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Timolol maleate, a β blocker eye drop, improved edema in a retinal vein occlusion model

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Purpose: To investigate the therapeutic effects of eye drops, namely, timolol maleate, a β-adrenergic receptor antagonist, and latanoprost, a prostaglandin F2α analog, on retinal edema in a murine retinal vein occlusion (RVO) model.
Methods: An RVO model was established using laser-induced RVO in mice, which were administered timolol maleate and latanoprost eye drops several times after venous occlusion. Subsequently, the thickness of the inner nuclear layer (INL) and the expression levels of such genes as *Vegf* and *Atf4*, which are stress markers of the endoplasmic reticulum, were examined. Primary human cultured retinal microvascular endothelial cells (HRMECs) were treated with timolol under hypoxic conditions, after which the gene expression pattern was investigated. Importantly, an integrated stress response inhibitor (ISRIB) was used in the RVO model, he known *ISRIB*, which suppresses the expression of *ATF4* in retinal edema.
Results: Increased INL thickness was suppressed by timolol eye drops, as were the expressions of *Vegf* and *Atf4*, in the RVO model. However, latanoprost eye drops did not induce any change in INL thickness. In HRMECs, hypoxic stress and serum deprivation increased the *Vegf* and *Atf4* expressions; in response, treatment with timolol suppressed the *Vegf* expression. Furthermore, the ISRIB decreased the *Vegf* expression pattern and edema formation, which are associated with RVO.
Conclusions: These results indicate that timolol eye drops may be a potential option for RVO treatment.

Retinal vein occlusion (RVO) is the second most common form of retinal vascular disease, associated with edema, retinal hemorrhage, and the formation of non-vascular areas, leading to vision loss [1]. Particularly, the severity of retinal edema is observed to correlate with the best-corrected visual acuity (VA); therefore, improving retinal edema is important to recovering VA [2].

Ranibizumab, an anti-vascular endothelial growth factor (VEGF) antibody, is used to treat RVO, as it improves VA by reducing retinal edema; however, it is a highly invasive treatment that requires monthly intravitreal (IV) administration [3]. Patients require multiple doses to ensure therapeutic and reduced adverse effects, such as retinal detachment, endophthalmitis, cataract formation, hypertension, and submacular hemorrhage [4-6]. Although anti-VEGF therapies are highly invasive, their efficacy is less than 50% in RVO patients [7], necessitating the development of less invasive RVO treatments, such as eye drops.

Therefore, we focused on two anti-glaucoma eye drops, namely, latanoprost and timolol maleate, the former of which is an analog of prostaglandin F2α (PGF2α) that is well tolerated as a first line in glaucoma treatment. Moreover, it has previously been reported to exhibit protective effects against pericyte loss in a murine model of diabetic retinopathy via the PI3k/Akt pathway [8,9]. Importantly, pericyte loss, which is also observed in RVO, contributes to the development of retinal edema by increasing vascular permeability [10,11]. Therefore, latanoprost, which protects against pericyte loss, may ameliorate retinal edema.

Another eye drop of interest, timolol maleate, a non-selective beta (β)-adrenergic blocker, regulates the expression of *VEGF*, which itself is known to normalize vascular permeability. Propranolol, a non-selective β blocker, is used to manage angina pectoris and hypertension, and it was previously reported to inhibit the induction of VEGF, whose involvement in angiogenesis and edema formation has been clarified in animal models of ischemic retinal disorders [12,13]. In addition, the efficacy of propranolol has been confirmed in patients with retinopathy of prematurity, in whom the induction of VEGF by hypoxic stress is considered important [14]. Based on these reports, we hypothesized that β blockers may be effective in the treatment of retinal edema in RVO, to which VEGF has been established as a contributing factor. Therefore, we investigated the effects of latanoprost and timolol maleate eye drops on retinal edema in an RVO murine model.

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METHODS

Animals: We purchased 8-week-old male ddY mice from Japan SLC (Hamamatsu, Japan) and caged them in our animal facilities, maintained at 23±3 °C under a 12 h:12 h light-dark schedule. The mice had access to food and water ad libitum. All procedures were performed in accordance with the Association for Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmic and Vision Research, and the experimental protocols were approved and monitored by the Institutional Animal Care and Use Committee of the Gifu Pharmaceutical University.

