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LABORATORY INVESTIGATION

Correlation Between Individual Differences in Intraocular Pressure Reduction and Outflow Facility Due to Latanoprost in Normal-Tension Glaucoma Patients

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Abstract

Purpose: The amount of intraocular pressure (IOP) reduction achieved by the use of latanoprost eyedrops varies among patients, and there are even nonresponders. This report examines whether there is any correlation between the amount of individual variability in IOP reduction and the uveoscleral outflow facility after latanoprost eyedrop instillation in normal-tension glaucoma patients.

Methods: Sixteen normal-tension glaucoma patients (mean age, 56.4 years) were enrolled in the study to investigate the relationship between the amount of IOP reduction and outflow facility. Before treatment, subjects underwent circadian IOP measurement and then tonography, and the outflow facility was calculated. Subsequently, patients began treatment once daily with latanoprost instillation in one eye. After 4 weeks of daily latanoprost treatment, circadian IOP was measured again.

Results: Mean pretreatment outflow facility was 0.23 ± 0.05µl/min per mmHg. On average, latanoprost instillation decreased IOP by 2.8mmHg, but the reduction varied among individuals from –0.3mmHg to 5.8mmHg. No significant correlation was noted between the outflow facility and the IOP decline associated with latanoprost.

Conclusion: Because there was no significant correlation between individual IOP reduction by latanoprost and outflow facility, the differences in substantial change in uveoscleral outflow after latanoprost administration may be one explanation for the individual variation in IOP reduction after treatment with this drug. **Jpn J Ophthalmol** 2006;50:20–24 © Japanese Ophthalmological Society 2006

Key Words: intraocular pressure reduction, latanoprost, normal-tension glaucoma, uveoscleral outflow facility

Introduction

Generally, intraocular pressure (IOP) is determined by aqueous humor flow volume through the ciliary epithelium, trabecular outflow, uveoscleral outflow, and episcleral venous pressure. These factors are summarized in the Goldmann formula $Fin = C(IOP - Pv) + Fu$, where Fin is aqueous humor production, C is outflow facility, Pv is episcleral venous pressure, and Fu is uveoscleral outflow.[1] The ocular hypotensive mechanism of latanoprost is thought to result mainly from increased uveoscleral outflow, reflecting degradation of the extracellular matrix (ECM) after matrix metalloproteinase (MMP) activation by the drug.[2–5]

Although a subgroup of glaucoma patients is known to be unresponsive to latanoprost administration, the mechanism is unknown. Despite some differences between reports in the definition of latanoprost nonresponders, 5% to 25% of patients fall into this group.[6–9] IOP reduction by

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latanoprost therapy is difficult to predict, as a poor response to latanoprost is not related to baseline IOP or patient age.[9] Therefore, we first studied whether an individual difference in latanoprost IOP reduction amount exists in normal-tension glaucoma patients. Then, we measured IOP and outflow facility in the subjects to investigate whether individual differences in IOP reduction and individual differences in outflow facility were correlated.

Patients and Methods

The subjects included six men and ten women in whom normal-tension glaucoma was diagnosed between January 2002 and March 2003 by the glaucoma clinic at Gifu University School of Medicine. Diagnosis was based on reproducible visual field defects of the retinal nerve fiber layer type, corresponding optic disc excavation, normal open angles, and IOP readings within the normal range. Subjects had no previous intraocular surgery and had never received any ocular hypotensive medications. All were Japanese. The study was performed in accordance with the principles of the Declaration of Helsinki. Informed consent was obtained from all patients after provision of detailed information about the study.

Before treatment, subjects underwent IOP measurement in a sitting position every 2h for 24h using a Goldmann applanation tonometer, and they also underwent tonography (STX; Inami, Tokyo, Japan). Measurements were obtained at room temperature. Tonography was performed in a quiet environment with subjects supine, arms at their sides, after topical application of oxybuprocaine (Benoxil; Santen, Osaka, Japan). All tonography was carried out between 3 to 5 P.M. by a single experienced examiner. After these measurements, patients began treatment once daily with latanoprost instillation (Xalatan; Pfizer, Tokyo, Japan) in the eye showing the worse mean deviation in the central 30-2 program for the Humphrey Field Analyzer. After 4 weeks of daily latanoprost treatment, IOP was measured by Goldmann applanation tonometry to construct a diurnal curve including findings at 10 A.M., 12 noon, 2 P.M., and 4 P.M.. The diurnal IOP and circadian IOP were the mean of intraocular pressure readings measured for 6h, from 10 A.M. to 4 P.M., and for 24h, respectively.

Demographic and clinical data for the patients are summarized as follows. Their mean age ± SD was 56.4 ± 14.5 years (range, 32–77). The mean refractive index was –2.3 ± 3.0D (range, –8.5 to +1.4). The mean deviation (MD) and corrected pattern standard deviation (CPSD) for the central 30-2 program of the Humphrey Field Analyzer before latanoprost eyedrop instillation were –10.92 ± 8.32 dB (range, –30.54 to –1.09) and 8.3 ± 3.8dB (range, 0.0–12.9), respectively.

Statistical analysis was carried out by a paired t test, an unpaired t test, or regression analysis, as appropriate for the items evaluated. The significance level adopted was $P < 0.05$ (two-tailed test).

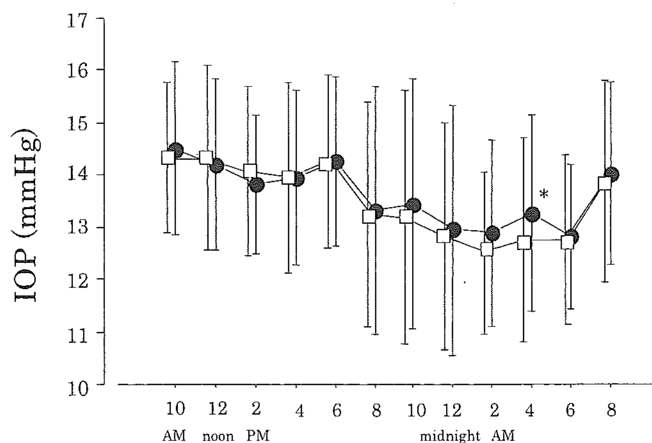

Figure 1. Pretreatment 24-h intraocular pressure (IOP) measurements. IOP did not differ significantly between the eye later to be treated with latanoprost and the fellow eye except at 4 A.M. (●, untreated eyes; □, eyes to be treated with latanoprost; *paired t test, $P = 0.0152$).

Results

The tonographically measured outflow facility was 0.23 ± 0.05 µl/min per mmHg (range, 0.14–0.31). Mean diurnal IOP, mean circadian variation, maximum and minimum circadian variation, and the circadian variation range of the pretreatment 24-h IOP were 14.1 ± 1.3mmHg (10.8–17.0), 13.6 ± 1.3mmHg (10.9–16.1), 15.6 ± 1.5mmHg (12–19), 11.4 ± 1.3 mmHg (10–14), and 4.2 ± 1.3mmHg (2–8), respectively.

IOP measured before treatment was higher in the daytime than at night. IOP in the eye assigned to subsequent latanoprost treatment did not differ significantly from that in the fellow eye at any time point except at 4 A.M. (paired t test, $P = 0.0152$; Fig. 1).

After 4 weeks of latanoprost administration, IOP was reduced significantly in the treated eye regardless of the time of measurement; IOP was approximately 2.8mmHg less than in the fellow eye (unpaired t test, $P < 0.0001$). This also was true for diurnal IOP, compared with pretreatment measurements, again by approximately 2.8mmHg (paired t test, $P < 0.0001$; Figs. 2, 3).

The average IOP reduction differed among individuals from –0.3 to 5.8mmHg. The relationship between the IOP reduction and the pretreatment outflow facility is shown in Fig. 4. The r^2 value was 0.083, and the slope of the regression line was small, indicating no statistical significance ($P = 0.2780$). On the other hand, the change in uveoscleral outflow (ΔFu) and the mean IOP reduction resulting from latanoprost were significantly correlated (Fig. 5).

Discussion

In the current study of normal-tension glaucoma patients, 4 weeks of latanoprost instillation resulted in an IOP reduction of approximately 2.8mmHg, compared with baseline

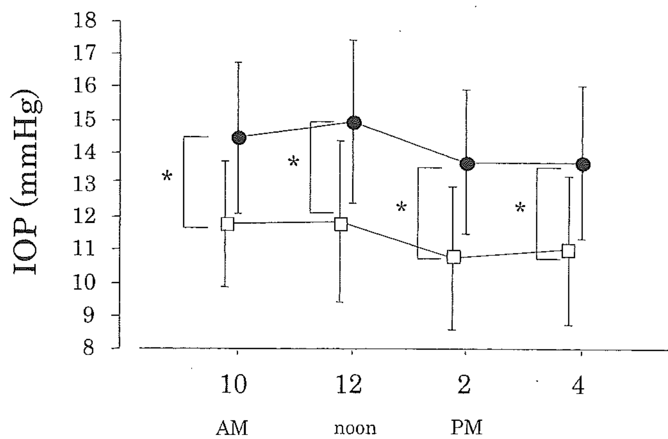

Figure 2. Diurnal IOP in the treated eyes after 4 weeks of daily latanoprost instillation was significantly lower than in the fellow eyes (●, untreated eyes; □, latanoprost-treated eyes; *unpaired t test, $P <$ 0.0001).

