

Neuroprotective Effects of Angiotensin II Type 1 Receptor (AT1-R) Blocker via Modulating AT1-R Signaling and Decreased Extracellular Glutamate Levels

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PURPOSE. To investigate the mechanism of the neuroprotective effects of the angiotensin II type 1 receptor (AT1-R) blocker against retinal ischemia-reperfusion injury in the rat.

METHODS. Retinal ischemia was induced by increasing intraocular pressure. Glutamate release from the rat retina and intravitreal PO₂ (partial pressure of oxygen) profiles were monitored during and after ischemia using a microdialysis biosensor and oxygen-sensitive microelectrodes. ELISA was used to measure changes in the expression of AT1-R. Retinal mRNA expressions of p47phox and p67phox were measured by real-time polymerase chain reaction. Reactive oxygen species (ROS) were measured using dihydroethidium.

RESULTS. Administration of candesartan, which is an AT1-R blocker (ARB), suppressed ischemia-induced increases in the extracellular glutamate. Candesartan also attenuated the increase in intravitreal PO₂ during reperfusion. AT1-R expression peaked at 12 hours after reperfusion. Although there was an increase in the retinal mRNA expression of p47phox and p64phox at 12 hours after the reperfusion, administration of candesartan suppressed these expressions. The production of ROS that was detected at 12 hours after reperfusion was also suppressed by the administration of candesartan or apocynin.

CONCLUSIONS. NADPH oxidase-mediated ROS production increased at 12 hours after reperfusion. Candesartan may protect neurons by decreasing extracellular glutamate immediately after reperfusion and by attenuating oxidative stress via a modulation of the AT1-R signaling that occurs during ischemic insult. (*Invest Ophthalmol Vis Sci.* 2012;53:4099–4110) DOI: 10.1167/iops.11-9167

Cell death of the retinal ganglion cells and the inner retinal neurons is a characteristic of retinal ischemia-reperfusion injury. It is believed that ischemia-induced injury may play an important role in various retinal diseases such as glaucoma, retinal vein occlusion, and diabetic retinopathy. At the present

time, the mechanism responsible for retinal ischemia-induced cell death has yet to be completely clarified. Reactive oxygen species (ROS) trigger ischemic cell damage and lead to the hypersecretion of glutamate and aspartate.¹ These excess amounts of glutamate that are produced during pathologic conditions such as hypoxia² and ischemia-reperfusion^{3,4} can ultimately lead to neuronal cell toxicity. In fact, the excess amount of glutamate that is produced during ischemia-reperfusion stimulates N-methyl-D-aspartate (NMDA), which is a subtype of the glutamate receptor,³ thereby inducing an influx of excess Ca²⁺ via the NMDA receptor-operated channels.^{5,6}

The renin-angiotensin system (RAS) is a major controller of systemic blood pressure. There are two angiotensin II (Ang II) receptor subtypes: Ang II type 1 receptor (AT1-R) and Ang II type 2 receptor (AT2-R).^{7,8} Since it has been shown that the major Ang II-related systemic functions are mediated by AT1-R signaling, this antagonist action has been widely used to treat hypertension and cardiac diseases. Ang II activates the nicotinamide adenine dinucleotide phosphate (NADPH)-dependent oxidase complex, which is a major source of superoxide (O₂⁻), and is upregulated in several pathologic conditions associated with oxidative stress.^{9,10} NADPH-oxidase-derived ROS plays a major role in Ang II signaling in the neurons.^{11–13} The oxidase itself is a complex enzyme that consists of a membrane subunit, cytochrome b₅₅₈ (gp91phox [NOX 2] and p22phox), and multiple cytosolic subunits (p47phox, p67phox, p40phox, and Rac-1). When stimulated, the cytosolic subunits migrate to the plasma membrane, which leads to the formation of a functional complex that generates ROS.¹⁴

We recently reported that the AT1-R antagonist had a neuroprotective effect against retinal ischemia-reperfusion injury.¹⁵ The purpose of the present study was to further investigate the mechanism of the protective effects of the AT1-R blocker, candesartan, on neuronal death in retinal ischemia-reperfusion injury.