Murine RVO model: In total, 70 mice were used and the preparation of RVO murine model was previously described in detail [15]. Briefly, mice were anesthetized intramuscularly (IM) with a mixture of ketamine (120 mg/kg IM; Daiichi-Sankyo, Tokyo, Japan) and xylazine (6 mg/kg IM, Bayer Health Care, Osaka, Japan). Their pupils were dilated with 1% tropicamide and 2.5% phenylephrine (Santen Pharmaceuticals Co., Ltd., Osaka, Japan), while hydroxyethyl cellulose (Senju Pharmaceutical Co. Ltd., Osaka, Japan) was applied to the corneas to prevent desiccation. Three retinal veins were photocoagulated by a 532-nm laser light applied at 50 mW, 5 s, and 50 µm (Phoenix Research Laboratories, Inc., Pleasanton, CA). The right eye of each animal was irradiated after an injection of rose bengal (8 mg/ml; Wako, Osaka, Japan) into the tail vein; then, 10–15 laser spots were applied to three retinal veins of each mouse at three disc diameters from the optic nerve head.

Drug administration: Timolol maleate 0.5%, latanoprost 0.005%, and vehicle solutions were kind gifts from Nitto Medic Co. Ltd. (Toyama, Japan), all of which were administered by eye drop (5 µl) immediately and at 3, 6, 12, and 18 h after laser irradiation. In another independent experiment, an integrated stress response inhibitor (ISRIB; 9 ng/2 µl; Cayman Chemical, Ann Arbor, MI) was injected (2 µl) into the vitreous body of the right eye immediately after laser irradiation using a sterile 34-gauge needle (Terumo, Tokyo, Japan) attached to a Hamilton glass syringe (701 N; Hamilton Co., Reno, NV). For controls, mice were IV injected with 2 µl of 0.01 M PBS into the right eye, after which 0.5% levofloxacin ophthalmic solution (Santen Pharmaceuticals Co., Ltd.) was applied topically to the treated eyes.

Histological analysis: The mice were euthanized by cervical dislocation under deep anesthesia, and each eye was enucleated. For histological analysis, the retina was immersed for at least 48 h at 4 °C in a fixative solution containing 4% paraformaldehyde. Six paraffin-embedded sections (5-µm thick) were cut parallel to the maximum circumference through the optic disc, and eye sections were prepared in a standard manner and stained with hematoxylin and eosin (H&E). The damage induced by RVO was evaluated using three H&E-stained sections from each eye for morphometric analysis, while light microscope images were photographed and the INL from the optic disc was measured in the photographs at 240-µm intervals.

For immunostaining, the retinal sections were permeabilized with 0.3% Triton-X 100 for 1 h at room temperature (RT). Subsequently, the sections were rinsed in PBS and then incubated in PBS containing 10% horse serum (Vector Laboratories, Burlington, VT). After washing with PBS, the sections were incubated with ATF4 rabbit monoclonal antibody (1:50; Cell Signaling Technology, Danvers, MA) overnight at 4 °C. The slides were then rinsed 2X with PBS and incubated with Alexa Fluor-546 donkey anti-rabbit IgG (1:1000, Thermo Fisher Scientific, Waltham, MA) for 1 h at RT. Nuclei were stained with Hoechst 33,342 (1:1000, Thermo Fisher Scientific) for 10 min at RT, and the slides were washed 3X with PBS and mounted using the Fluoromount mounting medium. Finally, images were photographed using the All-in-One Fluorescence Microscope (model BZ-X710, Keyence, Osaka, Japan).

Cell cultures: Primary human retinal microvascular endothelial cells (HRMECs; Cell Systems, Kirkland, WA) were cultured, as previously described in detail [16], and were maintained in a complete classic medium supplemented with CultureBoost-R (Cell Systems), 100 µg/ml streptomycin (Meiji Seika Pharma Co., Ltd., Tokyo, Japan), and 100 U/ml penicillin (Meiji Seika Pharma Co., Ltd.). Before seeding the cells, culture dishes and well plates were precoated with an attachment factor (Cell Systems). Thereafter, the cells were maintained at 37 °C in a humidified atmosphere containing 5% CO_2. Passages 6–10 were used in the experiments.

The HRMECs (n = 4 or n = 8) were seeded at 2 × 10^4 cells/well in 24-well plates and incubated for 24 h; then, the initial medium was exchanged with a medium containing 10% fetal bovine serum (FBS) without CultureBoost-R. At 24 h following the medium exchange, the medium containing 1% FBS or without FBS was changed and timolol was added before hypoxia. Thereafter, the cells were cultured under 1% O_2 for 6 h [17].

RNA extraction and RT-PCR: Total RNA was isolated from the retina and cultured cells using a NucleoSpin RNA kit (Takara Bio Inc., Shiga, Japan) following the manufacturer's protocol, and the RNA concentration in the extract from cells was determined using NanoVue Plus (GE Healthcare Japan, Tokyo, Japan). Further, cDNA was synthesized using a PrimeScript RT Reagent Kit (Takara Bio Inc.), and the glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) gene

was used as a housekeeping gene. In addition, SYBR Premix Ex TaqII (Takara Bio Inc.) and the TP 8000 Thermal Cycler Dice Real-Time system (Takara Bio Inc.) were used, and the PCR primer sequences of the *Vegfa* (Gene ID: 22339, OMIM 192240), *Il6* (Gene ID: 16193 OMIM 147620), *Tnf-a* (Gene ID: 21926 OMIM 191160), *Atf4* (Gene ID: 11911 OMIM 604064), and *Gapdh* (Gene ID:14433 OMIM 138400) genes are cited in Table 1.