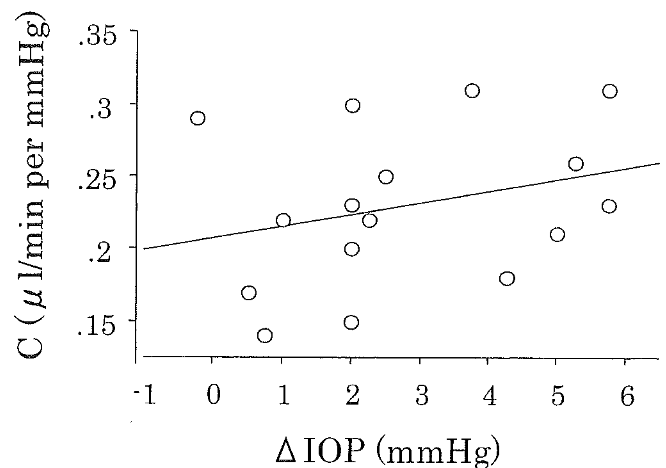

Figure 4. No correlation was seen between outflow facility (C) and the mean IOP decrease associated with latanoprost (n = 16; r^2 = 0.083, P = 0.2780).

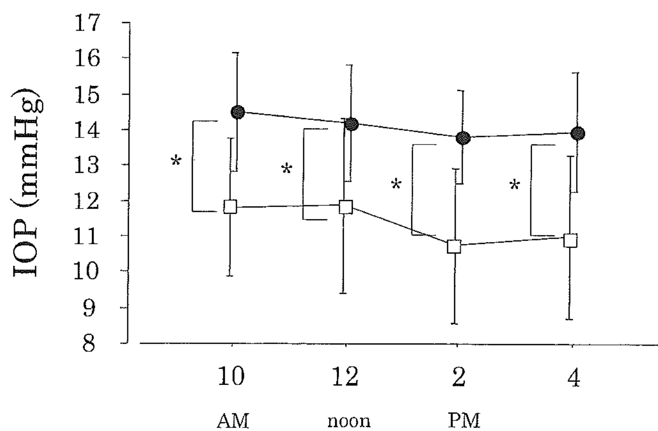

Figure 3. Diurnal IOP before and after 4 weeks of daily latanoprost instillation. Latanoprost administration significantly reduced IOP (●, before treatment; □, after treatment; *paired t test, $P < 0.0001$).

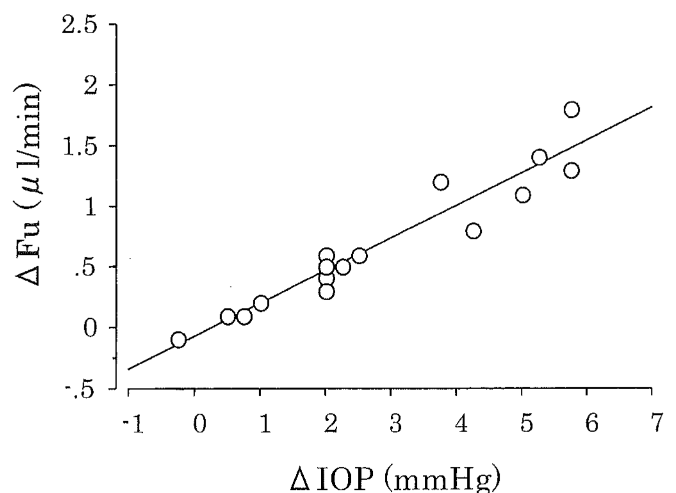

Figure 5. Correlation between and mean latanoprost-induced IOP reduction was significant (n = 16; y = 0.269x − 0.072; r^2 = 0.917, $P < 0.0001$).

measurements or with measurements in untreated contralateral eyes. Furthermore, the value of ΔFu correlated strongly with IOP reduction, suggesting that latanoprost-induced IOP reduction may depend chiefly on uveoscleral outflow. However, we could not predict the amount of IOP reduction by latanoprost prior to its administration.

Latanoprost (13,14-dihydro-17-phenyl-18,19,20-trinor-prostaglandin F_2 -isopropyl- ester), a prostaglandin F_2 derivative and selective FP receptor agonist, activates MMP. This promotes metabolic turnover and remodeling of the ECM adjacent to ciliary muscle cells, increasing uveoscleral outflow and thus reducing intraocular pressure.[2,3] Using tonography and fluorophotometry, most investigators have found no significant change in outflow facility or aqueous humor flow volume related to the instillation of latanoprost.[1,4,5,10] To our knowledge, only one study tonographically demonstrated the improvement of outflow by latanoprost.[10] The analysis performed in the current study was based on the first point of view, since most animal and human studies have indicated that IOP reduction with latanoprost monotherapy results almost exclusively from increased uveoscleral outflow.

Latanoprost instilled once daily causes a 25% to 35% IOP reduction in normal volunteers and in patients with ocular hypertension and primary open-angle glaucoma.[1,6–9,11–14] The drug continues to exert its ocular hypotensive effects throughout a 24-h period.[15–17] Our results were in agreement with such findings. Even eyes with

Table 1. Goldmann equation of aqueous humor dynamics and derived equations

Derivation steps	Equations
Goldmann equation	$Fin = C(IOP - Pv) + Fu$
Step 1, solve for IOP	$IOP = (Fin - Fu)/C + Pv$
Step 2, substitute changed values	$IOP¢ = (Fin - Fu¢)/C + Pv$
Step 3, calculate the difference and simplify	$DIOP = DFu/C$

It is assumed that the uveoscleral outflow, *Fu*, changes to *Fu¢* when *IOP* changes to *IOP¢* and that other parameters remain constant.

Fin, aqueous humor production; *C*, outflow facility; *IOP*, intraocular pressure before latanoprost; *IOP¢*, intraocular pressure after latanoprost; *Pv*, episcleral venous pressure; D*IOP*, *IOP¢* – *IOP; Fu*, uveoscleral outflow before latanoprost; *Fu¢*, uveoscleral outflow after latanoprost; D*Fu*, *Fu¢* – *Fu*.

normal-tension glaucoma showed a significant mean reduction in IOP of approximately 2.8mmHg (19.9%) after 4 weeks of latanoprost therapy, compared with the untreated eyes and the fellow eyes. In previous reports, latanoprost reduced IOP in normal-tension glaucoma patients by up to 3.6mmHg (21% to 24%).[18,19] One might expect that future therapy with a combination of agents could reduce IOP more than 30% from baseline values even in normal-tension glaucoma patients.

Aqueous humor dynamics in the steady state are defined by the Goldmann equation,[1] $Fin = C(IOP - Pv) + Fu$. If it is assumed that Pv, Fin, and C do not change between before and after latanoprost instillation and that uveoscleral outflow (Fu) changes to $Fu¢$ when IOP changes to $IOP¢$, then $\Delta IOP = \Delta Fu/C$, where $\Delta IOP = IOP¢ - IOP$ and $\Delta Fu = Fu¢ - Fu$ (Table 1). Thus, a change in the outflow facility causes changes in IOP variation, even if ΔFu is constant.

A significant correlation was evident between ΔFu and mean IOP reduction resulting from latanoprost ($y = 0.269x - 0.072$; $r^2 = 0.917$, $P < 0.0001$) (Fig. 5). Therefore, the more latanoprost reduced the IOP, the more uveoscleral outflow increased. Values of ΔFu showed a considerable range, (–0.1 to 1.8).

The correlation coefficient (r^2) between the amount of latanoprost intraocular pressure reduction (ΔIOP) and the amount of uveoscleral outflow increase (ΔFu) indicates that the two are closely related. On the other hand, outflow facility, C, was not significantly correlated with the reduction of intraocular pressure induced by latanoprost (ΔIOP). These results indicate that the IOP reduction by latanoprost in each subject depended essentially on uveoscleral outflow. Furthermore, the relative increase in uveoscleral outflow after latanoprost instillation (ΔFu) varied considerably, from –0.1 to 1.8. This may be one explanation for the variation in the amount of IOP reduction achieved by this drug.

Of the 16 patients, 1 (6.25%) had approximate ΔFu values of 0 or less, suggesting that in some individuals latanoprost fails to enhance uveoscleral outflow. Linden and Alm[20] proposed in 1998 that variation in the increase in uveoscleral outflow might reflect variation in sensitivity of the FP receptor. Alternatively, Gandolfi and Cimino[21] hypothesized that some latanoprost nonresponders might have limited ability to activate the drug by de-esterification. Finally, Tsubai et al.[22] suggested that even with pharmacologic MMP activation, metabolic turnover and remodeling of the ECM adjacent to ciliary muscle cells may differ among individuals, possibly contributing to variation in IOP reduction.

In conclusion, individual differences in increased uveoscleral outflow seemed to be responsible for individual differences in the amount of IOP reduction by latanoprost. The mechanism, however, remains unclear, and further investigation is required to address this issue.

