Route of Penetration of Topically Instilled Nipradilol into the Ipsilateral Posterior Retina

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Purpose. To investigate how topically instilled nipradilol penetrates the ipsilateral posterior retina-choroid in normal rabbit eyes.

Methods. Albino rabbits were used. Topical instillation (1%, 100 μL) or intracameral (0.1%, 100 μL) or sub-Tenon injection (0.1%, 10 μL) of [^{14}C]nipradilol was performed in one eye. Ocular and periocular distribution and the concentrations of [^{14}C]nipradilol were determined by whole-head autoradiography, the results of which were validated by measurements in isolated tissues. In addition, the unchanged form of nonradiolabeled nipradilol in the posterior retina after topical instillation (1%, 100 μL) was quantified by liquid chromatography-tandem mass spectrometry (LC/MS/MS).

Results. Autoradiography revealed that the nipradilol concentration after topical instillation was higher in the ipsilateral posterior retina-choroid than on the contralateral side (142.9 ng/g vs. 108.3 ng/g, $P = 0.026$), and in the periocular tissue around the optic nerve insertion on the ipsilateral side than on the contralateral side (207.1 ng/g vs. 141.1 ng/g, $P < 0.001$). After intracameral injection, radioactivity was observed only in anterior, but not posterior parts of the eye. Radioactivity was observed only in the ipsilateral posterior retina-choroid and periocular tissues around the optic nerve insertion after sub-Tenon injection. The results in the isolated tissues validated autoradiographic evaluations.

Conclusions. These results suggest that diffusion from posterior periocular tissues across the posterior sclera may be the main route for local penetration of the instilled drug to reach the posterior retina-choroid in albino rabbits. (*Invest Ophthalmol Vis Sci.* 2009;50:2839–2847) DOI:10.1167/iovs.08-2922

Local penetration of topically instilled drugs into the posterior segments of the eye at pharmacologically active levels is of clinical interest. It was thought that topical instillation could not convey drugs to the posterior segments of the eye at pharmacologically active levels because of the long diffusion.[1–3] In recent studies, however, many investigators, but not all, have suggested that topically instilled ophthalmic drugs could reach the posterior segments at pharmacologic levels in experimental animals.[4–10] We previously reported that unilateral instillation of nipradilol, which has vasodilating effects,[11] inhibited intravitreously injected endothelin (ET)-1–induced constriction in only the ipsilateral retinal arteries in albino rabbit eyes.[12] A similar result was obtained by Ishii et al.,[13] who used iganidipine, a water-soluble dihydropyridine-derivative Ca^{2+} channel blocker in pigment rabbits and monkeys. These results suggest that the local penetration route is very important for some topically instilled ophthalmic drugs to reach the posterior vitreous–retina interface in pharmacologically active levels, since systemic absorption alone cannot explain the significant effect in only the ipsilateral eye; however, the penetration route of topically instilled drugs to the ipsilateral posterior parts is unknown.

Previous studies suggested three possible local penetration routes from the tear layer to the ipsilateral posterior retina: (1) a periocular and transposterior sclera route: conjunctival cul-de-sac → periocular Tenon tissue → posterior sclera → posterior choroid, and then the retina[14,15]; (2) a transvitreous route: cornea → anterior chamber → vitreous → posterior retina[4,5,7]; and (3) an uveal route: cornea → anterior chamber → anterior choroid → posterior choroid, and then the retina (Fig. 1).[16] Permeability of isolated tissue such as the cornea, sclera, or conjunctiva to drugs has been well documented,[17–20] but to determine the distribution of drugs after topical instillation in physiological states, experiments using living eyes are needed.

Nipradilol has nonselective β-, and selective α_1-receptor, blocking properties[21–26] with nitric oxide (NO) donative action[11] and a molecular weight (326.35) and octanol/buffer ratio (0.79 at pH 7.3) close to those of timolol,[27] a representative antiglaucoma ophthalmic solution. In an attempt to elucidate the penetration route of topically instilled drugs to the posterior parts of the eye, we determined the ocular and periocular distribution and the concentration of radiolabeled total nipradilol after its topical instillation, intracameral injection, and sub-Tenon injection in albino rabbits by whole-head autoradiography.

Methods

All experiments were performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Twenty-nine male Japanese White (JW) rabbits (10 weeks old; Japan Laboratory Animals Inc., Tokyo, Japan) were used to evaluate the penetration pattern and the concentration of [^{14}C]nipradilol by topical, intracameral, or sub-Tenon injection. Another six rabbits were used to measure the concentration of unchanged, nonradiolabeled nipradilol in the isolated posterior retina. In addition, three male Sprague-Dawley rats (7 weeks old; Japan Laboratory Animals, Inc.) were used to make standard curves to quantify the radioactivity concentration of [^{14}C]nipradilol.