Statistical analyses: All values are expressed as mean ± standard error of the mean (SEM), and the significance of the differences was determined using the student's *t* test, Dunnett's test, or the Tukey–Kramer test, with statistical significance set at $p<0.05$.

Table 1. Primer sequences used in real-time PCR.

Gene name	Primer sequence (5'-3')
Vegfa	F: ACATTGGCTCACTTCCAGAAACAC
	R: GGTTGGAACCGGCATCTTTATC
Il6	F: TCTGCAAGAGACTTCCATCCAGT
	R: TCTGCAACTGCATCATCGTTGT
Tnf-α	F: GAGTGACAAGCCTGTAGCC
	R: CTCCTGGTATGAGATAGCAAA
Atf4	F: AGGAGTTCGCCTTGGATGCCCTG
	R: AGTGATATCCACTTCACTGCCCAG
Gapdh	F: GGGATGGTCCTTGCATCAGAA
	R: ACTGGTAGCCACTGGTCTGGTTG

Note: F=forward primer, R=reverse primer

RESULTS

Timolol maleate eye drops improved retinal edema in a murine RVO model: We first investigated the effects of timolol maleate and latanoprost eye drops on retinal edema, both of which were administered immediately, 3, 6, 12, and 18 h after laser irradiation. H&E staining was performed on the retina samples 24 h after occlusion, and INL thickness was measured, an increase in which due to venous occlusion was significantly reduced by the timolol eye drops; however, the latanoprost eye drops did not alter INL thickness when compared to the vehicle group (Figure 1).

Timolol maleate eye drops reduced the Vegf and Atf4 gene expressions: To elucidate the mechanism of the edema-ameliorating effect of timolol eye drops in the RVO model, we used real time (RT)–PCR to confirm the changes in the expression patterns of inflammatory cytokines and *Vegf*. Gene expression alterations in the retinas 12 h after venous occlusion were confirmed using RT–PCR, and the *Vegf* expression was suppressed by timolol. In contrast, the expression levels of inflammatory cytokines, such as tumor necrosis factor alpha (TNF-α) and interleukin 6 (IL-6), were not altered by timolol treatment.

We have recently shown that the ER stress inducer thapsigargin stimulates the expression of *Vegf* in HRMECs [16], as well as that timolol suppressed tunicamycin-induced retinal damage [8]. Therefore, we hypothesized that timolol, a β blocker, would decrease the expression of *Vegf* by suppressing ER stress. In addition, we confirmed changes to the gene expression of *Atf4*, an ER stress marker, which was upregulated in the retina of the RVO model and whose increase was suppressed via timolol administration (Figure 2).

Timolol suppressed the Vegf expression in HRMECs under hypoxia: The *Vegf* expression, induced by hypoxia-only stress, was not suppressed by timolol treatment, and moreover, hypoxic stress alone did not alter the *Atf4* expression (Figure 3A). In contrast, hypoxia in combination with serum starvation stress enhanced the expressions of *Atf4* and *Vegf*, the latter of which was suppressed by timolol treatment (Figure 3B).

ISRIB attenuated the formation of retinal edema via Vegf downregulation: The *Atf4* expression was examined by immunostaining, and it was localized in all retinal layers following RVO induction (Figure 4A). Next, to clarify the relationship among ER stress, *Vegf*, and retinal edema, we investigated the effect of a small molecule, N, N'-trans-(cyclohexane-1,4-diyl)-bis-(2-(4-chlorophenoxy) acetamide, an ISRIB, on retinal edema, as it inhibits downstream eukaryotic initiation factor 2 alpha (elF2α) and the consequent *Atf4* expression. We thus found that the IV administration of the ISRIB reduced the vascular occlusion-associated increase in INL thickness (Figure 4B), and it suppressed any increases in the *Vegf* gene expression in the retinas of RVO models. Similar to timolol, the ISRIB did not alter the expression levels of proinflammatory cytokines, such as TNF-α and IL-6 (Figure 4C).

DISCUSSION

Our data show that the topical administration of timolol maleate, a β blocker, ameliorated retinal edema caused by venous occlusion in a murine RVO model. Previous studies using RVO murine models have only investigated the effects of the IV administration of a drug (anti-VEGF and caspase inhibitor) or eye drop reagent [18,19]; thus, our data have demonstrated for the first time that timolol, an eye drop approved in many countries, could improve the pathogenesis of RVO.