MATERIALS AND METHODS

Animals

Male Sprague-Dawley rats weighing 200 to 250 g were obtained from Charles River Japan (Yokohama, Japan). Rats were permitted free access to standard rat food (Oriental Yeast Co., Ltd., Tokyo, Japan) and tap water. Animal care and all experiments were conducted in accordance with the approved standard guidelines for animal experimentation of the Kagawa University Faculty of Medicine, and adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

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Drug

AT1-R antagonist candesartan was obtained from a commercial source (Toronto Research Chemicals, North York, Ontario, Canada). Candesartan was administered intraperitoneally (IP) 30 minutes before the induction of ischemia or orally on a daily basis via the use of feeding needles.

Ischemia

Rats were anesthetized via an IP injection of 50 mg/kg pentobarbital sodium (Abbott Laboratories, Abbott Park, IL) in conjunction with topically administered 0.4% oxybuprocaine hydrochloride. The anterior chamber of the right eye was cannulated with a 27-gauge infusion needle connected to a reservoir containing normal saline. The intraocular pressure (IOP) was raised to 130 mm Hg for 45 minutes by elevating the saline reservoir. After ischemia, the needle was withdrawn, and reflow of the retinal circulation was documented visually. Only the right eye of each rat was subjected to ischemia. Retinal ischemia was indicated by whitening of the iris and fundus. The left eye of each rat served as the nonischemic control. Rectal and tympanic temperatures were maintained at approximately 37°C, using a feedback-controlled heating pad (Bio Research Center Co. Ltd, Nagoya, Japan) during the operation. After restoration of blood flow, temperature continued to be maintained at 37°C.

Measurements of Glutamate in the Vitreous Body

Measurements were performed using a previously described method.¹⁶ Briefly, a dialysis electrode (Microdialysis Biosensor, Applied Neuroscience, London, UK) that was composed of a platinum wire located within a hollow semipermeable dialysis membrane (500 Dalton) with an outside diameter of 230 μ m was used for the measurements. Two additional electrodes, which included a reference electrode (Ag/AgCl) and a counter electrode (Ag) that was comprised of an electrochemical cell, were collectively installed in a glass capillary that was located away from the main sensing area. For the glutamate measurements, the probe was filled with 10 mM phosphate-buffered saline (PBS, pH 7.4) with or without glutamate oxidase (100 U/mL; Yamasa Co., Ltd., Chiba, Japan) and then perfused at a flow rate of 0.2 μ L/min by means of a microinfusion pump (IP-2 Microinfusion Pump; Bio Research Center). Each of these substances is able to diffuse across the dialysis membrane into the electrode, with the respective oxidase then producing hydrogen peroxide that can be detected by the electrode. The current that is detected by the electrode is sent to an amplifier and is recorded on a polygraph in real time. In each experiment before and after *in vivo* measurements of glutamate and H₂O₂ were determined with and without glutamate oxidase solution in the test tube, respectively. Regression lines were then plotted based on known concentrations. Current detected by the electrode was sent to an amplifier (EPS-800; Eicom, Kyoto, Japan), with a change of 1 nA equivalent to 8 μ M of glutamate. At 30 minutes prior to the ischemia, 1 mg/kg candesartan or saline was administered, with the glutamate monitored in the candesartan and control groups before, during, and after the ischemia. Body temperature was maintained at around 37°C during the measurements. Regression lines were calculated based on the known glutamate concentrations in each of the experiments.

Measurements of PO₂ in the Vitreous Body

Intravitreal oxygen was monitored using a 0.1-mm-diameter PO₂ (partial pressure of oxygen) probe (Intermedical Co., Ltd., Nagoya, Japan). After the fiber-optic probe was dipped into an oxygen-saturated PBS solution, the value of the amplifier was adjusted to 155 mm Hg. Using the same technique, the probe was then inserted into the vitreous for the glutamate measurements.

ELISA for AT1-R

The eyes were immediately enucleated at 15 minutes, 1 hour, 3 hours, and 12 hours after 45 minutes of ischemia, with the retina then carefully isolated. The retinas were put into buffer (IBLysis-I; Immuno-Biological Laboratories [IBL], Takasaki-Shi, Japan) and homogenized. Samples were centrifuged at 10,000 rpm for 10 minutes, with the supernatant fluid then removed and put into individual wells of a 96-well plate. After adding 10 μ g/mL rabbit anti-AT1-R antibody (Santa Cruz Biotechnology, Santa Cruz, CA) to each well, the plates were covered and kept at 4°C overnight. On the next day, the wells were washed with PBST (PBS + 0.05% Tween 20), followed by the addition of horseradish peroxidase (HRP)-conjugated anti-rabbit antibody and a further incubation at 4°C for 1 hour. Subsequently, after washing the samples with PBST and then adding 100 μ L substrate (TMBS; IBL), samples were placed in the dark at room temperature for a 30-minute period to allow for development. After the reaction was stopped with 100 μ L 1 N H₂SO₄, optical density (OD450) measurements were immediately performed.