Standard Curve for Autoradiographic Measurements

[^{14}C]nipradilol (code CFQ11032, radiochemical purity 98%, specific radioactivity 1.55 MBq [43 μCi]/mg) was obtained from Amersham Pharmacia Biotech (Buckinghamshire, UK). To determine the [^{14}C]nipradilol concentration in the biocomponent of the rabbit whole-head

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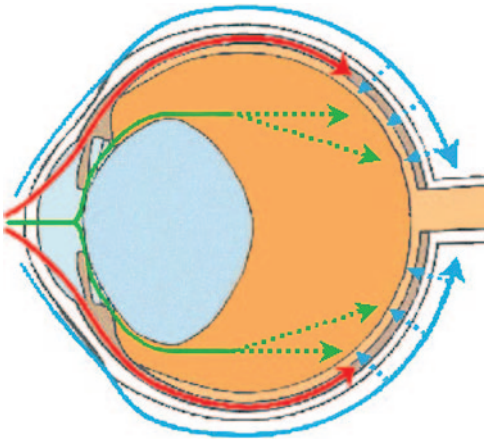

FIGURE 1. Three possible local penetration routes of topically instilled nipradilol from the tear layer to the ipsilateral posterior retina-choroid. (1) Periocular and transposterior scleral route (*blue arrow*); conjunctival cul-de-sac → periocular Tenon tissue → posterior sclera → posterior choroid, and then retina; (2) transvitreous route (*green arrow*); cornea → anterior chamber → vitreous → posterior retina; (3) uveal route (*red arrow*); cornea → anterior chamber → anterior choroid → posterior choroid, and then the retina.

autoradiogram, we established standard samples under similar conditions using rat liver homogenate. In detail, rats were euthanatized and the liver was isolated, homogenized, and mixed with five concentrations (10, 100, 1,000, 10,000, and 100,000 Bq/g) of [^{14}C]nipradilol. These standard samples were dissolved in 2 mL of tissue solubilizer (Soluene-350; Perkin Elmer, Wellesley, MA), and the concentration of [^{14}C]nipradilol in each standard sample was measured with a liquid scintillation counter (Tri-Carb Liquid Scintillation Analyzer 2700TR; Perkin Elmer). These samples were mounted in a whole-head tissue block made with a 3% carboxymethyl cellulose (CMC) gel described later, and cut into 30-μm sections at −15°C with a cryomacrotome (Cryomacrocut; Leica Micro Systems, GmbH, Nussloch, Germany). Each section was exposed to an imaging plate (BAS-III; Fuji Photograph Film, Tokyo, Japan) for 72 hours to develop an autoradiogram visualized with a bioimaging analyzer (Fujix BAS2500; Fuji Photograph Film). This device can detect the radioactivity concentration in each section as the intensity of photostimulated luminescence (PSL) and visualize the radioactivity concentration as a color image. In addition, each section was exposed again to an imaging plate for 2 weeks to develop an autoradiogram for radioactivity that could not be detected after 72 hours' contact. Finally, we made two standard curves for autoradiographic measurements.

Topical Instillation and Intracameral and Sub-Tenon Injection Studies

In the topical instillation study, [^{14}C]nipradilol (1%, 100 μL, 1.5 MBq [41 μCi]/dose) was instilled into the lower cul-de-sac of the right eye. Ten milligrams per kilogram indomethacin (Sigma-Aldrich, St. Louis, MO) was injected into the abdominal cavity 2 hours before the intracameral or sub-Tenon injection. For the intracameral injection, 100 μL of aqueous humor was carefully and slowly drained with a 30-gauge needle, and replaced with an equal volume of [^{14}C]nipradilol (0.1%, 0.15 MBq [4.1 μCi]/dose) in artificial aqueous humor (Opeguard; Senju Pharmaceutical, Inc., Osaka, Japan) in the right eye. For the sub-Tenon injection, a 30-gauge needle was inserted subconjunctivally at the 11 o'clock position 2 mm from the limbus, and 10 μL [^{14}C]nipradilol (0.1%, 0.015 MBq [0.41 μCi]/dose) in artificial aqueous humor was injected into the sub-Tenon space under the superior rectus muscles in the right eye. The contralateral eye was left untouched to serve as a control. Fifteen and 60 minutes after the application, the rabbits were euthanatized by an intravenous injection of pentobarbital sodium (Nembutal; Abbott Laboratories, North Chicago, IL). The autoradiography method was essentially the same as described by Ullberg[28] and used previously.[14] Briefly, the head was immersed in hexane (minus 80°C using solid carbon dioxide) for 20 minutes. Immediately thereafter, the rabbit was decapitated, and the head was stored overnight at −15°C in a cryomacrotome container, to evaporate the hexane from it. After the fur was removed, the head was mounted in 3% CMC gel to make a tissue block and cut in 30-μm sequential sections with a cryomacrotome. Sections cut through the midline of lenses and optic nerve heads (ONHs) were used for autoradiography. Each section was exposed to an imaging plate for 72 hours or 2 weeks to develop an autoradiogram, visualized with a bioimaging analyzer.

In the central part of the cornea, the anterior chamber, posterior side of the lens, posterior side of the vitreous, equatorial and posterior retina-choroid, and periocular tissues around the equator and around the optic nerve insertion, quantitative analysis was performed of the [^{14}C]nipradilol concentration on the treated and contralateral control sides at 60 minutes after drug application. All measurements were performed with the highest resolving power of the image analyzer (1 PSL = 50 μm). The positions of the equatorial and posterior retina-choroid were defined as the 2-mm length of the equator and next to the ONH of the retina-choroid, respectively. Periocular tissue around the equator was defined as an approximately 1-mm^2 extraocular triangular area between the orbital bone and sclera. Periocular tissue around the optic nerve insertion was defined as an approximately 1-mm^2 area just behind the sclera around the optic nerve insertion. (See Fig. 3A for the location of each position on the treated eye.) Densitometric readings obtained from autoradiography by a masked investigator were converted into radioactivity with a standard curve developed on an imaging plate from rat liver homogenate as described earlier and quantified by the following equation:

$$\text{Concentration of radioactive nipradilol} = (\text{PSL} - \text{BG})/\text{A}$$

where PSL is photostimulated luminescence, BG is background PSL, and A is the area of each tissue on the bioimaging analyzer.