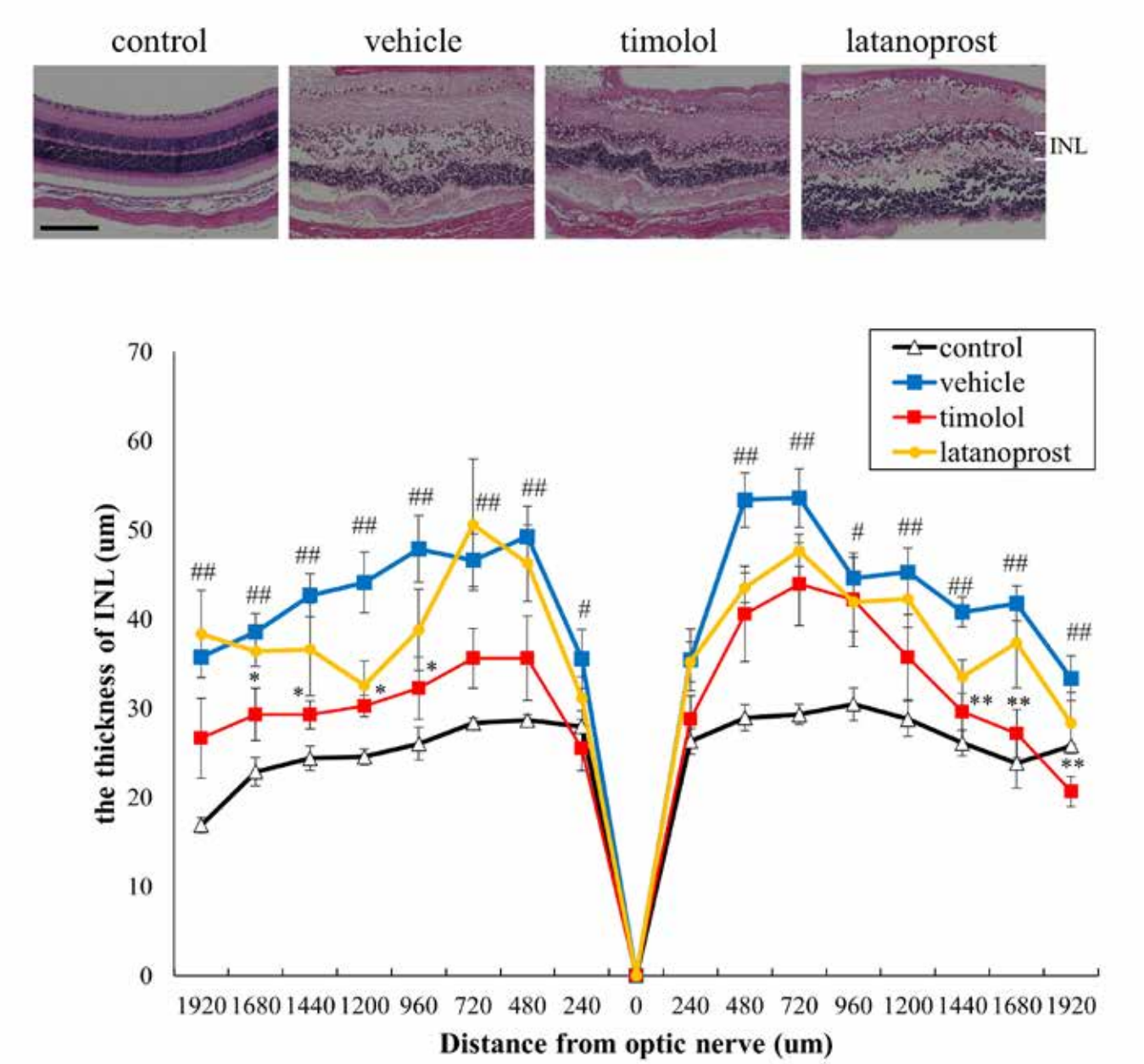

Figure 1. Effect of timolol and latanoprost eye drops on retinal edema; H&E staining with the quantitative data of INL thickness of the RVO retina after topical administration of timolol maleate and latanoprost. The scale bar is 50 µm. Data are presented as mean ± SEM (n = 7–17). **p*<0.05, ***p*<0.01 versus vehicle, #*p*<0.05, ##*p*<0.01 versus control (Tukey's test).

In the present study, latanoprost eye drops did not improve retinal edema, which was ameliorated by timolol. Meanwhile, a previous report suggested that latanoprost improved pericyte loss [9] and another that latanoprost treatment increased the *Vegf* expression in the chick chorioallantoic membrane [20], which may result in an edema-counteracting effect. Because no improvement in edema was observed following topical administration, as mentioned above, the effect of latanoprost in the experiments on the mechanism of edema improvement was not investigated.