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Action of biologically active peptides on monkey iris sphincter and dilator muscles

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Abstract

Biologically active peptides modulate pupillary responsiveness in many non-primate mammals. We examined the action of seven different peptides on iris sphincter and dilator muscles of rhesus monkey. Iris sphincter and dilator muscle preparations from rhesus monkeys were mounted in an organ bath, and tension changes were recorded by an isometric transducer. Electrical field stimulation (100 Hz, 0·3 msec, 10 V) was applied through a pair of platinum plate electrodes. Monkey iris sphincter and dilator muscles produced simple cholinergic and adrenergic excitatory responses respectively to electrical field stimulation. Strong field stimulation did not elicit slow Substance P (SP) mediated contractions like those in rabbit iris sphincter. Exogenously applied pituitary adenylate cyclase-activating peptide (PACAP) enhanced in a concentration-dependent manner (0·3 nM–0·1 μM) the sphincter response to field stimulation, while neuropeptide Y (NPY) and somatostatin (SRIF) attenuated it. These three peptides did not affect sphincter contractions induced by acetylcholine, and therefore were acting at presynaptically. SP, calcitonin gene-related peptide (CGRP), vasoactive intestinal polypeptide (VIP) and galanin (GAL) had no effect (at 0·1 μM) on iris sphincter. None of seven exogenously applied peptides had an effect on monkey iris dilator muscle. The innervation of primate irises may be relatively simple compared to non-primates because each of the peptides in this study can modulate miosis or mydriasis in non-primate mammals. Future studies will be expected on the functional significance of species differences in iridial innervation.
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Keywords: iris sphincter; iris dilator; rhesus monkey; peptide; isometric contraction

1. Introduction

The innervation of the ocular pupillary control system differs considerably among species. Universally, the iris sphincter and dilator muscles are innervated by cholinergic and adrenergic excitatory nerve fibers respectively. While these fibers induce muscle contraction, adrenergic and cholinergic inhibitory innervations produce muscle relaxation when iris sphincter and dilator muscles of dogs (Yoshitomi and Ito, 1986), cats (Schaeppi and Koella, 1964a,b), rats (Narita and Watanabe, 1981, 1982) and cattle (Suzuki et al., 1983) are contracted by electrical field stimulation. Although exogenously applied noradrenaline relaxes the monkey iris sphincter (van Alphen, 1976), little is known about the inhibitory response to field stimulation in this species. Understanding these responses can provide new insights to the physiological control of the pupillary reflex in vivo.

As with autonomic agents, iris muscle responsiveness varies among species with respect to peptidergic agents. The rabbit iris muscles are affected by substance P (SP) (Ueda et al., 1981), vasoactive intestinal polypeptide (VIP) (Hayashi et al., 1982, 1983), neuropeptide Y (NPY) (Piccone et al., 1988), calcitonin gene-related peptide (CGRP) (Haruno et al., 1996), pituitary adenylate cyclase-activating peptide (PACAP) (Yoshitomi et al., 2002), somatostatin (SRIF) (Yamaji et al., 2003b), and galanin (GAL) (Yamaji et al., 2003b). Almegård et al. (1992) showed that cholecystokinin and vasopressin contract

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the monkey iris sphincter muscle directly but SP, VIP, CGRP, SRIF and GAL have no action on it. The possibility of muscle relaxation induced by these agents still remains untested. Moreover, Firth et al. (2002) recently demonstrated rich innervation of SRIF-like immunoreactive axons within the iris sphincter muscle in monkey, necessitating further physiological studies. Therefore, this study utilized electrical field stimulation of rhesus iris sphincter and dilator muscles and direct stimulation with autonomic and peptidergic agents to determine which agents may modulate miosis and/or mydriasis at pre- or post-synaptic sites.

2. Materials and methods

2.1. Isolation and incubation of muscle specimens

The experiments were performed on seven male rhesus monkeys weighing 6·1–6·4 kg. All animals were treated in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. The monkeys were sacrificed by pentobarbital sodium (Abbott Laboratories, North Chicago, IL). The eyes were immediately enucleated and placed in Krebs solution composed of (mM): NaCl, 94·8; KCl, 4·7; $MgSO_4$, 1·2; $CaCl_2$, 2·5; KH_2PO_4, 1·2; $NaHCO_3$, 25·0; and glucose, 11·7 and gassed with 95% O_2 and 5% CO_2. After removal of the cornea, a ring-shaped iris sphincter muscle specimen (1 mm in width) or radial-shaped dilator muscle specimen (45 degrees in sector) was prepared according to the method previously reported (Yamaji et al., 2003a). The ends of each specimen were tied with 8–0 braided silk thread and mounted vertically in an organ bath (1·5 ml) by suspending one end to the isometric tension transducer (Nihon Kohden Co., TB-612T) and securing the other end to bottom of the bath. Oxygenated Krebs solution warmed to 37°C was perfused continuously (4·2 ml/min) throughout the experiment. The sphincter and dilator muscle specimens were stretched to 10 and 4 mm respectively. These lengths were defined from the length-tension relationship of each specimen obtained in the same manner as described previously for rabbits (Yamaji et al., 2003a). These lengths produce the maximum response to each stimulus. For approximately 1 hr after stretching, the sphincter and dilator muscle tones gradually declined, reaching steady-states at 13·4±5·1 and 35·4±10·5 mg, respectively.

2.2. Electrical field stimulation experiments

Electrical field stimulation was applied through a pair of platinum electrodes with 11 mm separation and placed in the organ bath so that the current pulse passed transversely across the tissue. The stimulation consisted of 10 pulses delivered at 0·3 msec/pulse, 100 Hz, 10 V, and applied every 1·5 min (Yamaji et al., 2003b). After starting the electrical stimulation, the amplitude of response gradually increased and then stabilized within 1 hr. When stable responses were attained, the following experiments were performed.

The specificity of cholinergic and adrenergic responses to field stimulation was determined by addition of 10 μM atropine and 10 μM phentolamine to the incubation media of sphincter and dilator muscles respectively (Yoshitomi et al., 1985, 1986). In rabbit sphincter muscle, strong electrical stimulation consisting of 100 pulses produces slow contractions mediated by SP in addition to fast cholinergic contractions (Yoshitomi et al., 2002). This phenomenon was examined in monkey iris sphincter by comparing the responses evoked by both 10 and 100 pulses.

The effects of seven peptides known to alter contractions of rabbit iris sphincter and dilator muscles were tested in this study ($n=4$). To determine if the peptide solutions were active, they were first applied to rabbit iris smooth muscles before executing the monkey experiments. In the monkey experiments, prior to each peptide application, the muscle was stimulated for 20 min with 10 pulses to achieve normal contractions. The peptide was then added to the perfusion medium, and the contractions were recorded for another 20 min with the same stimulation. Since the effect of each peptide reached steady-state in the first 10 min, stable effect was recorded for the last 10 min of incubation period. A peptide washout period of at least 1 hr, during which electrical stimulation was maintained, followed each test period. Normal contractions were reestablished for 20 min prior to the next test period. The concentration of each peptide was 0·1 μM, which was the highest concentration used in the rabbit experiments (Haruno et al., 1996; Yoshitomi et al., 2002; Yamaji et al., 2003b). The effect of each peptide was quantified by calculating the average ratio of 3 responses taken just before peptide application and 3 responses just before peptide washout. For controls ratios were taken from the responses of the first and last 20 min in the placebo trial. Concentration-response curves ranging from 1 nM to 0·1 μM were also obtained ($n=4$). Each specimen produced the curves for all peptides which were found to be effective at 0·1 μM.

2.3. Direct contraction experiments

These experiments were performed to determine if the peptides effective in the electrical field stimulation experiments were acting on pre- or postsynaptic sites. For many species, acetylcholine has low potency on the iris sphincter muscle (Harris et al., 1974; Hasegawa et al., 1987; Patil et al., 2003; Suzuki and Kobayashi, 1989; Yoshitomi et al., 2001). Therefore the concentration-response relationship of acetylcholine on the monkey iris sphincter was generated by applying this agonist cumulatively (1 μM–10 mM). Based upon these results, 0·3 mM acetylcholine was chosen to elicit a submaximal contraction to determine the effect of exogenously applied peptides. At this concentration, it took about 15 min to reach a steady contraction, whereupon

0·1 μM ($n=4$) of each peptide was added to the solution for 20 min. Then the peptide was washed out for at least 1 hr prior to the next peptide trial.

2.4. Data analysis

We applied one way ANOVA followed by Dunnet's test as a post hoc test, with $\alpha=0\cdot01$ as the acceptable level of significance, to compare the control and peptide-applied contractions. Concentration-response curves were calculated by fitting a sigmoidal function to the individual experimental data. The parameters of sigmoidal function were estimated using BFGS, a nonlinear optimization method (Gill et al., 1981). The median effective concentration (EC_{50}) was estimated from the function as that which produced 50% of contractile amplitude. All other results are expressed as means ± SD.

2.5. Chemicals

The following drugs and chemicals were used in this study: NPY, SRIF, GAL, CGRP, SP, VIP and PACAP 27 (all from Peptide Institute, Inc., Osaka, Japan), and phentolamine, atropine and acetylcholine (all from Wako Chemical Inc., Osaka, Japan). Peptides were prepared in aliquots and stored at −30°C.

3. Results

3.1. Electrical field stimulation experiments

Electrical field stimulation evoked a simple monophasic excitatory response in both sphincter and dilator muscles (Fig. 1). Inhibitory responses were not observed in monkey. The sphincter and dilator responses were blocked by application of 10 μM atropine and 10 μM phentolamine respectively (Fig. 1). The results indicate that the monkey sphincter and dilator muscles are innervated mainly by excitatory cholinergic and adrenergic nerve fibers, respectively. In response to strong field stimulation of 100 pulses, the sphincter muscles had only fast contractions (Fig. 2) in contrast to rabbit sphincter where both fast cholinergic and slow SP-mediated contractions occur (Yoshitomi et al., 2002).

In the experiment where seven peptides were applied, 0·1 μM PACAP significantly enhanced the response about 28·0±9·2%, while 0·1 μM NPY and SRIF significantly attenuated it about 35·5±3·4 and 12·3±7·4% respectively ($n=4$, $P<0\cdot01$, Fig. 3, Fig. 4 left). The concentration-response relationships (1 nM–0·1 μM) for these peptides were monotonic and well fitted by a sigmoidal function (Fig. 5). CGRP, SP, VIP and GAL had no significant effect on the sphincter response at 0·1 μM (Fig. 4 left). The dilator muscle contractile response to field stimulation was not affected by any peptides we examined (Fig. 4 right).