Determination of nipradilol concentrations in the posterior vitreous and retina-choroid isolated from enucleated frozen eyes after topical instillation or intracameral or sub-Tenon injection of [^{14}C]nipradilol was also performed to validate the autoradiographic measurement results. We enucleated the eye through the orbital skin, bisected the frozen globe into anterior and posterior cups, and isolated the tissue from the vitreous side on which radioactivity was thought to be lowest.[12,14] Briefly, topical instillation or intracameral or sub-Tenon injection of [^{14}C]nipradilol was performed as described earlier and, 60 minutes after application, each globe was enucleated with the eyelids and surrounding tissues, as a pouch and frozen immediately. Posterior surrounding connective tissues were removed, and the frozen globe was cut circle-wise with a specially designed small circular saw to divide the posterior from the anterior cups at a radius of 5 mm with the optic nerve at the center. Vitreous and then retina-choroid were exfoliated from the vitreous side of the posterior cup. All procedures were performed under frozen conditions to avoid possible contamination during tissue isolation. In addition, it was confirmed that no radioactivity was present in the hexane used to freeze the eyeballs. Finally, isolated vitreous and retina-choroid were dissolved in a tissue solubilizer and radioactivity was measured with a liquid scintillation counter.

Concentration of Unchanged Nipradilol in the Isolated Posterior Retina

This experiment was performed to confirm the presence of the pharmacologically active form of nipradilol in the ipsilateral retina after its unilateral instillation. Sixty minutes after the unilateral topical instillation of nonradiolabeled nipradilol (1%, 100 μL), the rabbit was euthanatized by intravenous injection of pentobarbital, both eyes were enucleated, and the posterior pole retina was isolated. This procedure

was performed in unfrozen conditions to isolate the retina from the choroid or additional ocular tissues. In detail, each enucleated eyeball was washed quickly in ice-cold physiologic saline. The anterior aqueous humor was drained, and the cornea containing a high concentration of nipradilol was excised. Next, the lens was removed, and about a 2-mm width of the scleral ring containing the corneoscleral limbus was excised together with the iris and ciliary body. The vitreous was then removed. The retina was isolated at a radius of 5-mm with the optic nerve for its center from the posterior cup of the sclera from the vitreous side where the concentration of nipradilol should be lowest.[12,14] We changed all instruments at each step to avoid contamination. Thus, nipradilol in the isolated posterior retina was extracted, and the concentration of the unchanged form was measured by LC/MS/MS.

Statistical Analysis

All data are presented as the mean ± SEM. Nipradilol concentrations between the same area on both sides of the retina, retina-choroid, and periocular tissue were compared by paired *t*-test. In addition, the concentrations of nipradilol between the equatorial and posterior part of the retina-choroid or periocular tissue in the same eye were compared by paired *t*-test. $P < 0.05$ was considered significant.

Results

Standard Curve for Autoradiographic Measurements

After 72 hours' contact of sections with an imaging plate, we detected standard spots of 100 Bq/g and higher. Thus, we first made a standard curve using 100, 1,000, 10,000, and 100,000 Bq/g. This curve covers from 1.53 to 1,267.48 and from 68.6 to 60,666.3 for (PSL − BG)/A and nanogram-equivalent per gram, respectively (Fig. 2A). In addition, after 2 weeks' contact, we detected standard spots ranging from 10 to 10,000 Bq/g, which covers from 0.44 to 322.76, and from 6.20 to 4,549.16 for (PSL − BG)/A and nanogram-equivalent per gram, respectively (Fig. 2B). Therefore, we used the first standard curve (Fig. 2A) for 1.53 (PSL − BG)/A and higher, and the second (Fig. 2B) for less than 1.53 (PSL − BG)/A. The upper and lower detection limits were 1267.48 (PSL − BG)/A and 0.44 (PSL − BG)/A, respectively.

Topical Instillation Study

Figure 3A shows frozen sections of rabbit heads obtained through ONHs of both eyes. There were no nonspecific reactions that were not attributable to radioactivity with the present method (data not shown). Autoradiograms corresponding to Figure 3A at 15 minutes after instillation are shown in Figure 3B. At this time point, the level of radioactivity was highest in the cornea and conjunctiva, followed by the iris, anterior chamber, and lens in the anterior part of the instilled side. Weak but significant radioactivity was observed along the posterior retina-choroid of the instilled side. Outside the eye, the anterior periocular tissues of the instilled side and Harder's gland on both sides, seen as a crescent-shaped area in the posterior part of the orbit, showed relatively high radioactivity (Fig. 3B).

Sixty minutes after instillation, there was still high radioactivity in the anterior parts of the instilled eye. Higher radioactivity in the posterior retina-choroid was detected on the instilled side than on the contralateral side and tended to be higher at 15 minutes. Radioactivity of Harder's gland was similar on both sides (Fig. 3C). Nipradilol concentration in periocular tissue around the optic nerve insertion was higher on the ipsilateral side than the contralateral side (207.1 ± 23.9 and 141.1 ± 25.5 ng/g, $P < 0.001$). Radioactivity was higher in periocular tissue around the equator than around the optic nerve insertion on the ipsilateral side ($P = 0.015$), but no such difference was seen on the contralateral side. Nipradilol concentration in the ipsilateral posterior retina-choroid was 142.9 ± 20.2 ng/g, which was significantly higher than that on the contralateral control side (108.3 ± 11.5 ng/g, $P = 0.026$). In samples isolated from enucleated frozen eyes, a significant concentration of nipradilol was detected in the posterior retina-choroid on both sides, but the nipradilol concentration on the instilled side (97.7 ± 17.8 ng/g) was significantly higher than that on contralateral control side (61.9 ± 9.7 ng/g, $P = 0.047$; Table 1). The relatively high concentration of nipradilol on the contralateral ocular and in periocular tissues is probably attributable to the small-distribution volume in rabbits.[12]