Fluorescent Labeling of ROS

To investigate the production of ROS, we injected 5 mg/kg dihydroethidium (DHE; Sigma-Aldrich, Tokyo, Japan) in 5% DMSO in PBS IP 30 minutes prior to enucleation of the eyeballs. A 0.3-mL aliquot of distilled water was administered IP 30 minutes before ischemia or 11 hours after reperfusion. A 0.3-mL aliquot of 1 mg/kg candesartan was administered IP and 0.5 μ L of 2 mM apocynin (Extrasynthese, Genay Cedex, France), which is a specific inhibitor of NADPH oxidase, was injected into the vitreous at 11 hours after the reperfusion. Eyes were enucleated at 1 hour or 12 hours after reperfusion and then embedded in optimal cutting temperature compound (Sakura Finetek, Torrance, CA), after which cryosections (20 μ m) were prepared. Sections were examined with a microscope (Radiance 2100/Rainbow, Carl Zeiss, Munich, Germany) using a laser set (excitation laser 514 nm; emission laser more than 580 nm).

mRNA Expression of NADPH Oxidase Components

p47phox and p67phox mRNA expression levels in the retinal tissues were quantitatively analyzed by real-time transcription polymerase chain reaction, according to a previously described method.¹⁸ Data were compared with the retinal expression of GAPDH in the controls and then expressed as the relative difference at 15 minutes, 3 hours, or 12 hours after reperfusion when treated with vehicle or at 12 hours after reperfusion when treated with candesartan. Oligonucleotide primers for p47phox and p67phox were synthesized based on previously published sequences.¹⁶

Histologic Detection of Released H₂O₂

In vitro detection was done by applied immunohistochemical staining using HRP (anti-mouse IgG, peroxidase-linked species-specific F(ab')₂ fragment from sheep; Amersham, Piscataway, NJ) and diaminobenzidine (DAB) solution (1 mg/mL; Vector Laboratories Inc., Burlingame, CA) and glutamate oxidase (GO) (100 U/mL) (Yamasa Co., Ltd., Okayama, Japan).¹⁷ The retina was quickly removed and immersed in ice-cold artificial cerebrospinal fluid (ACSF) with 10 mM glucose bubbled in a gaseous mixture of 95% O₂ and 5% CO₂. The composition of ACSF was as follows (in mM): 124 NaCl, 4.4 KCl, 2.5 CaCl₂, 1.3 MgSO₄, 1 NaH₂PO₄, and 26 NaHCO₂. The retina was incubated in a dish with 500 μ L of ACSF that also contained 0.04% each of GO and HRP, maintained at 37°C for at least 30 minutes before the experiment. To examine the H₂O₂ released, 5 μ L of DAB solution was added to the retina followed by observation under a stereoscopic microscope for the development of a brown color, as the DAB solution undergoes a polymerization reaction to yield the brown color upon binding to H₂O₂.¹⁸ For ischemia induction, the