3.2. Direct contraction experiments

The effect of NPY, SRIF and PACAP on acetylcholine-induced contractions was examined. A concentration-response curve for acetylcholine was generated for sphincter muscle (Fig. 6). The EC50 estimated from the sigmoidal function was 0·54 mM (±0·15, 95% confidence interval). Since acetylcholine contraction was continuous for 35 min in each trial (see detail in Methods), partial contraction by 0·3 mM was chosen to avoid desensitization and fatigue of the muscle. After the application of NPY, SRIF and PACAP, acetylcholine contractions were 99·1±4·7%, 100·4±7·1% and 96·2±7·6% ($n=4$) respectively. Thus, none of the three peptides affected the response amplitude induced by acetylcholine ($P>0\cdot05$). This suggests that NPY, SRIF and PACAP act on presynaptic sites.

4. Discussion

Electrical stimulation evoked cholinergic excitatory contraction in sphincter muscle and adrenergic excitatory contraction in dilator muscle. Histological investigations have revealed that the iris sphincter muscle is innervated by both adrenergic and cholinergic neurons in the monkey (Nomura and Smelser, 1974) and human (Ehinger, 1966). Electric stimulation of human iris sphincter muscle, however, does not produce the adrenergic inhibitory response (Yoshitomi et al., 1988). This observation is consistent with our finding in monkey iris sphincter muscle. Moreover, similar electric stimulation developed similar amplitudes of cholinergic contractions (about 50 mg) in human (Yoshitomi et al., 1988) and monkey iris sphincter muscles. Thus, the iris sphincter muscle component of the pupillary reflex in humans and monkeys may be controlled principally by cholinergic innervation.

Monkey iris dilator muscle produced only an adrenergic excitatory response that was considerably stronger than that generated by human dilator, which produced only a few milligrams tension (Yoshitomi et al., 1985). This may relate to the fact that human iris dilator muscle also produced a cholinergic inhibitory response in addition to adrenergic excitatory response (Yoshitomi et al., 1985). Some of these differences may be attributed to different experimental techniques. In any case, the differences will need to be resolved by direct comparisons using iris dilator muscle from a variety of primates. In addition, to confirm the involvement of cholinergic and adrenergic receptors in monkey iris sphincter and dilator muscles, carbachol- and phenylephrine-induced muscle contraction should be performed in the presence and absence of atropine and phentolamine.

In previous reports, the effects of peptides on iris muscles were examined mainly in rabbit tissue, and each of the seven peptides used in this study had significant effects on the iris

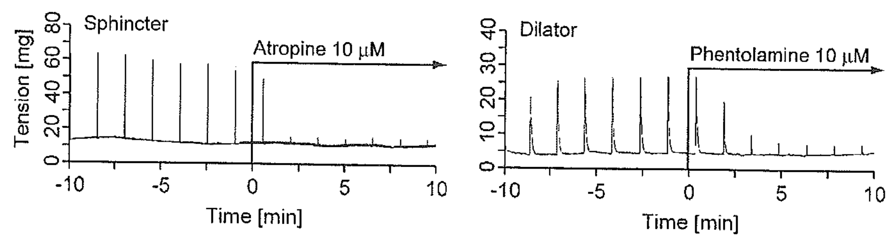

Fig. 1. Response and specificity of iris sphincter and dilator muscles to field stimulation. The sphincter and dilator muscles responded to 10-pulse field stimulations with sharp monophasic contractions. The contractions were abolished by 10 μM atropine applied to the sphincter (left) and 10 μM phentolamine applied to the dilator (right). The antagonists were present continuously after $t=0$ min.

sphincter and/or dilator muscles (Ueda et al., 1981; Hayashi et al., 1982, 1983; Piccone et al., 1988; Haruno et al., 1996; Yoshitomi et al., 2002; Yamaji et al., 2003b). SP mediates irritative ocular responses (Bill et al., 1979), and in rabbit induces strong miosis by sphincter muscle contraction (Ueda et al., 1981). Rabbit iris sphincter muscle is richly innervated with SP-like immunoreactive nerves; however, this innervation in monkey is far less dense than in rabbit (Stone et al., 1982). Consistent with these observations, monkey iris sphincter did not produce a SP-mediated response to relatively strong electrical stimulation like that present in rabbit (Ueda et al., 1981). Isolated iris sphincter muscles from baboon and human also do not respond to exogenously applied SP up to 5 μM (Unger and Tighe, 1984), and this is also consistent with our results here. CGRP coexists with SP in trigeminal nerve fibers (Kuwayama and Stone, 1987). Although CGRP inhibits iris dilator muscle contraction in rabbit (Haruno et al., 1996), this effect was not observed in monkey at the concentration we examined. This is consistent with the absence of specific binding sites for CGRP in monkey iris (Heino et al., 1995). VIP, which relaxes the iris sphincter and dilator muscles in rabbit (Hayashi et al., 1982, 1983) where there is a high density of binding sites (Denis et al., 1991), was ineffective in our experiments. This is consistent with the low levels of immunoreactivity for VIP in monkey iris muscles (Stone, 1986). For each peptide that did not elicit a detectable effect at 0·1 μM, it may be useful to determine if other concentrations do.

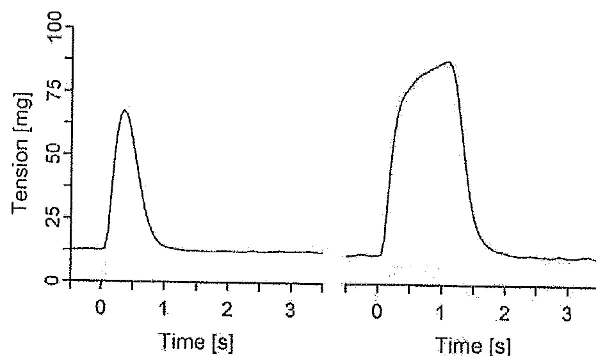

Fig. 2. Effect of weak and strong field stimulation on iris sphincter muscle. The responses to weak field stimulation (left) (10 pulses) and strong stimulation (right) (100 pulses) were both rapid and monophasic, lacking a slow component typical of the SP-mediated response. Each figure is the averaged curve of 5 responses. The gray bar at the bottom of each figure indicates the duration of electrical stimulation.

PACAP enhanced the field stimulation-mediated contraction of the monkey sphincter. PACAP-like immunoreactive nerves are localized in primate trigeminal ganglion (Tajti et al., 1999) along with cholecystokinin (Ghilardi et al., 1992), CGRP (Tajti et al., 1999), and SP (Tajti et al., 1999). Thus, PACAP, and possibly cholecystokinin, which causes contraction of human iris sphincter muscle (Almegård et al., 1992), may act in concert with several peptides of trigeminal origin to induce or modulate miosis in primates.

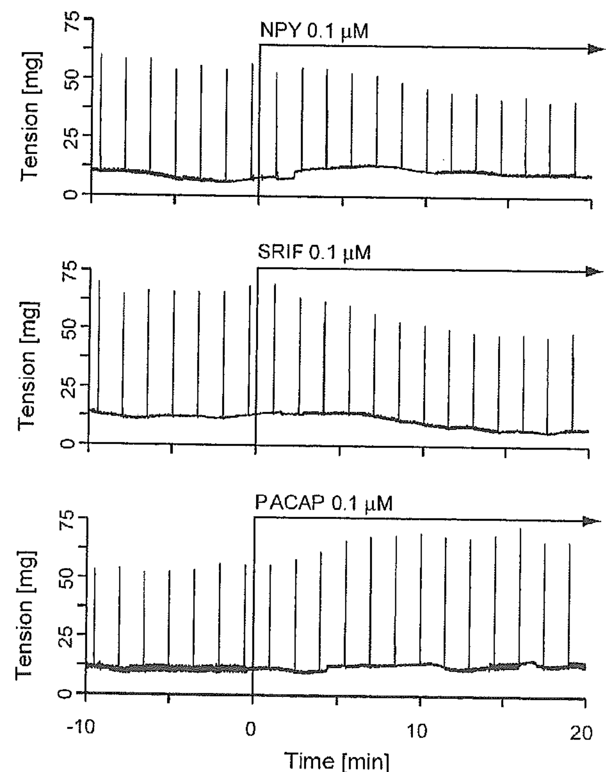

Fig. 3. Effect of NPY, SRIF and PACAP (0·1 μM) on field stimulation-induced contractions of sphincter muscles. Stimulation (10 pulses) was applied every 1·5 min. The peptide was present continuously after $t=0$ min. NPY and SRIF attenuated the sphincter response, while PACAP enhanced it without effect on the basal tone. Each peptide was applied to the same specimen after at least a 1 hr washout period.

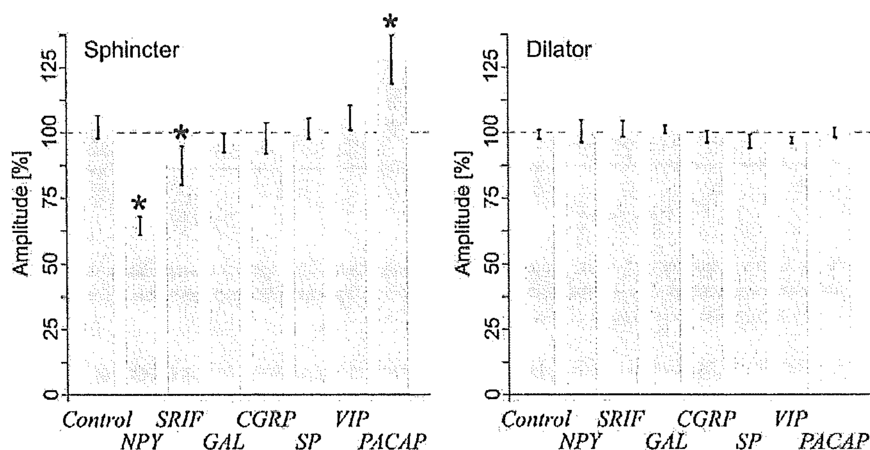

Fig. 4. Effect of seven peptides on sphincter and dilator muscles. The amplitude [%] in each specimen was calculated as the ratio of the response before (100%) and after drug application. The attenuation by 0·1 μM NPY and SRIF were 35·5 and 12·3% respectively, while the enhancement by PACAP was 28·0%. CGRP, SP, VIP and GAL had no significant effect on the sphincter. None of seven exogenously applied peptides had an effect on dilator muscle. $n=4$, $*P<0·01$.