In-vitro and in-vivo studies have suggested that the pharmacological effects of timolol in an RVO model are mediated by suppression of the *VEGF* and *ATF4* expressions, induced by ischemic stress. Retinal edema in the RVO model is thought to be caused by increased vascular permeability induced by *Vegf* [15]; in fact, previous reports have implicated

β-adrenergic receptors in the regulation of the *VEGF* expression. For example, hypoxic stress increases the level of catecholamines, which are ligands for β-adrenergic receptors [21], and norepinephrine has also been reported to lead to increases in an oxygen-induced retinopathy (OIR) model, considered an animal model of ischemic retinal disorder [22]. Furthermore, it has been reported that β-adrenergic receptor antagonists suppress the expression of *Vegf* and pathological angiogenesis in OIR and choroidal neovascularization (CNV) models of retinal ischemia [13,23]. Consistent with previous reports [13,23], we found in this study that a β-adrenergic receptor antagonist, timolol, reduced the *Vegf* expression and

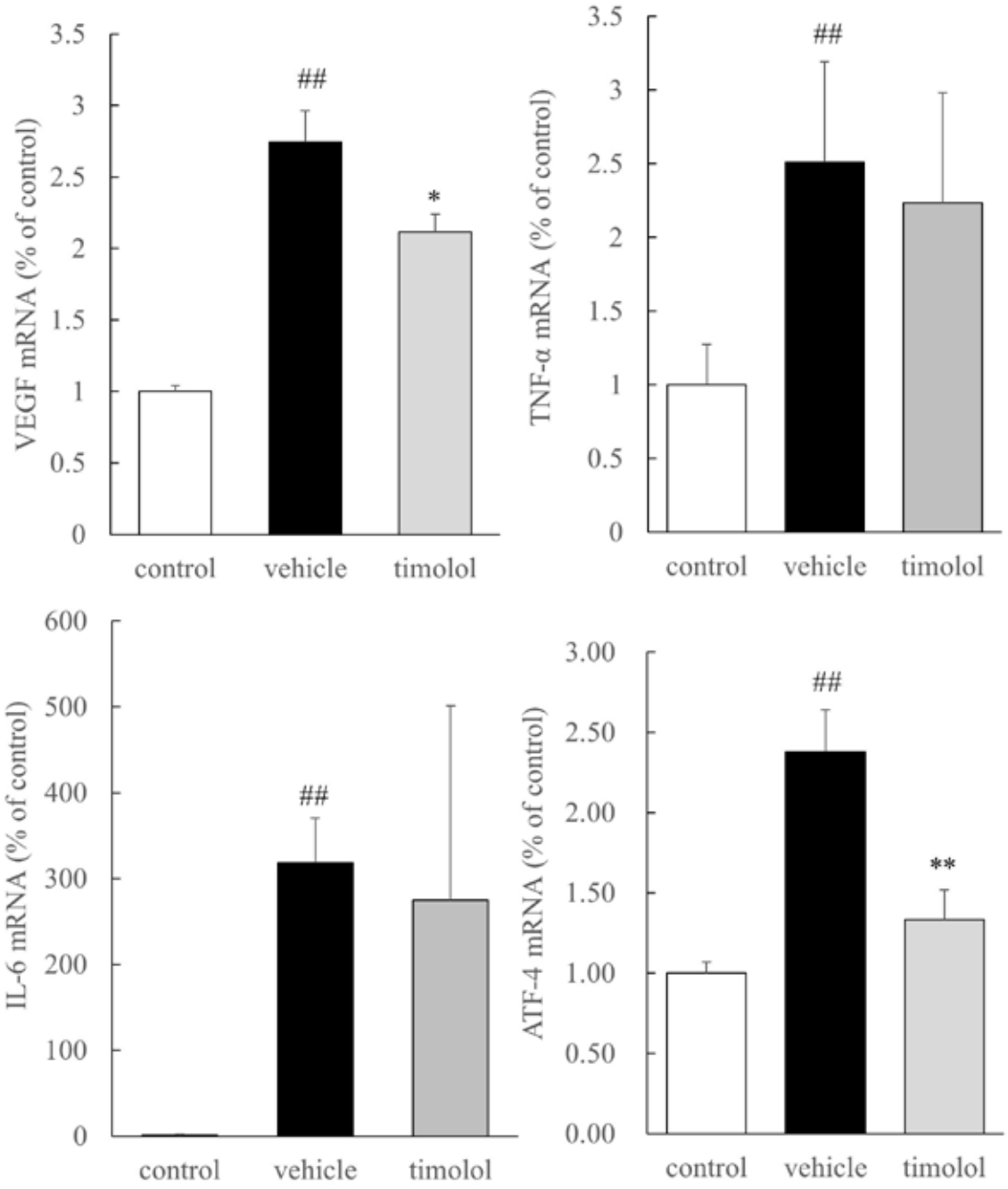

Figure 2. Expressions of RVO-related genes after timolol eye drops in the retina of RVO mice. Expression levels of *Vegf*, *Atf4*, *Il6*, and *Tnf-α* in the occluded retinas after topical administration of timolol maleate. Results are presented as mean ± SEM (n = 5, 6). *p<0.05, **p<0.01 versus vehicle, $^{\#\#}p$<0.01 versus control (Tukey's test).

