05$ (paired t-test with Bonferroni's correction) compared with value in vehicle-treated eyes.

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the treated eye; and (3) effects other than the adrenergic receptor blocking effect of timolol that was systemically absorbed or penetrated locally. It is quite likely that systemically absorbed timolol reached a sufficient plasma concentration to act as a systemic beta blocking agent in the present study. According to Vareilles et al., [35] after a single instillation of 0.5% timolol into the rabbit eye, the ocular plasma concentration was 188 ng/mL at 30 minutes, which was much higher than 9 ng/mL at which timolol antagonized the positive inotropic effect of isoproterenol on isolated cat heart papillary muscles.[36] Further, in the present study, a significant reduction in pulse rate and IOP in the vehicle-treated eye was encountered after a single instillation of timolol. A significant reduction in the IOP in the vehicle-treated contralateral eyes was also encountered in the 20-day experiment. The vascular beta-2 receptor blocking effect, itself, is expected to cause vasoconstriction.[4,5,37] Under the condition that the total blood flow is not significantly changed, vasoconstriction may increase the blood velocity and consequently the NB. However the above cited result obtained after ET-1 injection eliminates this possibility: Vasoconstriction induced by ET-1 reduced both the NB and blood flow rate determined with the hydrogen gas clearance method in the ONH. The increase in the NB is thought to be indicative of not only the tissue blood velocity but also the blood flow rate through the ONH. Therefore, explanations (1) and (2), above, seem unapplicable.

In our previous report where OPP (calculated as BP_m − IOP) was decreased by increasing the IOP, we found that NB_{av} obtained from ONH tissue showed autoregulation against acute reduction in the OPP.[27] However, chronic reduction in the OPP might have different effects. In the present 20-day instillation experiment, OPP could not be calculated because blood pressure was not measured. However, the bilateral difference in IOP in the timolol-treated rabbits should be the same as in OPP. The difference in IOP between the timolol- and the vehicle-treated eyes was approximately 2 mmHg after 20-day treatment. On the other hand, OPP was approximately 70 mmHg (Table 1). Maepea[38] reported that the pressure in the anterior ciliary artery near the limbus was approximately 70% of the BP_m in monkey eyes. If we assume this relationship also in the posterior ciliary artery that supplies the ONH in rabbits,[39] the OPP in the ONH would be approximately 50 mmHg. In either case, the bilateral difference in OPP (≃4%) seems to be insufficient to fully account for that in $NB_{av(ONH)}$ (≃13%).

After instillation of timolol in one eye of a rabbit, the concentration of the drug in the posterior pole of that eye is higher than in the plasma or in the posterior pole of the contralateral eye,[40] suggesting that a part of the instilled drug reaches the posterior pole of the instilled eye by diffusion. Using high performance liquid chromatography, Acheampong et al.[41] recently reported that the optic nerve concentration after a single instillation of 0.5% timolol in albino rabbits was a maximum of 17 μmol/L, being about 30 times higher for timolol than for levobunolol, whereas the concentration in the aqueous was comparable between them. Thus, the above data may indicate that topical timolol has very high availability in the optic nerve as compared with other ocular beta-blockers. Although the data on timolol concentration in the posterior segment of the eye after long-term repeated instillations were not available, carteolol concentration in the choroid-retina and optic nerve after 20-day twice daily instillations were reported to be about 10 times those after single instillation.[42,43] Thus, it may be possible that the concentration of timolol after 20-day treatment in the ONH tissue in the timolol-treated eyes might have reached a level of 100 μmol/L, being much higher than in the plasma or the posterior pole of the vehicle-treated contralateral eyes. Hester et al.[44] reported that timolol has weak vascular relaxing properties similar to Ca^{2+} channel blockers in the porcine long posterior ciliary artery at a concentration of 100 μmol/L or higher. Although the present result that $NB_{av(ONH)}$ increased only in the timolol-treated eyes after 20-day treatment needs further study, it may be partly explained by a local effect other than beta-blocking activity on the ONH vasculature exerted by locally penetrating timolol. This beneficial direct or indirect effect of topical timolol in at least some parts of the ocular circulation has been observed also by other investigators: A single instillation of timolol reportedly caused a significant increase in the human ophthalmic artery pressure determined with compression ophthalmodynamometry;[23] a single or 2-week instillation of timolol reportedly caused a significant increase in the human retinal blood flow rate determined with laser Doppler flowmetry and monochromatic fundus photography;[12,14] or a significant reduction in the resistive index of the human central retinal artery and posterior ciliary arteries determined with color Doppler ultrasound.[22] As discussed above, the timolol concentration in the plasma was thought to reach a concentration sufficient to exert beta-activity in the present animals. Lack of effect on the $NB_{av(ONH)}$ in the contralateral vehicle-treated

eyes may suggest that vasoactive beta-2 receptors in the rabbit ONH vasculature have little physiological influence on controlling the ONH tissue blood flow.

There are species differences in ONH vasculature between rabbits and man, and rabbits may not be a good model for estimating drug effects on the human ONH circulation. However, the rabbit ONH vasculature has some similar features with that of primates;[39] the principal blood supply of the rabbit ONH is derived from the short posterior ciliary arteries by the arterial circle. Further, ocular penetration of topically applied beta-blockers including timolol and betaxolol does not differ greatly between rabbit and human eyes.[40,41,45,46] Thus, the effect of topical timolol in ONH microcirculation presently observed and probably attributable to a locally penetrating drug would be an interesting subject to be studied also in human eyes.

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