Based upon the prominent NPY-like immunoreactive innervation of monkey dilator muscle (Stone et al., 1986), we expected a significant response to NPY. To our surprise, this was not evident. In isolated rabbit iris dilator muscle, NPY enhances phenylephrine-induced contractions, but has no direct effect on basal tone and does not alter the electrically induced contraction (Piccone et al., 1988). In the current experiments, we did not determine the effects of phenylephrine on monkey dilator muscle or test the possibility that NPY modulates the response to it. Not only NPY but also other peptides should be examined for effect on sphincter and dilator muscles after acetylcholine or phenylephrine treatment. These will be the subjects of future experiments. Compared to the dilator, the distribution of NPY-like immunoreactive nerve fibers in monkey sphincter muscle is modest (Stone et al., 1986). Our physiological results showed that NPY inhibits the sphincter muscle contraction. This suggests that functionally NPY-like containing fibers may modulate miosis or enhance mydriasis.

In rabbit, SRIF inhibits cholinergic transmission at pre-synaptic sites less than GAL does (Yamaji et al., 2003b). Here we showed that SRIF also inhibits pre-synaptic cholinergic transmission in monkey sphincter muscle and could attenuate miosis, while GAL was not effective in either muscle. These results are consistent with immunohistological observations (Firth et al., 2002) that showed SRIF-like immunoreactive axons are abundant in sphincter muscle and less so in dilator muscle, and GAL-like immunoreactive axons are only occasionally observed in either muscle in monkey.

In conclusion, we investigated the autonomic responsiveness of monkey iris muscles to electrical field stimulation and the ability of peptides to modulate the responses. There are significant species differences in peptidergic innervation of monkey and rabbit irises. The effects of histamine (Yoshitomi et al., 1995) and bradykinin (El Sayah and Calixto, 2003) may also be important. Examining the reasons for the differences may lead to

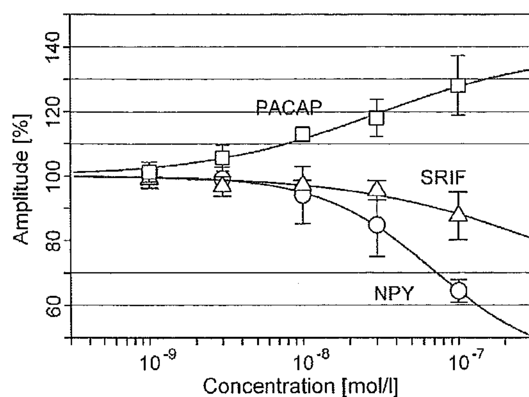

Fig. 5. Concentration-response relationship of NPY, SRIF and PACAP on the iris sphincter muscle response to electrical field stimulation. Each curve was fitted by a sigmoidal function to the data using a nonlinear optimization method. $n=4$ for each peptide.

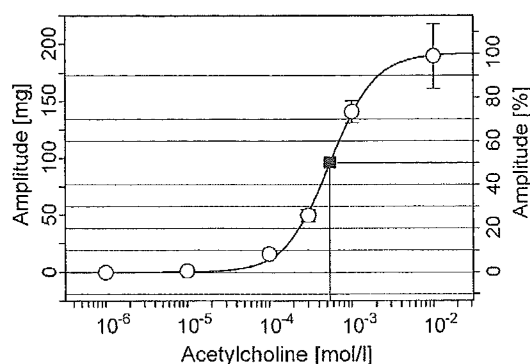

Fig. 6. Concentration-response relationship of acetylcholine on the iris sphincter muscle. Acetylcholine was applied cumulatively. Amplitude [%], indicated on the right hand axis, was normalized to the estimated sigmoidal function shown by the solid curve. The filled square indicates the median EC_{50}, 0·54 mM of which 95% confidence intervals are ±0·15 mM. $n=4$.

a better understanding of the innervation in pupillary control systems.

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Prostaglandins E_1 and E_2, but not $F_{2\alpha}$ or Latanoprost, Inhibit Monkey Ciliary Muscle Contraction

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ABSTRACT *Purpose*: To investigate the effects of prostaglandin (PG) E_1, E_2, $F_{2\alpha}$, and latanoprost acid on the electrically evoked contractile response of isolated rhesus monkey ciliary muscle. *Methods*: Longitudinal ciliary muscle preparations from rhesus monkeys were mounted in an organ bath, and tension changes were recorded by an isometric transducer. Electrical field stimulation (100 Hz, 0.3 ms, 10 V) was applied through a pair of platinum plate electrodes. *Results*: The ciliary muscle produced atropine-sensitive excitatory contraction in response to field stimulation. PGE_1 and PGE_2 (1 μM) attenuated the contraction to levels that were 68% and 65.1%, respectively, of the normal amplitude. However, $PGF_{2\alpha}$ and latanoprost acid (1 μM) did not significantly change the response amplitude. *Conclusions*: Our results indicate that $PGF_{2\alpha}$ and latanoprost acid do not interact with the prostanoid receptor involved at the pre- and/or postsynaptic site. Therefore, it is unlikely that the hypotensive action by these agents is due to relaxation of the ciliary muscle.

KEYWORDS ciliary muscle; intraocular pressure; latanoprost; prostaglandin; uveoscleral outflow

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INTRODUCTION

Prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) and its analogue latanoprost lower intraocular pressure (IOP) by increasing the uveoscleral outflow.[1–3] These agents increase production of matrix metalloproteinases,[4,5] thereby reducing collagen types I, III, and IV, which are key elements in the extracellular matrix between ciliary muscle bundles.[6,7] This structural change causes the enlargement of space between bundles, thus enhancing uveoscleral outflow.[8,9] These morphological changes occur after topical application of $PGF_{2\alpha}$ for four days.[8] In contrast to these long-term actions, a hypotensive effect by $PGF_{2\alpha}$ and latanoprost can be seen within a couple of hours after a topical application.[10,11] It is reasonable, therefore, to assume that different mechanisms underlie the long-term actions and the acute hypotensive action caused by $PGF_{2\alpha}$ and latanoprost.

Pilocarpine-induced contraction of the ciliary muscle nearly blocks the uveoscleral outflow.[12] The hypotensive action by $PGF_{2\alpha}$ is also blocked by pilocarpine,[1,13] suggesting that uveoscleral outflow is dependent on the degree

of the ciliary muscle tone. $PGF_{2\alpha}$ induces relaxation in monkey ciliary muscle, although the maximum reduction was less than 20% of the contractile force induced by a near maximal carbachol dose.[14] Latanoprost might be expected to relax the ciliary muscle because it binds much more selectively to the FP receptor than $PGF_{2\alpha}$ does.[3] However, there are no published data regarding the effects of latanoprost on ciliary muscle. Also, little is known about the effect of PGs on the ciliary muscle response to electrical field stimulation. Electrical field stimulation excites the intrinsic nerves and is a suitable method for examining the physiological effects of different treatments on the ciliary muscle. Here we have used the field stimulation method to clarify the effects of PGs, including latanoprost acid, on isolated monkey ciliary muscle.

MATERIALS AND METHODS

Isolation and Incubation of Muscle Specimens

All animals were treated in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Male rhesus monkeys weighting 4.6–6.4 kg were sacrificed by pentobarbital sodium (Abbott Laboratories, North Chicago, IL, USA). The eyes were immediately enucleated and placed in Krebs solution composed of (in mM) NaCl (94.8), KCl (4.7), $MgSO_4$ (1.2), $CaCl_2$ (2.5), KH_2PO_4 (1.2), $NaHCO_3$ (25.0), and glucose (11.7) and gassed with 95% O_2 and 5% CO_2. The ciliary muscles were dissected in the same manner as previously described for the bovine eye.[15] Briefly, we prepared longitudinal ciliary muscle specimens of approximately 2 mm in width and 5 mm in length. Previous reports showed that circular and longitudinal ciliary muscle have the same contractile properties and action in response to $PGF_{2\alpha}$ treatment.[14] The increase of uveoscleral outflow occurs primarily by morphological changes in the anterior portion of the longitudinal muscle.[8,9] Thus, we chose the longitudinal muscle for these experiments. Each end of the muscle specimen was tied with braided silk thread and vertically mounted in an organ bath (1.5 ml). One end was suspended from the isometric tension transducer (Nihon Kohden Co., TB-612T; Tokyo, Japan) and the other end was secured to the bottom of the bath. Initial tension was set at about 150 mg,[14] which gradually relaxed to a steady state of about 70 mg after 0.5–1 hr. Oxygenated Krebs solution warmed to 37°C was perfused continuously (4.2 ml/min) throughout the experiment.