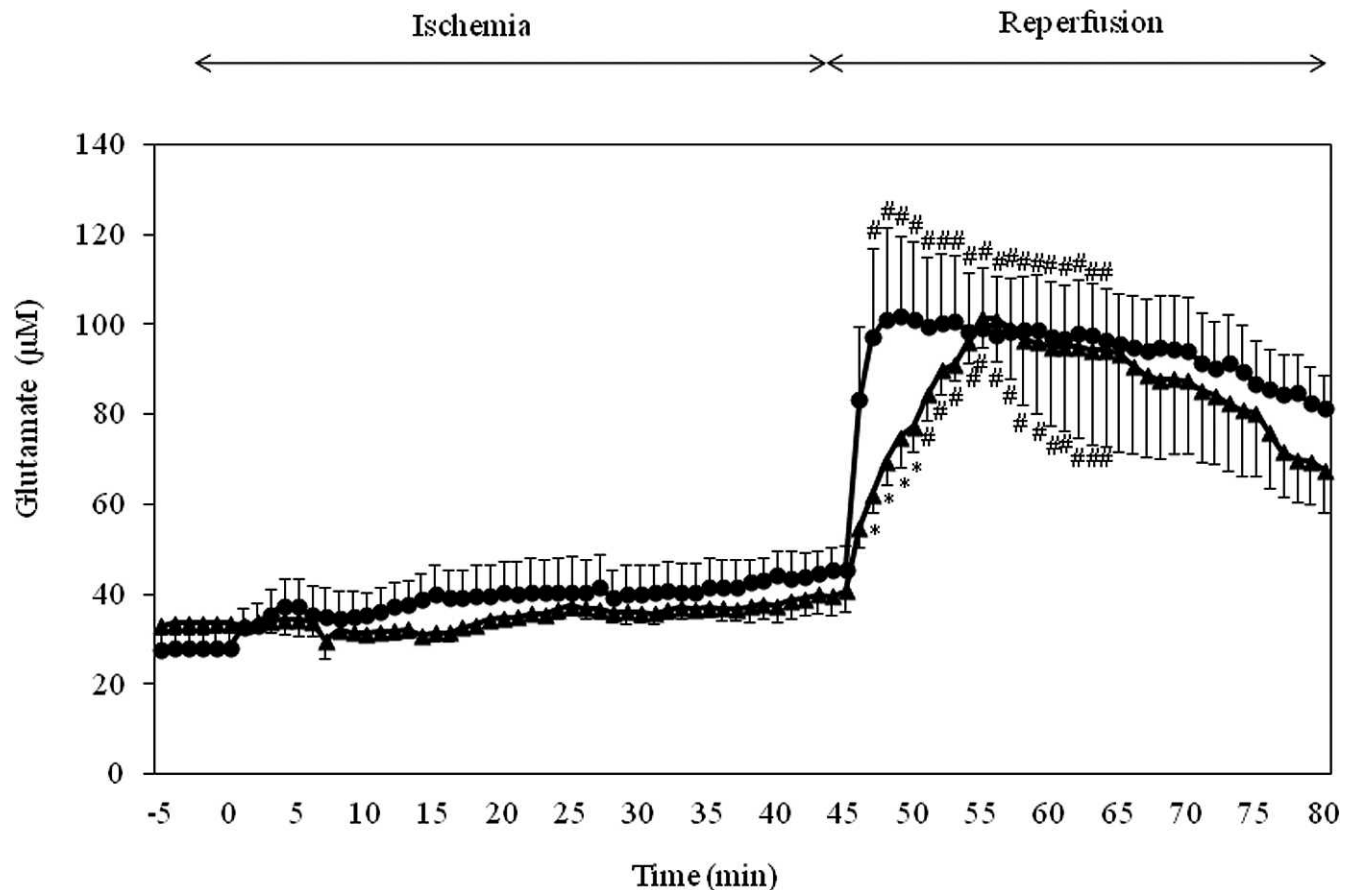


FIGURE 1. Effect of candesartan on the release of glutamate from the rat retina. Ischemia was induced by elevating the intraocular pressure for 45 minutes. Candesartan was administered 30 minutes before ischemia and glutamate was measured by an electroenzymatic method of microdialysis. Candesartan suppressed the release of glutamate. Data represent the mean $\pm$ SEM: Control (solid circle); Candesartan (solid triangle). * $P < 0.05$ vs. control (independent Student's *t*-test). # $P < 0.05$ vs. before ischemia (one-way ANOVA).

solution was changed to ACSF bubbled with 100% N₂ gas. A stereoscopic microscope (Leica MZ FL II; Leica Microsystems, Tokyo, Japan) was used with analytical software (Olympus CCD, DP70 and DP70-WPCP; Olympus Inc., Tokyo, Japan).

Histologic Examination

For the histologic examination, rats were anesthetized by IP injection of pentobarbital sodium (50 mg/kg) 7 or 21 days after ischemia. The anterior segments including the lens were removed. The posterior eye cups were embedded in paraffin, and thin sections (5 μ m in thickness) were cut using a microtome. The sections were carefully cut to include the full length from superior to inferior along the vertical meridian through the optic nerve head. Each eye was mounted on a silane-coated glass slide and then stained with hematoxylin and eosin. Scleral thickness was measured to confirm that the sections were not oblique sections.