inhibited edema formation in a rodent RVO model. However, this model might have increased catecholamine levels, as might the OIR model, due to the impaired blood flow and hypoxic stress response caused by vascular occlusion in the RVO model.

Previous reports have shown that β-adrenergic receptors are expressed in vascular endothelium, Müller, and bipolar cells [24]. Further, in primary cultured human umbilical vein endothelial cells (HUVECs), hypoxic stress enhanced the nuclear translocation of hypoxia-inducible factor 1-alpha (HIF-1-α), which is known to regulate the *Vegf* expression,

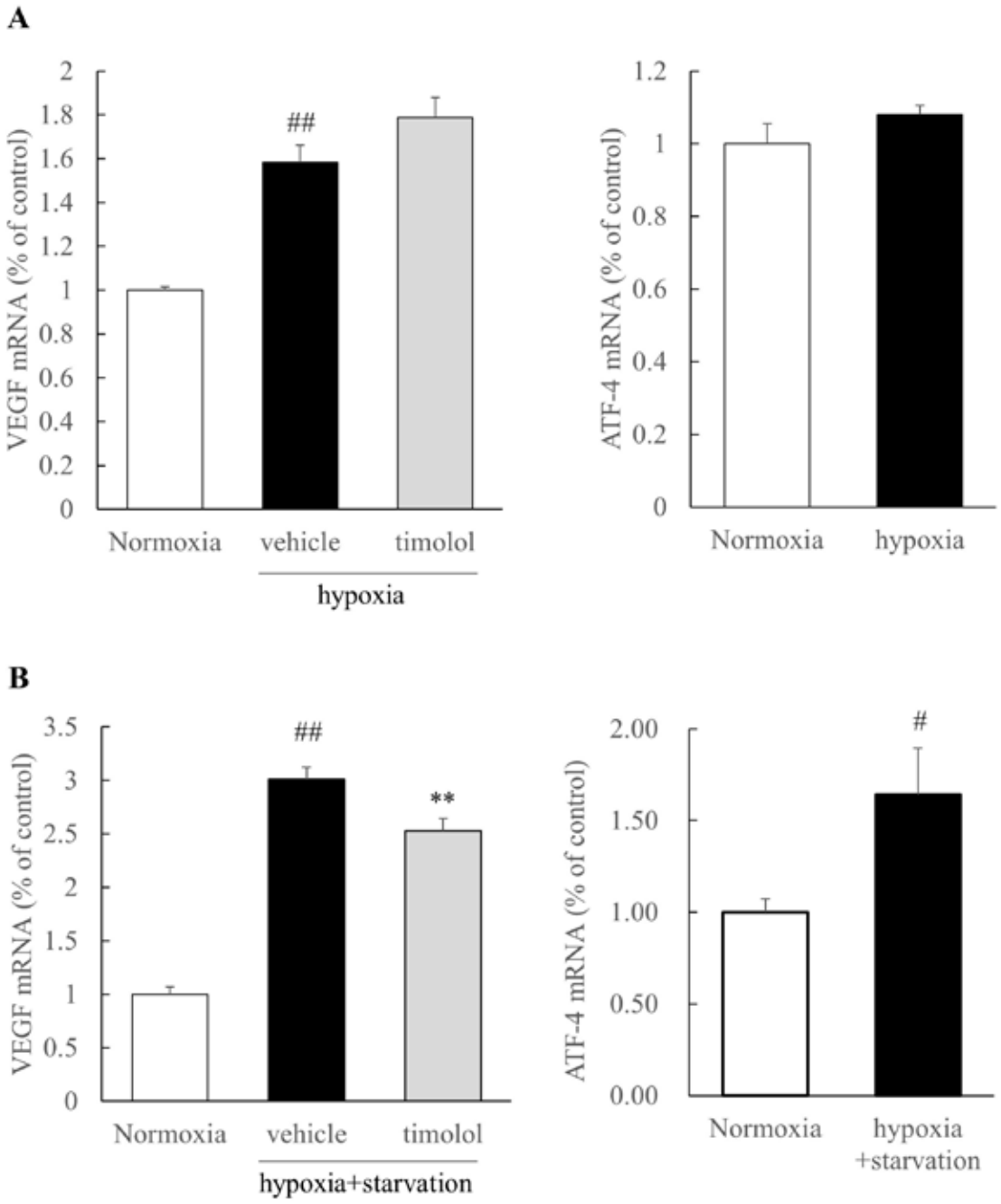

Figure 3. Effects of timolol on gene expressions in HRMECs under hypoxia. Expressions of *Vegf* and *Atf4* in HRMECs cultured under hypoxic conditions (**A**) and under hypoxia and serum starvation (**B**) and treated with timolol for 6 h. Results are the mean ± SEM (n = 4–8). **p*<0.05, ***p*<0.01 versus vehicle, $^{\#\#}p$<0.01, $^{\#}p$<0.05 versus normoxia (Tukey's test or student *t* test).