Electrical Field Stimulation Experiments

Electrical field stimulation was applied through a pair of platinum electrodes placed 11 mm apart in the organ bath so that the current pulse passed transversely across the tissue. The stimulation, consisting of 100 pulses delivered at 0.3 ms/pulse, 100 Hz, and 10 V, was applied every 1.5 min. Prior to each PG application, the muscle was stimulated for 20 min to achieve normal contractions. The PG was then added to the perfusion medium, and the contractions were recorded for another 20 min using the same stimulation. The control data were obtained by recording the responses for 40 min in order to compare the responses in the first 20 min with those in the last 20 min. A washout period of at least 1 hr followed each PG application. Then, the normal contractions were repeated, and a different PG was applied. In addition to $PGF_{2\alpha}$ and latanoprost acid, PGE_1 and PGE_2 were also tested as agonists for different prostaglandin receptor subtypes. To produce the maximum relaxation, we used a concentration of 1 μM for each reagent.[14] Different concentrations ranging from 1 nM to 1 μM was also examined in case of latanoprost acid.

Chemicals

The following drugs and chemicals were used in this study: atropine (Wako Chemical Inc., Osaka, Japan), PGE_1, PGE_2, $PGF_{2\alpha}$, and latanoprost acid (all from Sigma Chemical Co., St. Louis, MO, USA).

Results

The ciliary muscle produced a simple monotonic excitatory response to the field stimulation. This response was abolished by the application of 1 μM atropine, suggesting that the response was cholinergic in origin (Fig. 1). PGE_1 and PGE_2 (1 μM) inhibited the response to field stimulation. However, inhibition was not observed after application of $PGF_{2\alpha}$ or latanoprost acid (1 μM). None of the agent had an effect on the basal tone (Fig. 2). Because the responses to PGE_1 and PGE_2 became stable within 10 min after the application, the averaged amplitude response from 10 min to 20 min (about 7 responses) was compared with the

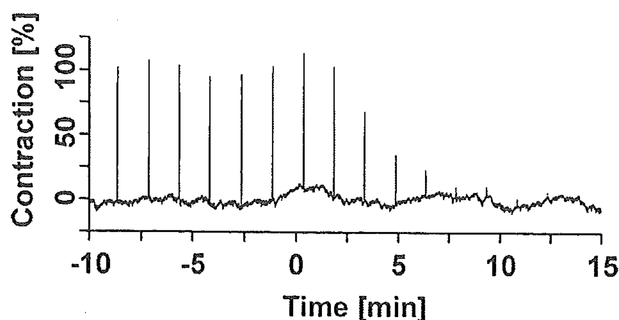

FIGURE 1 Parasympathetic innervation of ciliary muscles. Response of ciliary muscle to electrical field stimulation was abolished by 1 μM atropine. The antagonist was present continuously after $t = 0$ min. Stimulation consisting of 100 pulses was applied every 1.5 min.

control (Fig. 3). PGE_1 and PGE_2 significantly attenuated the ciliary muscle response to 68% and 65.1%, respectively, of the normal contraction amplitude. Although $PGF_{2\alpha}$ also tended to inhibit the response, its influence was not statistically significant. Latanoprost acid had no significant effect in concentrations ranging from 1 nM to 1 μM (Table 1).

DISCUSSION

This study demonstrates that whereas PGE_1 and PGE_2 attenuate the monkey ciliary muscle contraction evoked by electrical field stimulation, $PGF_{2\alpha}$ and latanoprost acid do not significantly affect the muscle response. A previous report showed that $PGF_{2\alpha}$ has the potential to induce relaxation of carbachol precontracted ciliary muscle in rhesus monkeys.[14] The maximum effect occurred when the dose was higher than 100 nM $PGF_{2\alpha}$ and the reduction was less than 20% of the near maximal contraction of 1 μM carbachol. Although the effect of $PGF_{2\alpha}$ in this study was not significant, the same trend from the previous study[14] can be observed. Latanoprost acid is a much more selective PG F (FP) receptor agonist than $PGF_{2\alpha}$.[3] If the muscle relaxation is mediated by the FP receptor, then latanoprost acid should have a greater relaxation effect at lower doses than $PGF_{2\alpha}$. However, the difference between the control and 1 μM latanoprost acid treatment data was less than 0.5%. Thus, latanoprost acid was not active in the ciliary muscle. A previous immunohistochemical study reported a low abundance of FP receptor in monkey ciliary muscle.[16] In human tissue, FP receptor is expressed more in the circular muscle of the ciliary body than in the longitudinal muscle,[17] although both vectors of ciliary muscles have the same responses to $PGF_{2\alpha}$ in monkey.[14] $PGF_{2\alpha}$ is active not only at FP receptors, but also at PG E (EP) and PG T (TP) receptors.[3,18] All factors considered, it is likely that the relaxation by $PGF_{2\alpha}$ in monkey ciliary muscle was mediated by EP receptors, as shown in cat ciliary muscle.[21] In fact, in contrast to $PGF_{2\alpha}$ and latanoprost acid, PGE_1 and PGE_2 clearly reduced the muscle contraction, suggesting that the EP receptor is functionally active in this tissue. EP_2 and EP_4 receptors are coupled to G_s and increase cyclic AMP concentration,[19] and the presynaptic EP_3 receptor inhibits the response to field stimulation.[20] Thus, these receptors may mediate the relaxant effect of PGE_1 and PGE_2, although this study did not identify which subtype is involved.

The relatively long-term hypotensive effect of $PGF_{2\alpha}$ and latanoprost on IOP is thought to result

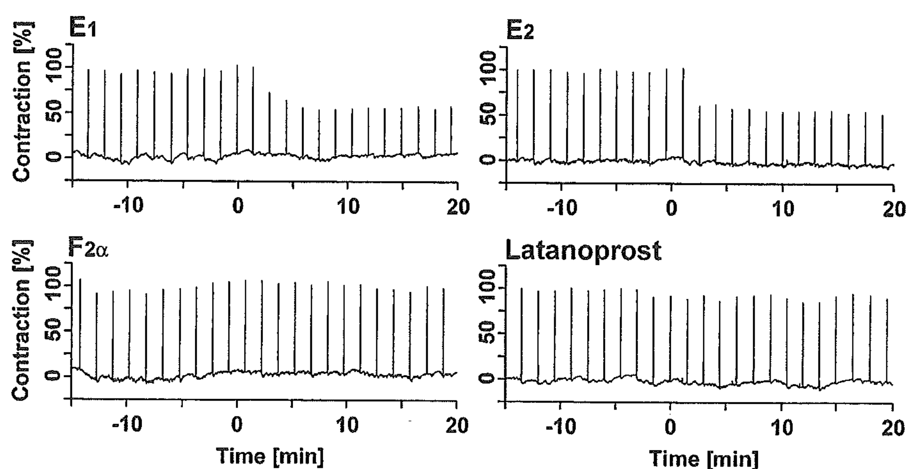

FIGURE 2 Responses of ciliary muscle before and after application of prostaglandins. Stimulation (100 pulses) was applied every 1.5 min. The prostaglandin was present continuously after $t = 0$ min. Each prostaglandin was applied to the same specimen after a washout period of at least 1 hr. Prostaglandins E_1 and E_2 clearly inhibit the ciliary muscle response to field stimulation.

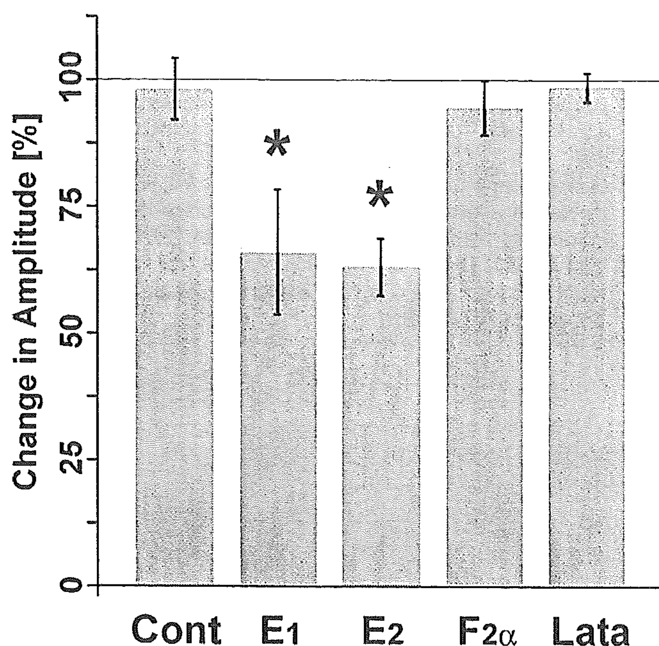

FIGURE 3 **Effect of prostaglandins on ciliary muscle. Prostaglandins E_1 and E_2 significantly attenuated the ciliary muscle response to 68% and 65.1%, respectively, of the normal contraction amplitude (*$p < 0.01$). Cont = control; Lata = latanoprost acid. Data are given as mean ± SD, n = 6.**

from the widening of space between ciliary muscle bundles.[8,9] However, short-term hypotensive action occurs within a couple of hours after treatment with these reagents.[10] Several papers hypothesized that the acute hypotensive action originates from the relaxation of the ciliary muscle.[1,13,14,21] Topical application of 10 μg $PGF_{2\alpha}$ isopropylester is enough to reduce IOP in monkeys.[11] This dosage corresponds to about 2.5 μM[14] by assuming that 1% of a topical dose penetrates[22] into 100 μl of the anterior chamber.[23] That is, the concentration adopted in this study can induce IOP reduction *in vivo*. However, $PGF_{2\alpha}$ and latanoprost acid have no significant effect on ciliary muscle contraction, suggesting that ciliary muscle relaxation cannot be a major cause of the acute hypotensive action. We currently do not have an alternative hypothesis to explain the acute hypotension. There still is a possibility that the lytic enzymes are responsible for the acute hypotension. In fact, mRNA of metalloproteinase increased at 4 or 6 hr after exposure to latanoprost acid.[24] To our knowledge, there is no published data for shorter periods of time that exactly correspond to the acute action. Further investigation will be needed to determine the mechanism of IOP reduction via $PGF_{2\alpha}$ or latanoprost.