Morphometric analysis was performed to quantify ischemic injury. Five sections were selected randomly in each eye. A person with no prior knowledge of the treatments performed all of the light microscopic (magnification, $\times 10$ to $\times 100$; Olympus BX-51, Olympus Inc.) examinations. A microscopic image of each section within 0.5 to 1 mm superior of the optic disc was scanned. In each computer image, the thickness of the inner plexiform layer (IPL) and inner nuclear layer (INL) were measured. For each animal, this parameter in the right eye was normalized to that in the intact left eye and shown as a percentage.

Retrograde Labeling of Retinal Ganglion Cells

At 7 days prior to sacrifice, hydroxystilbamidine (Molecular Probes Inc., Eugene, OR) was injected bilaterally into the superior colliculi of anesthetized rats. The skull was exposed and kept dry and clean. After identifying and marking the bregma, a small window was drilled in the scalp of both the right and left hemispheres. The windows were drilled to a depth of 3.6 mm from the surface of the skull and located at 6.8 mm behind the bregma on the anteroposterior axis, and 1.5 mm lateral to the midline. Using a commercial syringe (Hamilton Co., Reno, NV), 1.5 μ L of 2% hydroxystilbamidine was slowly injected into the bilateral superior colliculi. After suturing the skin over the wound, antibiotic ointment was applied.

Tissue Preparation and Assessment of Labeled Retinal Ganglion Cell Survival

Animals were euthanized using an overdose of pentobarbital sodium (Nembutal) at 1 week after fluorescent dye (Fast Blue; Polysciences Inc., Warrington, PA) application. Whole, flat-mounted retinas were then assayed for retinal ganglion cell density. Rat eyes were enucleated and fixed in 4% paraformaldehyde for 10 hours at room temperature. After removal of the anterior segments, the resultant posterior eyecups were left in place. Subsequently, four radial cuts were made in the periphery of the eyecup, with the retina then carefully separated from the retinal pigment epithelium. To prepare the flat mounts, the retina was dissociated from the underlying structures, flattened by making four