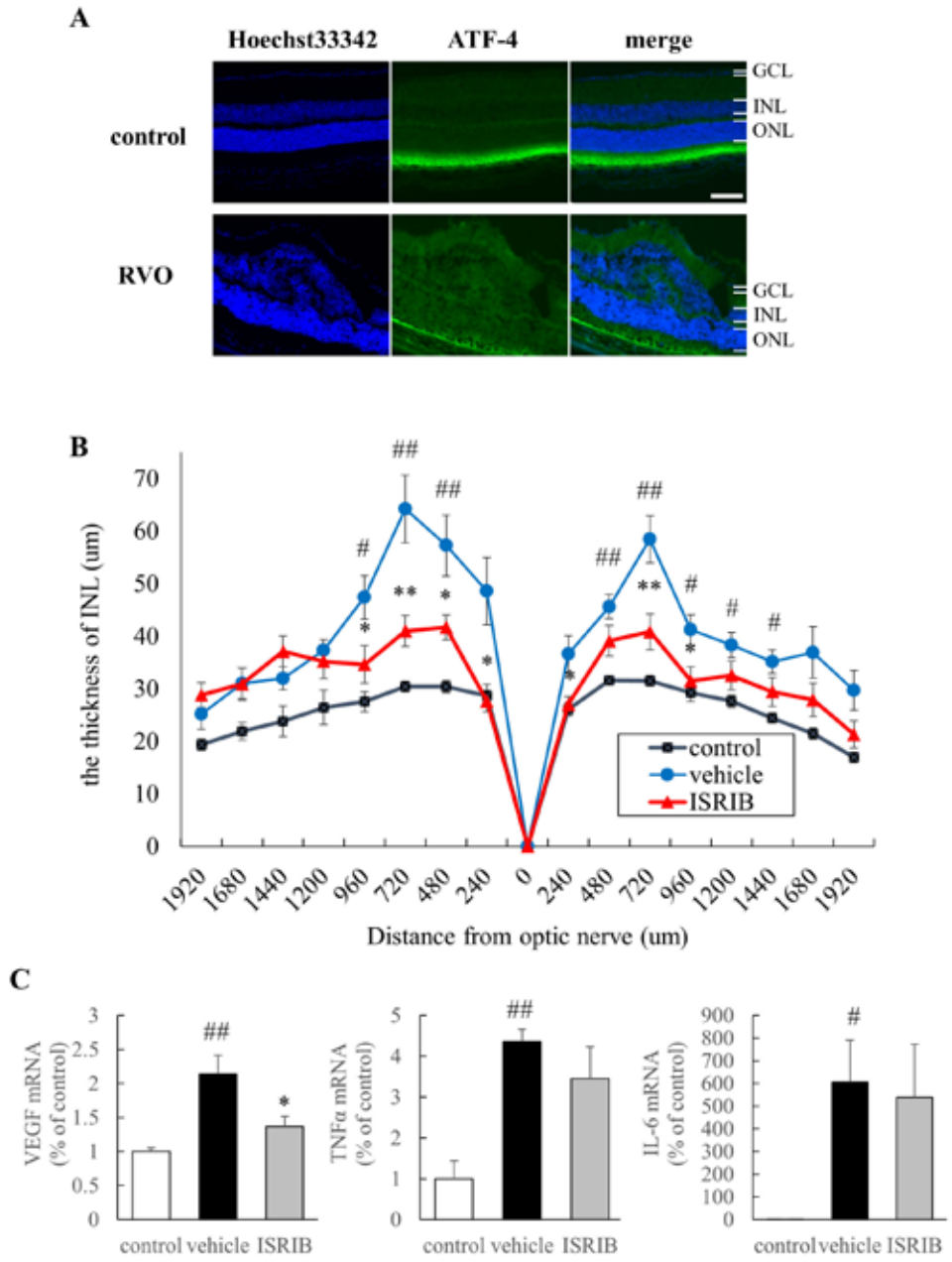

Figure 4. Role of *Atf4* in an RVO murine model. (A) Immunohistochemistry of ATF4 (green) and Hoechst 33,342 (blue) 24 h after occlusion. GCL; ganglion cell layer, INL; inner nuclear layer, ONL; outer nuclear layer (B) Quantitative data of INL thickness of the occluded retinas after IV injection of ISRIB. Results are the mean ± SEM (n = 5–12). **p*<0.05, ***p*<0.01 versus vehicle, $^{\#}p$<0.05, $^{\#\#}p$<0.01 versus control (Tukey's test). (C) Expressions of *Vegf*, *Tnf-α*, and *Il6* in occluded retinas 12 h after ISRIB injection. Results are the mean ± SEM (n = 5–12). **p*<0.05, ***p*<0.01 versus vehicle, $^{\#}p$<0.05, $^{\#xss\#}p$<0.01 versus control (Tukey's test).

whereas propranolol treatment suppressed this increase in HIF-1-α in the nucleus [25]. Therefore, we hypothesized that β-adrenergic receptors in retinal vascular endothelial cells regulate the increase in the *Vegf* expression in response to hypoxic stress, and as such, we investigated whether timolol suppresses the increased *Vegf* expression in HRMECs under hypoxic conditions. First, hypoxic stress among the HRMECs cultured in the medium containing serum increased the *Vegf*

expression, but it was not suppressed by timolol. Moreover, hypoxic stress in the medium containing a serum did not increase the expression of *Atf4*, which is known to be induced by endoplasmic reticulum (ER) stress and was suppressed by the in-vivo topical administration of timolol. In contrast, the application of hypoxic stress to HRMECs cultured in a medium without serum increased the *Vegf* expression, similar to the result obtained for the serum-containing conditions. Meanwhile, timolol supplementation suppressed the *Vegf* expression, which is regulated by ER stress; for instance, Yasuda et al. reported that treatment with thapsigargin, an ER stress inducer, increased the *Vegf* expression in HRMECs, and the ISRIB suppressed angiogenesis in a CNV model [16]. Furthermore, β-adrenergic receptors have been reported to be associated with ER stress. For example, isoproterenol, a β-adrenergic receptor agonist, increases the level of GRP78, an ER stress-related protein, and induces ER stress [26,27]. The present study also revealed that edema formation and an increased *Vegf* expression in the RVO model were inhibited by the ISRIB, which is known to ameliorate ER stress. Thus, timolol appears to improve retinal edema by suppressing the *Vegf* expression induced by ER stress in the retinas of the RVO model.