TABLE 1 **Effect of Different Concentrations of Latanoprost Acid on Ciliary Muscle Contraction**

Latanoprost acid (mol/L)			
10^{-9}	10^{-8}	10^{-7}	10^{-6}
95.6 ± 3.2%	100.0 ± 2.7%	99.1 ± 3.8%	98.6 ± 2.9%

There is no significant difference between the control and each of latanoprost acid concentration (P > 0.05). Data are given as mean ± SD, n = 6.

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Morphometric evaluation of changes with time in optic disc structure and thickness of retinal nerve fibre layer in chronic ocular hypertensive monkeys

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Abstract

We examined the time course of changes in optic disc structure by means of a scanning laser ophthalmoscope (Heidelberg Retina Tomograph, HRT) in ocular hypertensive (experimental glaucoma) monkeys, and clarified the relationships between the histological RNFL thickness and HRT parameters. Further, the time course of changes in retinal nerve fiber layer (RNFL) thickness in individual eyes was measured using a scanning laser polarimeter with fixed corneal polarization compensator (GDx FCC). In the present study, two separate experiments were carried out. A chronic intraocular pressure (IOP) elevation was induced by laser trabeculoplasty in the left eye in 11 cynomolgus monkeys. In Experiment 1, the HRT and GDx parameters were measured 12 weeks after the laser treatment in 10 eyes in five monkeys. In Experiment 2, the time course of changes in the HRT and GDx parameters was examined before and 1, 3, 4, 5, 6, 8, 10, 12, 14, and 16 weeks after the laser treatment in 12 eyes in six monkeys. The retardation values (thickness parameters) obtained from the GDx were used to derive thickness and ratio parameters in the superior, inferior, nasal and temporal quadrants. Ratio parameters were expressed as a ratio of superior and inferior quadrant to nasal quadrant. After the last measurements, each eye was enucleated, and retinal cross sections were prepared for histological analysis.

In the left (hypertensive) eyes, IOP was persistently elevated throughout the observation periods in both Experiments 1 and 2. In the HRT measurements in Experiment 1, seven out of eight global topographic parameters (exception, disc area) were statistically different between the hypertensive and control eyes 12 weeks after the laser treatment. In Experiment 2, the HRT parameters changed in a time-dependent manner, but each of them almost plateaued at about 4 weeks after the laser treatment. Significant correlations were seen between the histological mean RNFL thickness at 1.5 disc diameters from the optic disc margin and the HRT parameters in 21 eyes from 11 monkeys in Experiments 1 and 2. Especially good correlations with histological mean RNFL thickness were seen for the rim volume and cup volume.

In Experiment 1, good correlations were found between GDx ratio parameters and histological RNFL thickness in individual right control eyes ($n=5$). In individual left experimental glaucoma eyes of Experiment 2 ($n=6$), GDx ratio parameters declined in a time-dependent manner alongside the IOP elevation.

In conclusion, alongside the IOP elevation, time-related changes in optic disc topography and RNFL thickness were demonstrated in monkey eyes using HRT and GDx. HRT (rim and cup) parameters showed good correlations with histological RNFL thickness, and significant interrelations.
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Keywords: ocular hypertensive monkey; optic disc; retinal nerve fibre layer; scanning laser ophthalmoscope; scanning laser polarimeter

1. Introduction

Open-angle glaucoma (OAG) is a slowly progressive and chronic disease. To diagnose OAG, especially in its early stage, evaluation of the appearance of the optic nerve head and peripapillary retina is most important. For objective

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examination of these structures, the confocal scanning laser ophthalmoscope (Heidelberg retina tomograph, HRT; Heidelberg Engineering GmbH, Heidelberg, Germany) and scanning laser polarimeter (GDx; Laser Diagnostic Technologies, San Diego, CA, USA) have been developed. Analysis of HRT images allows quantitative three-dimensional optic disc topography (e.g. measurement of cup volume and rim volume), and HRT has been widely used as a research tool in topographical analysis of the optic nerve head (Malinovsky, 1996). The GDx is a scanning laser polarimeter for measuring the thickness of the peripapillary retinal nerve fibre layer (RNFL), which is directly affected by glaucoma, and this instrument can provide an objective assessment of optic nerve fibre layer thickness. The data obtained from these devices show correlations with the visual field defect in patients with OAG (Iester et al., 1997; Chen et al., 1998), and they can discriminate with certain sensitivity and specificity between eyes with glaucoma and normal eyes (Weinreb et al., 1995a; Essock et al., 2000; Wollstein et al., 2000).

Experimentally, the laser-induced ocular hypertensive monkey is a widely used animal model of glaucoma (Pederson et al., 1984; Fukuchi et al., 1992; Quigley et al., 1996). Morgan et al. (1998) reported that in a single normal monkey, the value for RNFL thickness obtained from the Mark II Nerve Fiber Analyzer (NFA, Laser Diagnostic Technologies), a prototype GDx, correlated with the histological one, while Yücel et al. (1998) noted that HRT parameters correlated with the number of optic nerve axons in 10 laser-induced ocular hypertensive monkeys. However, these studies were carried out after the glaucomatous changes had been established, and there is little information in the literature as to how the RNFL thickness alters alongside the IOP elevation. In a longitudinal study, Burgoyne et al. (1995) made a detailed examination of optic disc surface changes using digital ocular funduscopy and confocal scanning laser tomography (TopSS, Laser Diagnostic Technologies), and concluded that early changes in the optic disc surface are unlikely to be due to axon loss alone, but to damage to the load-bearing connective tissues of ONH. However, to our knowledge, no study has yet examined both the optic disc surface and RNFL thickness changes with time alongside an IOP elevation. Further, few studies have compared the in vivo HRT findings obtained just before sacrifice with findings derived from post-mortem histological examination of the retinal nerve fibre layer.

The current study had two aims. One was to examine how optic disc topography and RNFL thickness might alter alongside a chronic IOP elevation in laser-induced ocular hypertensive (experimental glaucoma) monkey eyes. The other was to compare optic disc topography (as determined using HRT) just before sacrifice with the histological findings obtained in the same eyes in a large enough number of monkey eyes for the comparison to be scientifically valid.

2. Materials and methods

2.1. Animals

A total of 11 young adult cynomolgus monkeys (*Macaca fascicularis*) weighing 4.0–6.0 kg aged 5–6 years (Keari Co. Ltd, Osaka, Japan) obtained from the same colony at the same time were used to keep the animal conditions uniformly. They were housed in an air-conditioned room at 24±2 °C with 60±10% humidity, and given food and water ad libitum. All investigations were in accordance with the guideline of the Statement on the Use of Animals in Ophthalmic and Vision Research, and were approved and monitored by the Institutional Animal Care and Use Committee of Santen Pharmaceutical Co. Ltd.

2.2. Induction of experimental ocular hypertension (experimental glaucoma)

An elevation of intraocular pressure (IOP) was induced in each monkey by applying the argon-laser photocoagulation burns to the trabecular meshwork of the left eye, with the right eye being used as an untreated control, as previously described (Quigley and Hohman, 1983). The laser irradiation was performed only on the left eyes, because we considered that laser irradiation on the ipsilateral eyes under the same conditions would facilitate accurate irradiation without technical dispersion. For the laser treatment, the animals were anaesthetized with an intramuscular injection of ketamine (8.75 mg kg^{-1}, Ketalar 50®; Sankyo, Tokyo, Japan) plus xylazine (0.5 mg kg^{-1}, Celactal®; Bayer, Leverkusen, Germany). Then, a single-mirror Goldmann lens filled with a hydroxyethylcellulose solution (Scopisol®15; Senjyu Pharmaceutical, Osaka, Japan) was placed on the eye to be treated. An argon blue/green laser was focused on the mid-portion of the trabecular meshwork, and a total of 150 laser-beam spots were applied around 360° (spot size, 100 μm; power, 1000 mW; exposure time, 0.2 sec) using an argon-laser photo-coagulator (Ultima 2000 SE®; Coherent, Inc., CA, USA) attached to a standard slit-lamp microscope (BQ 900; HAAG-STREIT, Köniz, Switzerland). Two weeks after the first treatment, the laser treatment was repeated so as to produce a maintained elevation in IOP. Time (in weeks) 'after the laser treatment' should be understood to date from the first of these treatments.

2.3. Experimental procedure

In this study, two separate experiments were carried out. In the first series (Experiment 1), five experimental glaucoma monkeys were used for comparison of the HRT and GDx results and fundus photographs with postmortem histological findings obtained 12 weeks after the laser treatment. IOP was measured (see below) before and at 3, 4, 6, 8, 10, and 12 weeks after the laser treatment. Baseline

IOP was taken as an average of three measurements made at 1-week intervals before the first laser treatment. All IOP measurements were carried out at the same time of day. After the last measurement, the animals were sacrificed, both eyes enucleated, and retinal cross sections prepared for histological analysis.

In the second series (Experiment 2), six monkeys were used for a study of the time course of changes in the HRT and GDx data in the experimental glaucoma eye (vs. values for the control eye) during a 16-week period after laser treatment. HRT and GDx data, and fundus photographs, were obtained before and at 1, 3, 4, 6, 8, 10, 12, 14, and 16 weeks after the laser treatment, while IOP was measured before and at 1, 2, 3, 4, 6, 8, 10, 12, 14, and 16 weeks after the laser treatment. Baseline values were taken as the average of three measurements made at 1-week intervals before the laser treatment. All IOP measurements were carried out at the same time of day. In the Experiment 2, after the last measurement, animals underwent the same procedures as in Experiment 1.