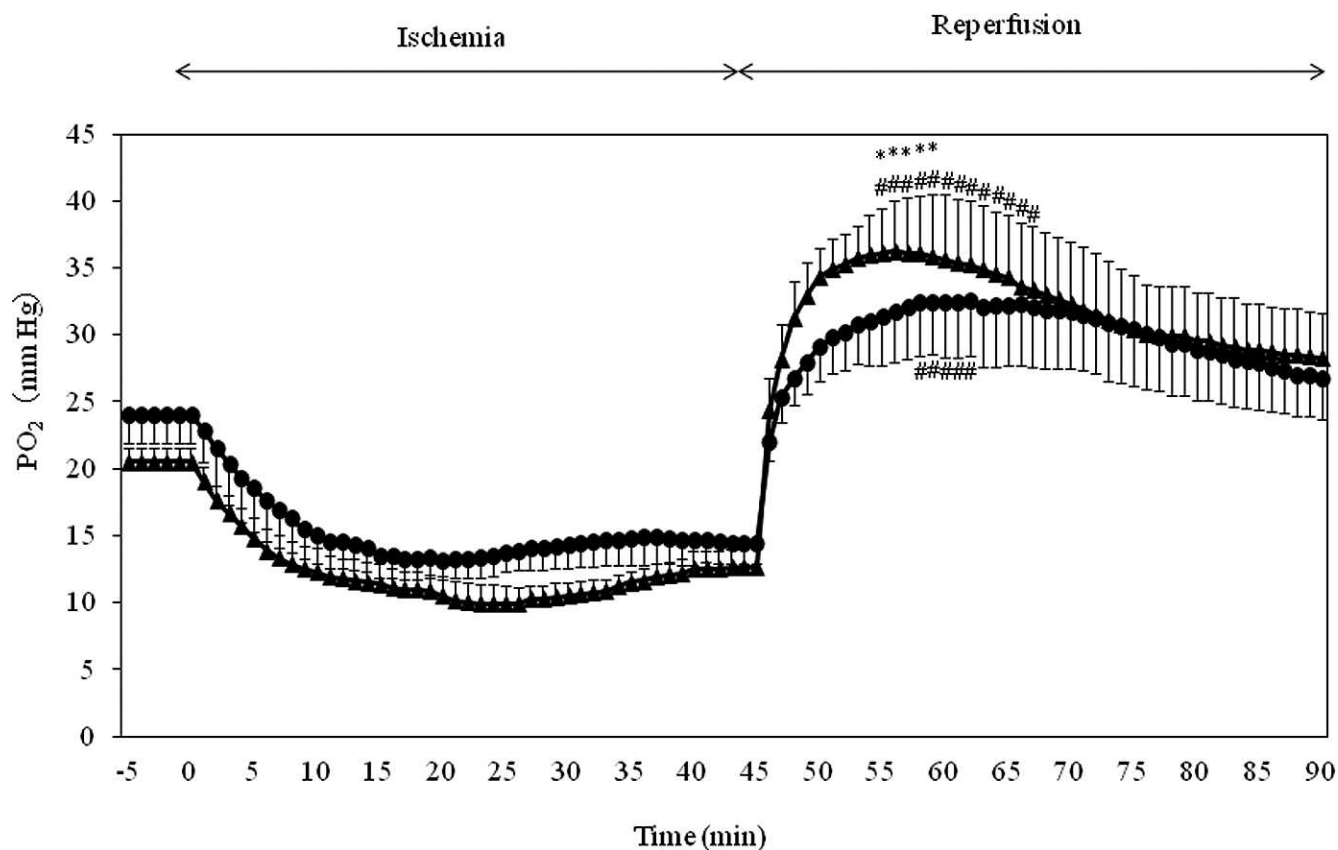


FIGURE 2. Effect of candesartan on PO₂ levels. Ischemia was induced by elevating the intraocular pressure for 45 minutes. Candesartan was administered 30 minutes prior to the ischemia. PO₂ levels, which were measured by a PO₂ probe, were significantly increased after ischemia and enhanced after candesartan administration. Data represent the mean $\pm$ SEM: Control (solid circle); Candesartan (solid triangle). * $P < 0.05$ vs. control (independent Student's t -test). # $P < 0.05$ vs. before ischemia (one-way ANOVA).

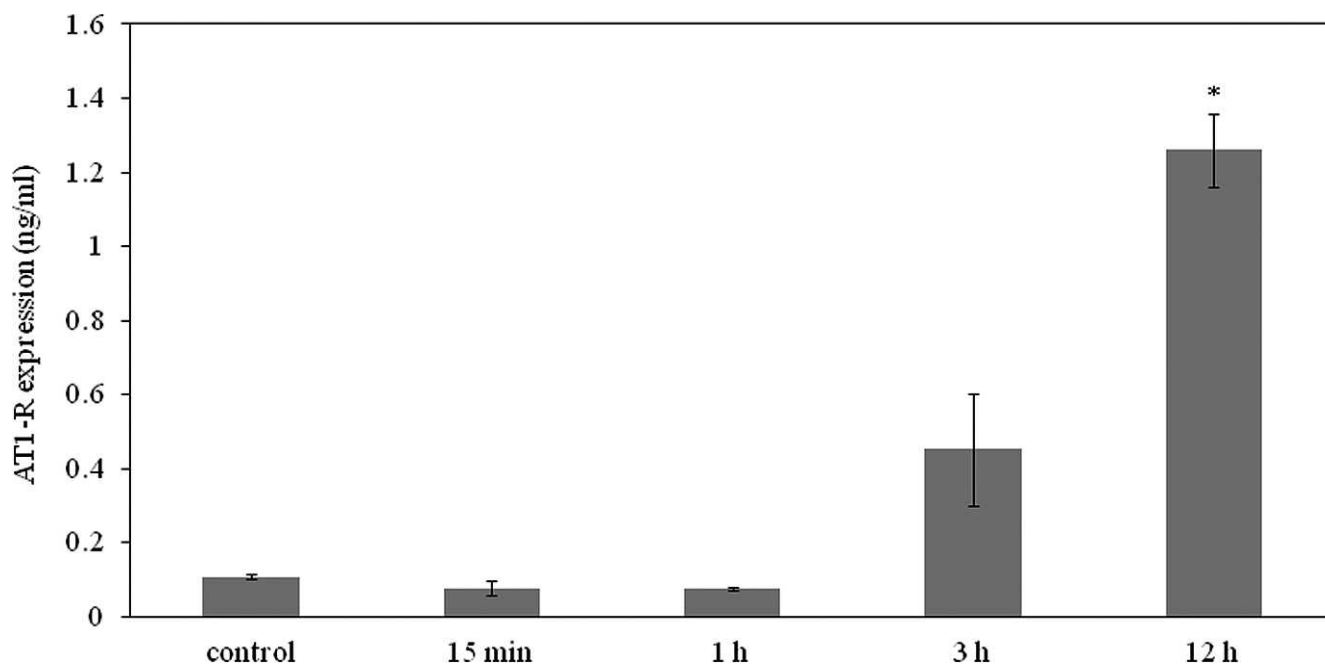


FIGURE 3. AT1-R expression of normal rat retina and rat retina at 15 minutes and 3, 6, and 12 hours after reperfusion. Data represent the mean $\pm$ SEM. * $P < 0.001$ vs. control (Dunnett's multiple comparison test).