Intracameral Injection Study

Autoradiograms corresponding to sections through ONHs at 15 and 60 minutes after the intracameral injection of [^{14}C]nipradilol are shown in Figures 3D and 3E, respectively. At 15 minutes after injection, high radioactivity was observed in the anterior chamber, iris, ciliary body, and cornea. No radioactivity was observed in the posterior part of the injected eye or in any contralateral tissues. Sixty minutes after injection, the distribution pattern of [^{14}C]nipradilol was almost the same as that at 15 minutes after injection. Nipradilol concentration in the cornea and anterior chamber measured by autoradiography were more than 600,000 ng/g and 23,090.6 ± 13,322.4 ng/g, respectively. Weak radioactivity was detected in periocular tissue around the equator (78.5 ± 26.6 ng/g) and equatorial retina-choroid (69.3 ± 10.5 ng/g). There was no significant radioactivity in the vitreous, posterior retina-choroid, or posterior periocular tissues on the injected side or in any of the examined parts on the contralateral side (Figs. 3D, 3E). There

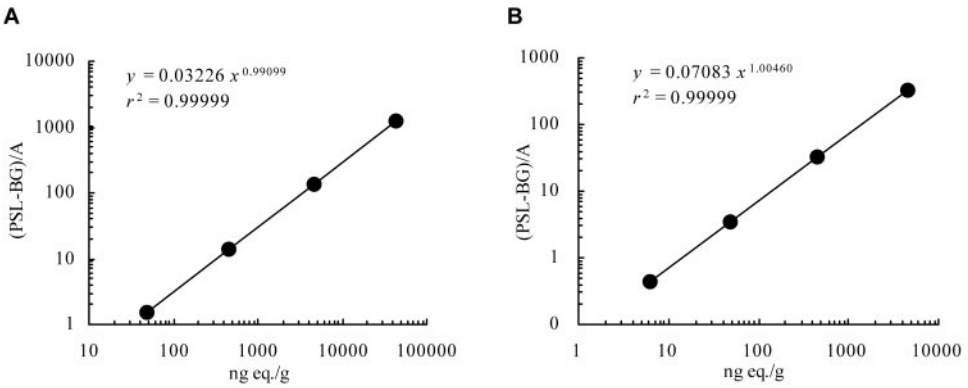

Figure 2. Logarithmic standard curves for the [^{14}C]nipradilol concentration between data obtained with the scintillation counter and PSL data from autoradiography: (**A**) 1.53 (PSL − BG)/A and over; (**B**) lower than 1.53 (PSL − BG)/A. Upper and lower detection limits were 1267.48 and 0.44 (PSL − BG)/A, respectively.