IOP was measured in both eyes of each animal using a calibrated pneumatonometer (Model 30 Classic Pneumatonometer; Medtronic Solan, FL, USA) under ketamine anaesthesia (8.75 mg kg^{-1}, i.m.), with local anaesthesia being produced using 0.4% oxibuprocaine hydrochloride (Benoxil® 0.4% solution; Santen Pharmaceutical Co. Ltd, Osaka, Japan).

Examinations (including refractometry, keratometry, fundus photography, confocal scanning laser imaging with HRT, and scanning laser polarimetry with GDx) were carried out under intramuscular ketamine (8.75 mg kg^{-1}) plus xylazine (0.5 mg kg^{-1}), and are described below in more detail.

2.4. Analysis of optic disc topography

For this analysis, the Heidelberg Retina Tomograph (HRT; Heidelberg Engineering, Heidelberg, Germany), a confocal scanning laser ophthalmoscope, was used. Just before image acquisition, the refraction and corneal curvature radius of each eye were measured using an Auto Ref/Keratometer (ARK-700A; NIDEK Co. Ltd, Aichi, Japan), and the values were entered into the Patient Data sub-menu for the examined eye to correct for magnification effects on the images. All measurements were made by the same operator (M.S.). Three images (10° field of view) of each eye were obtained by one experienced operator (M.S.), and for each pixel location, the mean of three topographic image height measurements was calculated, as previously described (Weinreb et al., 1993; Yücel et al., 1998). A good-quality mean topographic image was used; image quality of serial original images of each eye was judged qualitatively by two independent experienced observers (G.T., T.T.) and only three images judged to be of good quality were used for creating the mean image, and the mean image adopted was the one in which the optic disc morphology was clearly visible, with an overall standard deviation of less than 30 μm. The outline of the optic disc margin at the inner edge of the scleral ring was drawn by an experienced observer (G.T.) using a computer mouse system. Then, the Stereometric Measurements program (HRT software version 2.01; Heidelberg Engineering) calculated a number of predefined shape parameters.

2.5. Retinal nerve fibre layer analysis

A scanning laser polarimeter (Nerve Fiber Analyzer GDx; Laser Diagnostic Technologies, San Diego, CA, USA) was used for measuring the thickness of the retinal nerve fibre layer (RNFL). Details of its operation have been published elsewhere (Weinreb et al., 1990, 1995a,b). Briefly, the light source (a polarization-modulated laser beam; wave length, 780 nm) was directed at one point on the retina, and the reflected light (which double-passes the RNFL) was detected to obtain the retardation at that point. The laser beam was directed sequentially over each of a number of 256×256 pixels retinal locations with a field of view of approximately 15° to obtain a retardation map in which each pixel had a corresponding retardation value. All scannings were performed by the same operator (T.T.). Three retardation maps of the peripapillary retina were taken for each eye, and a baseline map was created by averaging the three retardation values corresponding to each pixel. The disc margin was drawn by an experienced operator (T.T.), who placed an ellipse to outline the inner margin of the peripapillary scleral ring. A good-quality image was used; image quality of serial original images of each eye was judged qualitatively by two independent experienced observers (G.T., M.S.) and only three images judged to be of good quality were used for creating the mean image, and the mean image adopted was the one in which the retardation map was clearly visible, with an overall standard deviation of less than 8 μm.

A measurement ellipse was then generated by the GDx instrument at 1.5–2.1 disc diameters away from and concentric with the margin of the optic disc. For analysis, we calculated thickness and ratio parameters from the pixels located within the measurement ellipse, as previously described (Xu et al., 1998). Retardation values (thickness parameters) were averaged over each 10° sector, for a total of 36 sectors. The 0° meridian is at 03:00 in the left eye and at 09:00 in the right eye. Data were downloaded into an attached personal computer and translated to a spreadsheet software program (Microsoft Excel 2000; Microsoft Corporation, Redmond, WA, USA) for analysis. The circumference was divided into four 90° quadrants: superior (50–139°), nasal (140–229°), inferior (230–319°), and temporal (320–49°), as previously described by Xu et al. (1998). In each of the nine 10° sectors in each quadrant, the superior, nasal, inferior, or temporal parameters were averaged to give the mean thickness parameter for that quadrant, and the sum of the superior and inferior thickness

parameters was termed S+I. The sum of the values obtained for the nine 10° sectors in the superior, inferior, or superior plus inferior quadrants was divided by the sum of those obtained for the nine 10° sectors in the nasal quadrant to give the ratio parameters, RaSN, RaIN, and Ra(SN+IN), respectively.

2.6. Histological examination

After the last measurements, animals were perfused via the common carotid artery with 0.5 l of 0.9% saline containing 10 U ml^{-1} heparin at room temperature, followed by 0.5 l of 4% paraformaldehyde in 0.01 mol l^{-1} phosphate-buffered saline (PBS; pH 7.4). This was done under deep sodium pentobarbital anaesthesia (30 mg kg^{-1}, i.v.; Nembutal; Abbott, North Chicago, IL, USA).

After enucleation, posterior portions of eyes were washed three times with PBS (pH 7.4), and cut to yield an area of retina with choroid and sclera (approximately 100 mm^2 and centred on the optic disc). They were fixed in 2.5% glutaraldehyde and 2% paraformaldehyde in PBS (pH 7.4) for at least 48 h at 4 °C, then embedded in paraffin. When posterior portions of the eyes were embedded in paraffin, they were aligned to correspond to the GDx image with blood vessels as an index in each eye. As shown in Fig. 1, retinal cross sections containing the optic disc were cut vertically at 100 μm intervals (each section, 3 μm thickness), then stained with haematoxylin and eosin. In five right control eyes in five monkeys in Experiment 1, the RNFL thickness from histological specimens was measured at seven locations between 1.5 and 2.1 disc diameters in 0.1 disc-diameter increments away from the outer edge of the optic disc rim, and the mean RNFL thickness in superior and inferior quadrants was calculated to allow comparison with the values for GDx parameters obtained in each disc diameter in the same eye just before sacrifice.

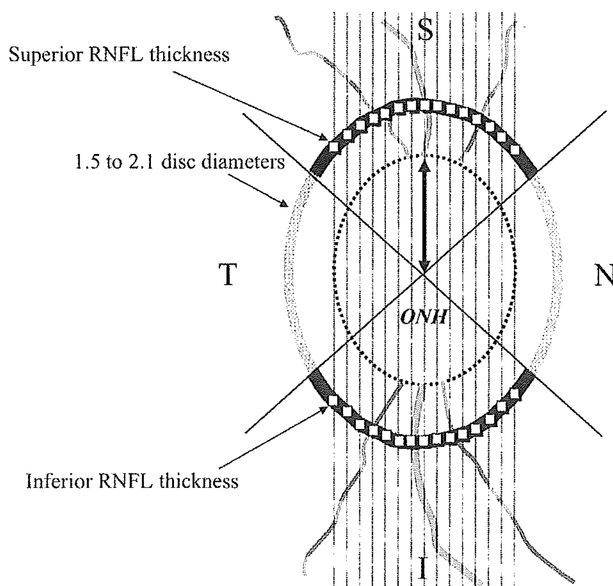

Fig. 1. Tissue processing for the analysis of histological mean retinal nerve fibre layer (RNFL) thickness. Vertical cross sections were prepared at 0.1 mm intervals, and their thickness at 1.5–2.1 disc diameters away from the optic nerve head (ONH) was measured in the superior (S) and inferior (I) quadrants. Mean thickness parameter was calculated for each of these quadrant. T, temporal quadrant; N, nasal quadrant.

The RNFL thickness in histological specimens was measured only at 1.5 disc diameters away from the edge of the optic disc in five left experimental glaucoma eyes (excluding one with corneal opacity) and six right control eyes of six monkeys in Experiment 2.

2.7. Statistical analysis

Data are expressed as mean ± SE. In Experiment 1, statistical analysis of the experimental glaucoma versus control eyes was performed using *t*-test with or without the modification of an Aspin-Welch as appropriate. A repeated-measures analysis with a general linear mixed-model for estimating an interaction effect between group (left experimental glaucoma and right control eyes) and time was used to examine the time course of changes in IOP in both experiments and in the HRT parameters in Experiment 2 using the Proc Mixed of the SAS package (SAS ver. 8.2, SAS Institute, Inc., Cary, USA). If the interaction effect was significant ($p<0.05$), the *t*-test with Bonferroni's correction was carried out at each time-point for comparison between the experimental glaucoma and control eyes, and a paired Dunnett's multiple-comparison test was performed for comparison between the pre-laser treatment and post-laser treatment values. Pearson correlation coefficient was calculated between the mean RNFL thickness in histological specimens and the HRT parameters or between GDx and HRT parameters in a total of 21 eyes of 11 monkeys, and between the mean RNFL thickness in histological specimens and the GDx parameters in each of five right control eyes in Experiment 1, after confirming no significant deviation from normal distribution on scatter plotting of data. Results were considered to show significant difference if $p<0.05$.

3. Results

3.1. Intraocular pressure (IOP)

In Experiment 1, the baseline IOP (before laser treatment) was 19.1 ± 0.7 mmHg ($n=5$) in the right eye and 18.7 ± 1.0 mmHg ($n=5$) in the left eye. In the experimental glaucoma eyes, IOP was persistently elevated throughout the 12 weeks after laser treatment, being statistically significant after 3, 4, 6, 8, 10, and 12 weeks versus the IOP of the right control eye or the pre-laser treatment value (Fig. 2). There was a significant interaction effect ($p<0.001$) between the group treatments and the time for IOP. In contrast, there were no significant changes