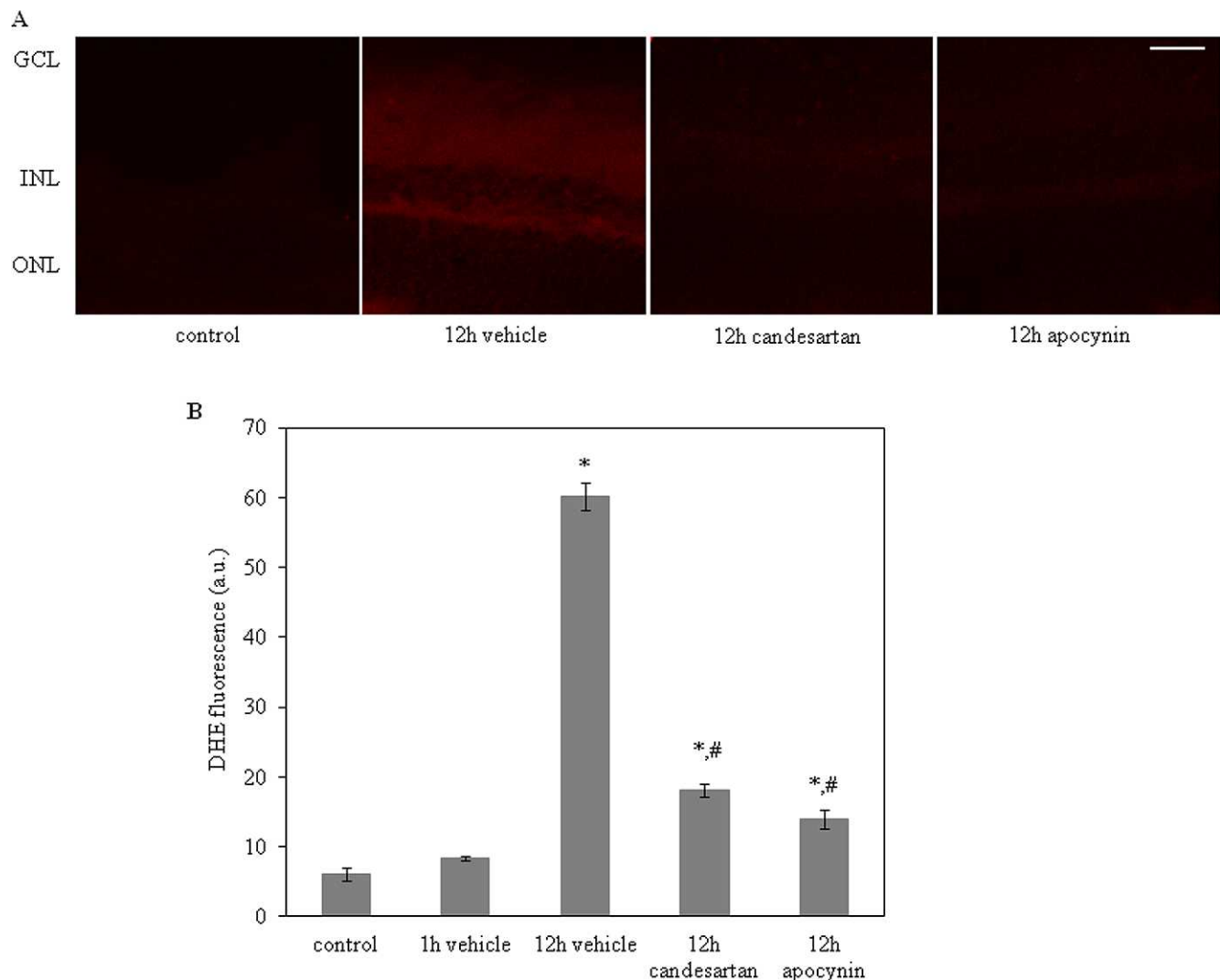


FIGURE 4. Effect of candesartan on the release of ROS. **(A)** ROS, which was detected by dihydroethidium (DHE), was upregulated in retinal neuronal cells in the retina at 12 hours after reperfusion. However, ROS levels decreased after candesartan pretreatment. Scale bar, 5 μ m. **(B)** Quantified specific retinal DHE fluorescence is expressed in arbitrary units (AU) for each of the groups. Data represent the mean $\pm$ SEM. * $P < 0.05$ vs. control. # $P < 0.05$ vs. 12 hours after reperfusion with vehicle (Tukey's honestly significant difference test).