Our findings demonstrate that timolol eye drops suppress increases in the *Vegf* expression and reduce retinal edema. Currently, the first-line treatment for RVO is the IV administration of anti-VEGF antibodies, but this option presents limitations related to drug adherence, including invasiveness, the high cost, and the need for hospital visits. Although timolol may not always exceed anti-VEGF antibodies in terms of effectiveness in RVO patients, it can nevertheless be administered via eye drop in a less invasive manner. That is, timolol drops can be administered at home after anti-VEGF antibody treatment in the hospital to extend the length of time between anti-VEGF treatments, or it may be used as a maintenance therapy for patients whose symptoms are stable due to anti-VEGF antibody treatment. Future studies are needed to confirm the effects of the combination of the IV administration of anti-VEGF antibodies and timolol (eye drop) in an RVO mouse model. In addition, the protective effects of timolol and latanoprost on retinal neurons should be examined, as previous reports have suggested that retinal cell death was induced in the RVO model, as it is in glaucoma models, and ER stress may be involved in this cell death [19,28]. Our previous study showed that timolol and latanoprost suppressed ER stress-, oxidative stress-, and ischemic stress-induced retinal neuronal cell death, suggesting that these eye dropsreduced cell death in RVO [8]. However, further studies are needed to ascertain the effects of timolol on retinal cell death. Taken together, these data suggest that timolol maleate eye drops may ameliorate retinal edema by suppressing the *Vegf* pathway and ER stress, potentiating their role as a new therapeutic option for RVO patients.

Although cell death was not examined in this study, a previous report showed that the number of terminal deoxynucleotidyl transferase dUPT nick end labelling (TUNEL)-positive cells increased in RVO models, and the death of these cells may be responsible for VA deterioration [19]. Moreover, timolol inhibited cell death caused by various forms of stress , including oxidative, ischemic, and ER stress, and it may also improve VA by inhibiting cell death, as well as edema formation [8]. Another limitation was the inability to investigate visual function directly, but timolol may induce improvements regardless, as it ameliorates the formation of edema, which is associated with reduced VA. Thus, further studies focusing on cell death and visual function are needed. In conclusion, we found that timolol maleate, a β-adrenergic receptor antagonist, reduced retinal edema by suppressing the *Vegf* expression and ER stress in a murine RVO model, making it a potentially beneficial option in treating RVO patients.

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