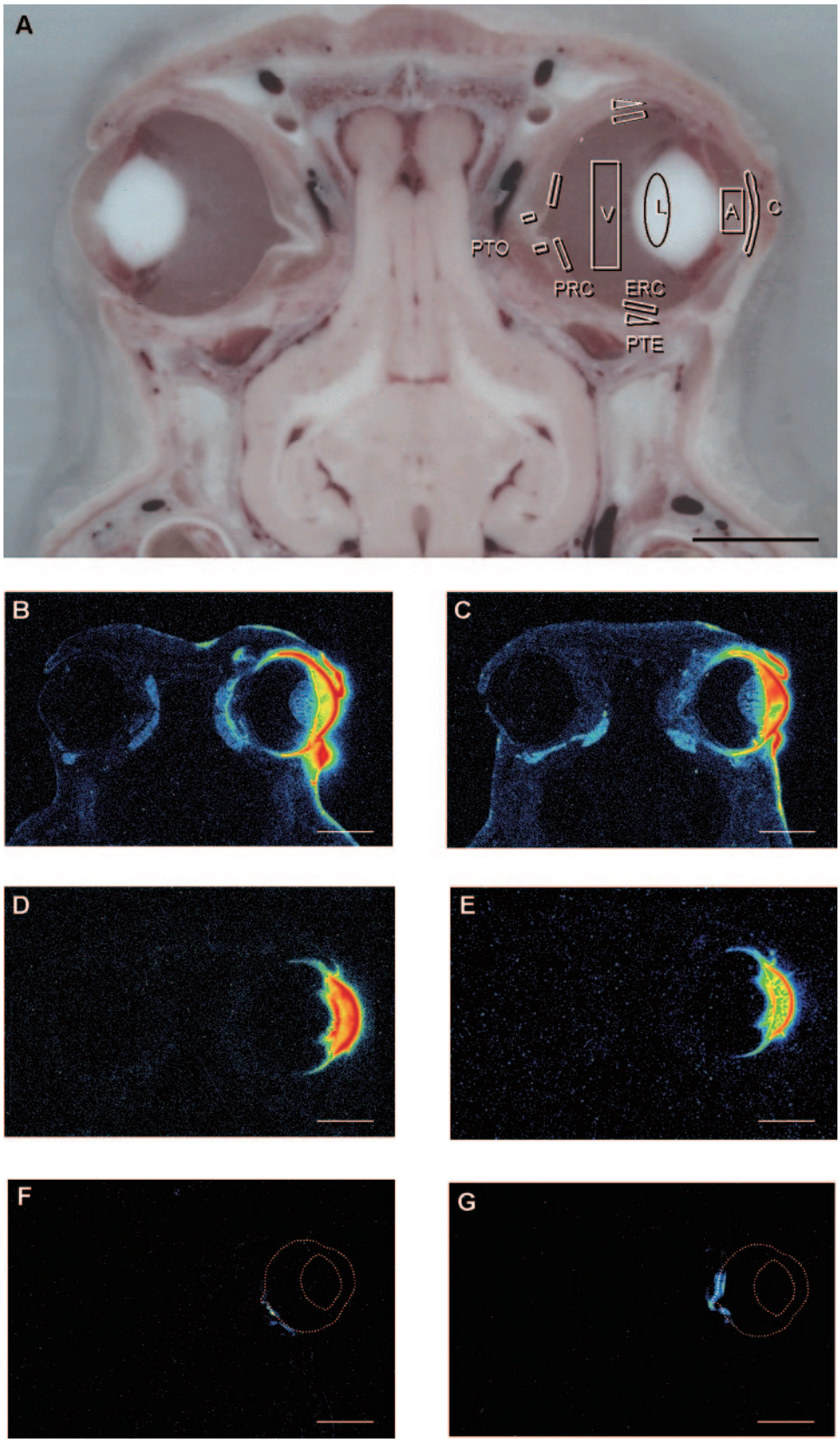

FIGURE 3. Image of the meridian section of a rabbit head and autoradiograms after topical instillation or intracameral or sub-Tenon injection of [^{14}C]nipradilol. (**A**) Meridian section of a rabbit head. Autoradiograms corresponding to sections (**A**) at 15 or 60 minutes after topical instillation or intracameral or sub-Tenon injection are shown in (**B**) and (**C**), (**D**) and (**E**), and (**F**) and (**G**), respectively. The area used to determine the concentration of total nipradilol in each tissue is enclosed in (**A**). C, cornea; A, anterior chamber; L, lens; V, vitreous; PTE, periocular tissue around the equator; PTO, periocular tissue around the optic nerve insertion; ERC, equatorial retina-choroid; PRC, posterior retina-choroid. [^{14}C]-nipradilol was applied onto the right side. Scale bar, 10 mm.

was no significant radioactivity in the posterior retina-choroid or posterior vitreous isolated from enucleated frozen eyes (Table 2), consistent with the results obtained from autoradiography.

Sub-Tenon Injection Study

Autoradiograms corresponding to sections through ONHs at 15 and 60 minutes after sub-Tenon injection of [^{14}C]nipradilol are

TABLE 1. Total Nipradilol Concentration in Posterior Tissue after Topical Instillation

	Enucleation–Freezing Method		Autoradiographic Method	
	Instilled Side	Contralateral Control Side	Instilled Side	Contralateral Control Side
Cornea	—	—	34,336.7 ± 3,946.5 (105.00 ± 5.40)	73.8 ± 21.5 (0.23 ± 0.03)
Anterior chamber	—	—	2,385.0 ± 349.1 (7.30 ± 0.48)	16.3 ± 3.3 (0.05 ± 0.00)
Lens	—	—	14.1 ± 0.6 (0.04 ± 0.00)	ND
Posterior side of vitreous	ND	ND	ND	ND
Equatorial retina-choroid	—	—	1,673.9 ± 269.8*† (5.12 ± 0.37)	108.1 ± 10.3 (0.33 ± 0.01)
Posterior retina-choroid	97.7 ± 17.8‡ (0.30 ± 0.07)	61.9 ± 9.7 (0.19 ± 0.04)	142.9 ± 20.2*§ (0.44 ± 0.03)	108.3 ± 11.5 (0.33 ± 0.02)
Periocular tissue around the equator	—	—	1,784.9 ± 376.7*‖ (5.46 ± 0.52)	131.3 ± 24.7 (0.40 ± 0.03)
Periocular tissue around the optic nerve insertion	—	—	207.1 ± 23.9* (0.63 ± 0.03)	141.1 ± 25.5 (0.43 ± 0.03)

Data are expressed as nanograms per gram (μM) of nipradilol concentration 60 minutes after topical instillation. —, exclusion from the respective method; ND, under the detection limit. $n = 5$–6.

* $P < 0.01$ vs. contralateral control side.

† $P < 0.01$ vs. posterior retina-choroid.

‡ $P < 0.05$ vs. contralateral control side.

§ $P < 0.05$ vs. periocular tissue around the optic nerve insertion.

‖ $P < 0.05$ vs. posterior tissue around the optic nerve insertion (paired *t*-test).

shown in Figures 3F and 3G, respectively. Fifteen minutes after injection, [^{14}C]nipradilol radioactivity was mainly detected in the retrobulbar space around the optic nerve insertion on the injected side (Fig. 3F). Sixty minutes after injection, radioactivity was detected around the optic nerve insertion and in the posterior part of the eye (Fig. 3G), and nipradilol concentration in the posterior retina-choroid was 131.3 ± 43.8 ng/g, whereas that in posterior periocular tissues around the optic nerve insertion was 224.4 ± 84.3 ng/g, significantly higher than that in the posterior retina-choroid ($P = 0.034$). There was no significant radioactivity in the anterior part on the injected or contralateral side. In samples isolated from enucleated frozen eyes after sub-Tenon injection, a significant level of nipradilol was detected in the posterior retina-choroid (35.5 ± 30.4 ng/g), but not in the posterior vitreous on the injected side. There was no radioactivity in the posterior retina-choroid or posterior vitreous on the contralateral side (Table 3).

Concentration of Unchanged Nipradilol in the Isolated Posterior Retina

Table 4 indicates the unchanged form of nipradilol concentration in the posterior pole retina after topical instillation of nonradiolabeled nipradilol (1%, 100 μL) in one eye. There was a fairly close relationship between standard sample concentrations and peak areas obtained from LC/MS/MS in the range from 0.5 to 100 ng/g tissue in the retina (Fig. 4). On the treated side of the posterior retina, the concentration of unchanged nipradilol was 68.9 ± 13.6 ng/g, significantly higher than that on the contralateral control side (57.5 ± 12.5 ng/g, $P = 0.008$).

DISCUSSION

The purpose of the present study was to assess the penetration route of topically instilled nipradilol to the posterior parts of

TABLE 2. Total Nipradilol Concentration in Posterior Tissue after Intracameral Injection

	Enucleation–Freezing Method		Autoradiographic Method	
	Injected Side	Contralateral Control Side	Injected Side	Contralateral Control Side
Cornea	—	—	>600,000 (>180)	ND
Anterior chamber	—	—	23,090.6 ± 13,322.4 (70.75 ± 40.82)	ND
Lens	—	—	ND	ND
Posterior side of vitreous	ND	ND	ND	ND
Equatorial retina-choroid	—	—	69.3 ± 10.5 (0.21 ± 0.03)	ND
Posterior retina-choroid	ND	ND	ND	ND
Periocular tissue around the equator	—	—	78.5 ± 26.6 (0.24 ± 0.08)	ND
Periocular tissue around the optic nerve insertion	—	—	ND	ND

Data are expressed as nanograms per gram (μM) of nipradilol concentration 60 minutes after intracameral injection. —, exclusion from the respective method; ND, under the detection limit. $n = 4$–5.

TABLE 3. Total Nipradilol Concentration in Posterior Tissue after Sub-Tenon Injection

	Enucleation–Freezing Method		Autoradiographic Method	
	Injected Side	Contralateral Control Side	Injected Side	Contralateral Control Side
Cornea	—	—	ND	ND
Anterior chamber	—	—	ND	ND
Lens	—	—	ND	ND
Posterior side of vitreous	ND	ND	ND	ND
Equatorial retina-choroid	—	—	ND	ND
Posterior retina-choroid	35.5 ± 30.4 (0.11 ± 0.09)	ND	131.3 ± 43.8 (0.40 ± 0.13)	ND
Periocular tissue around the equator	—	—	ND	ND
Periocular tissue around the optic nerve insertion	—	—	244.4 ± 84.3* (0.75 ± 0.26)	ND

Data are expressed as nanograms per gram (μM) of nipradilol concentration 60 minutes after sub-Tenon injection. —, exclusion from the respective method; ND, under the detection limit. $n = 4$–5.

* $P < 0.05$ vs. posterior retina-choroid (paired t-test).

the eye. It is controversial whether a topically instilled drug can penetrate locally to posterior segments of the eye at pharmacologically active levels.[1–10] We previously demonstrated that topical instillation of nipradilol (1%, 100 μL) penetrated locally to the ipsilateral posterior at pharmacologically active levels in normal albino rabbits[12] and monkeys[14]; however, the penetration route of topically instilled nipradilol to the ipsilateral posterior retina-choroid remains unknown.

Local penetration of topical drugs to the ipsilateral posterior retina-choroid could be via three potential routes: (1) periocular and transposterior scleral route,[14,15] (2) transvitreous route,[4,5,7] and (3) uveal route.[16] In this study, we mainly used whole-head autoradiography with a radiolabeled compound to visualize the distribution of nipradilol in intraocular as well as extraocular tissues. Further, to validate the autoradiography results, we used tissues isolated from frozen and unfrozen eyeballs. Our modified method using frozen eyeballs[12] has the advantage of minimizing contamination but, for the retina, accurate determination of the drug concentration in this tissue was difficult, since separating the retina from the underlying choriocapillaris was difficult in the frozen condition. In contrast, a method using unfrozen eyeballs, which requires very careful dissection of the tissues to avoid contamination, has the advantage of isolating only the retina and so that the drug concentration in it can be determined accurately. In the present study, we used the former method to determine total nipradilol levels in the posterior vitreous and retina-choroid, to confirm the autoradiographic determination when isolation of the signal from the retina only is difficult, and the latter to confirm that the drug penetrated not only the choroid, but also the retina, and that main radioactivity was not attributable to metabolized inactive nipradilol.

In the main part of this study, we examined the distribution of radiolabeled nipradilol after topical instillation. In the anterior parts of the eye, autoradiography revealed that radioactivity was highest in the cornea and conjunctiva, followed by the iris, anterior chamber, and ciliary body on the instilled side. In the posterior parts of the ipsilateral side, radioactivity was detected in the posterior retina-choroid and periocular tissue around the optic nerve insertion and was significantly higher than on the contralateral side. The difference in total nipradilol concentration between the ipsilateral and contralateral sides was 0.11 (0.44 - 0.33) and 0.20 (0.63 - 0.43) μM for the posterior retina-choroid and periocular tissue around the optic nerve insertion, respectively. Further, the bilateral difference in total nipradilol level in the posterior retina-choroid isolated from enucleated frozen eyes was significant, and the result agreed well with that of autoradiography (0.30 - 0.19 = 0.11 μM [enucleation method] vs. 0.44 - 0.33 = 0.11 μM [autoradiographic method]), indicating that a substantial amount of nipradilol reached the ipsilateral posterior retina-choroid not via the systemic circulation, but by local penetration.

On the contralateral control side in the topical instillation study, nipradilol concentrations in periocular tissues and retina-choroid of both equatorial and posterior parts ranged between 0.33 and 0.43 μM, and there was no significant difference between the equatorial and posterior parts of each tissue, suggesting that nipradilol in these tissues is attributable to

TABLE 4. Unchanged Nipradilol Concentration in the Retina

Instilled Side	Contralateral Control Side
68.9 ± 13.6* (0.21 ± 0.04)	57.5 ± 12.5 (0.18 ± 0.04)

1%, 100 μL nipradilol was instilled. Data are expressed as nanograms per gram (μM) of unchanged nipradilol in the retina at 60 minutes after topical instillation.

* $P < 0.01$ vs. contralateral control side (paired t-test, $n = 6$).

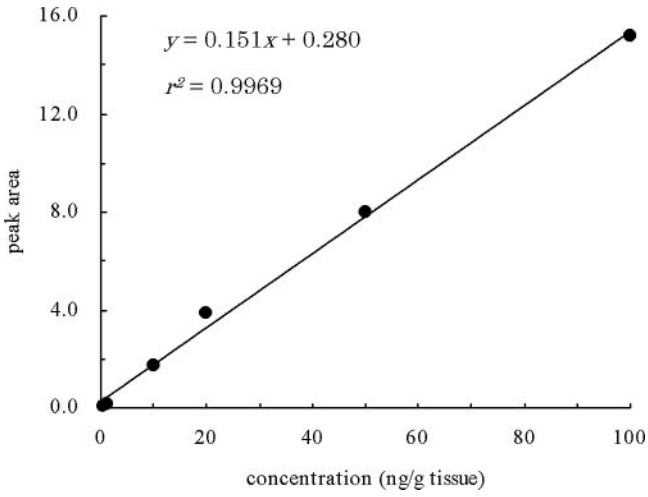

FIGURE 4. Logarithmic standard curves for unchanged nipradilol concentration between data obtained with the standard sample and peak areas in the retina. Upper and lower detection limits were 0.5 and 100.0 ng/g tissue.

circulating blood and is compatible with our previous monkey study.[14] On the other hand, on the instilled side, nipradilol concentrations in periocular tissue around the equator and the optic nerve insertion were significantly higher than on the contralateral control side, and that in the periocular tissue around the equator was significantly higher than around the optic nerve insertion, suggesting the local diffusion of topically instilled nipradilol from the equatorial to posterior periocular tissue on the instilled side.

Autoradiography results of topical, intracameral, and sub-Tenon studies showed that no radioactivity was found in the posterior side of the vitreous. In addition, no radiolabeled nipradilol was detected on the posterior side of the vitreous after topical, intracameral or sub-Tenon administration according to the enucleation study. Some tissues can accumulate higher concentrations than that in the fluids because of preferential partitioning and/or binding of drugs in tissues.[29] Kent et al.[7,9] reported that brimonidine was present at the low nanomolar-teens in the vitreous by 12 hours after topical instillation in humans. A similar result was reported by Acheampong et al.[5] in pigmented animal, and explained that drug binding to melanin act as the generation of a depot or slow-release site for the vitreous brimonidine. In the present study, however, vitreous fluid was not thought to be the dominant source of the drug in the posterior retina, since no radioactivity or drug was observed on the posterior side of the vitreous until at least 60 minutes after instillation.

On the other hand, since the concentration of nipradilol in the equatorial retina-choroid was significantly higher than that of the posterior retina-choroid, the possibility that nipradilol diffused via the uveal route could not be excluded. To clarify this point, we performed an intracameral injection study. We injected 0.1%, 100 μL of [^{14}C]nipradilol into the anterior chamber, which resulted in a concentration 100 times higher than the nipradilol concentration detected in the anterior chamber 60 minutes after topical instillation. Sixty minutes after the intracameral injection, the nipradilol concentration in the anterior chamber was still some 10 times higher than that after topical instillation. Even under this condition, radioactivity was observed only in the anterior parts of the eye, and no radioactivity was detected in the posterior retina-choroid, posterior periocular tissues, or vitreous, which was also confirmed by the results obtained from samples isolated from enucleated frozen eyes. These results indicate that drugs in the anterior chamber could not penetrate the posterior intraocular tissues at 60 minutes. Radioactivity detected in equatorial periocular tissue and retina-choroid after intracameral injection were presumably attributable to uveoscleral drainage of the aqueous humor containing a high concentration of nipradilol, and the uveoscleral flow-increasing effect of nipradilol in rabbits[23] may amplify the radioactivity of these tissues. These results strongly suggest that not only transvitreous diffusion, but also the uveal route makes no significant contribution to the local penetration of instilled drugs to the ipsilateral posterior retina-choroid.

The remaining possibility is the periocular and transposterior sclera route. Sponsel et al.[15] reported the accumulation of a greater quantity of β-blockers in periocular tissue after topical instillation in glaucoma patients and suggested that it could provide immediate and quantitative access of topical drugs to the posterior segment and proximal ocular vasculature, which agrees with the present results after topical instillation of nipradilol. A sub-Tenon injection study was performed to examine whether this route contributes to the penetration of topically instilled nipradilol to the ipsilateral posterior retina-choroid. Autoradiography revealed that significant radioactivity was detected only in the ipsilateral posterior retina-choroid and posterior periocular tissue around the optic nerve insertion, and total nipradilol concentration in posterior periocular tissue was significantly (approximately two times) higher than that of the posterior retina-choroid. In samples isolated from enucleated frozen eyes, nipradilol was also detected only in the ipsilateral posterior retina-choroid after sub-Tenon injection, but the concentration was thought to be rather low, even taking our current findings into account that autoradiographic determination tended to somewhat overestimate nipradilol concentration in the posterior retina-choroid. For the enucleation method, we cut the eyeball circle-wise at a radius of 5 mm with the optic nerve as its center to obtain a posterior cup. As shown in Figure 3G, this area also included the area where no apparent radioactivity was found, probably leading to a lower mean nipradilol concentration in the posterior retina-choroid with the enucleation method than with autoradiography.

After topical instillation, nipradilol concentrations in the posterior retina-choroid and posterior periocular tissue around the optic nerve insertion on the instilled side showed a significant difference ($P = 0.028$). On the other hand, nipradilol concentrations on the contralateral control sides of both tissues, presumably due to nipradilol in the circulating blood, showed no significant difference. Therefore, in the topical instillation study, the estimated nipradilol concentrations in the posterior retina-choroid and posterior periocular tissue around the optic nerve insertion on the instilled side not attributable to the drug in circulating blood were 0.11 (0.44–0.33) and 0.20 (0.63–0.43) μM, respectively. In the present topical instillation and sub-Tenon injection studies, the ratio of nipradilol concentration attributable to local penetration in the posterior retina-choroid to that in periocular tissue around the optic nerve insertion agreed well (0.55 = [0.11/0.20 in the topical instillation study] vs. 0.53 = [0.40/0.75 in the sub-Tenon injection study]). These results suggest that diffusion from posterior periocular tissues across the posterior sclera may be the main route for local penetration of the instilled drug to reach the posterior retina-choroid.

The unchanged nipradilol concentration on the posterior side of the retina was determined by using nonradiolabeled nipradilol. In the method using radiolabeled nipradilol, we obtained a total nipradilol concentration of 0.30 (enucleation–freezing method) to ~0.44 (autoradiographic method) μM in the ipsilateral posterior retina-choroid after instillation. Nipradilol in local tissues is thought to be mainly metabolized by reduced glutathione-dependent organic nitrate reductase and becomes desnitro-nipradilol,[30] which has much weaker pharmacologic activity.[22] We previously evaluated that 60 minutes after instillation, 47.3% of total nipradilol remained in the unchanged, active form in the instilled eye in albino rabbits[31]; therefore, 60 minutes after instillation, 0.30 to 0.44 μM of total nipradilol concentration in the ipsilateral posterior retina-choroid would roughly correspond to 0.15 to 0.20 μM of unchanged nipradilol. The unchanged nipradilol concentration in the isolated posterior retina obtained by the nonradiolabeled method was 0.21 μM, which roughly agrees with that obtained by using radiolabeled eyeballs. Further, the estimated bilateral difference in the unchanged nipradilol concentration in the retina-choroid determined by measuring radioactivity, 0.05 μM (bilateral difference in the total nipradilol concentration using both autoradiographic and enucleation methods, 0.11 μM, $\times$ the unmetabolized ratio of topical nipradilol in the instilled eye, 47%),[31] roughly agreed with that in the retina determined by liquid chromatography-tandem mass spectrometry (LC/MS/MS; 0.03 μM = 0.21–0.18 μM). This concentration of nipradilol has definite pharmacologic activity.[11,12]

This study was performed in albino rabbits and we must be very cautious in extrapolating these results to humans. Effects of melanin on ocular pharmacokinetics are well documented with brimonidine, an α2-adrenergic agonist[5,32] and β-block-

ers.[33] In addition, several reports have demonstrated that the presence of pigment in ocular tissues influences intraocular dynamics of antibacterial agents or chemotherapeutics.[29,34,35] In this study, however, we chose albino, not pigmented, rabbits to simplify the drug movement in the eye. Further, a high concentration of the drug in melanin-containing tissue, the choroid, may make it difficult to determine the drug level in the isolated retina in unfrozen conditions. In the present study, we found that a significant part of nipradilol in the retina after topical instillation is attributable to local penetration via the periocular and transposterior scleral route in albino rabbits, although whether this is also the case in pigment rabbits awaits future studies. Previous reports suggest that the finding obtained here in albino rabbits may not be very different from the results in pigment rabbits. Ishii et al.[13] reported that topically instilled iganidipine, a calcium antagonist, inhibited the intravitreously injected ET-1-induced constriction of retinal vessels, only on the ipsilateral side in pigment rabbits, and they pharmacologically determined the amount of iganidipine that locally penetrated the ipsilateral retina to be approximately 0.01 μM. A similar result was reported with bunazosin, an α-1 adrenergic antagonist, in pigmented rabbits.[36]

We have studied the periocular distribution of topically instilled nipradilol in cynomolgus monkeys[14] and found that periocular distribution of nipradilol on the instilled side is similar to that found in this study in albino rabbits. An increase in ONH blood flow induced by topical nipradilol after unilateral instillation was observed only on the ipsilateral side in humans,[14] which could not be attributed to nipradilol in circulating blood after unilateral instillation (0.2 ng/mL or lower).[37] Grover et al.,[38] recently documented that topical dorzolamide was effective in reducing cystoid macular edema in patients with retinitis pigmentosa. Since it is difficult to attribute this effect to systemically absorbed dorzolamide,[39] this result suggests that some topically instilled drugs can reach the posterior retina by local penetration also in human eyes. The present study showed that a significant amount of topically instilled drug reached the ipsilateral retina by local penetration, at least in albino rabbits, and this possibility may deserve to be studied in pigmented animals or humans.

In summary, the current results showed that, in normal albino rabbit eyes, some nipradilol after unilateral instillation penetrates to ipsilateral retrobulbar tissues by periocular diffusion and the posterior retina, crossing the posterior sclera and choroid, and that the amount of nipradilol thus reaching the ipsilateral posterior retina may be sufficient to exert pharmacologic actions.

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