radial cuts, and then spread on a gelatin-coated glass slide. Labeled retinal ganglion cells (RGCs) were visualized under a fluorescence microscope (Olympus BX-51/DP70; Olympus Inc.) with an ultraviolet filter (blue-violet: 395 to 440 nm). Fluorescence-labeled RGCs were counted in 12 microscopic fields of retinal tissue from 1 mm away from the optic disc. Image enhancement and analysis software (Image-Pro Plus, v 4.0, Media Cybernetics, Bethesda, MD) was used to count the total number of RGCs in each eye. Changes in the densities of the RGCs were expressed as the RGC survival percentage, which was based on a comparison between the surgical and contralateral control eyes. The specimens that were compared came from different retinal regions of the same animal.

Electroretinograms

Electroretinogram (ERG) responses were measured after overnight dark adaptation (at least 6 hours) using a recording device (Mayo Corporation, Aichi, Japan) 7 days after ischemia. Rats were anesthetized with an IP injection of 50 mg/kg pentobarbital sodium. Pupils were dilated with 0.5% tropicamide and 0.5% phenylephrine hydrochloride eye drops (Santen Pharmaceuticals, Osaka, Japan). All procedures were performed in dim red light, with all rats kept warm.

The light-emitting diode (LED) corneal electrode was set vertical to the cornea center. A reference electrode was set subcutaneously on the forehead and the ground connection was set on the base of the tail. An LED stimulator (LS-W) controlled the stimulus duration and intensity during the 11-step intensity series, which ranged from 0.0003 to 30 cds/m². The ERG response was amplified using an AC amplifier (ML135; Bio Amplifier, AD Instruments, NSW, Australia) with a bandwidth of 0.3 to 500 Hz and amplification of $\times 2000$. The ischemic damage to the retina was evaluated as the percentage of the a- and b-wave amplitudes of the ischemic right eyes as compared to the nonischemic left eyes.

Immunohistochemistry for AT1-R

Eyes were enucleated at 21 days after ischemia. Eyes were then fixed in 4% paraformaldehyde and embedded in paraffin. Retinal sections (5 μ m) were rinsed in 100% ethanol twice for 5 minutes each, followed by a separate 95% ethanol and 90% ethanol rinse for 3 minutes each. The sections were then washed using PBS, pH 7.4, three times for 10 minutes each and treated with 0.3% Triton X-100 in PBS, pH 7.4, for 1 hour. After further washing three times for 10 minutes each with PBS,

pH 7.4, sections were then blocked in 3% normal horse serum and 1% bovine serum albumin (BSA) in PBS for 1 hour, to reduce nonspecific labeling. Sections were incubated overnight at 4°C in a 1:100 dilution of rabbit polyclonal antibody against human AT1-R (Santa Cruz Biotechnology), as the primary antibody, in PBS containing 0.5% Triton X-100, 5% normal horse serum, and 1% BSA. Control sections were prepared by omitting both the primary antibody and the rabbit IgG (1:1000, Vector Laboratories Inc.), followed by incubation only in PBS containing 0.5% Triton X-100, 5% normal horse serum, and 1% BSA overnight at 4°C. After washing in PBS for 50 minutes, sections were then immersed in alkaline phosphatase (AP; Vectastain ABC-AP Kit; Vector Laboratories Inc.) for 30 minutes at room temperature, washed in PBS for 15 minutes, and processed using the avidin-biotin complex reagent (ABC Kit PK-6101; Vector Laboratories Inc.) for 1 hour at room temperature. Images were acquired using $\times 40$ objective lenses (DXM 1200; Nikon, Tokyo, Japan). A photographic editing program (Adobe Photoshop v. 5.0; Adobe Systems Inc., San Jose, CA) was used to adjust the brightness and contrast of the images.

Statistical Analysis

Fluorescence or brown color was quantified by automated image analysis software (Image-Pro Plus v. 4.0; Media Cybernetics). After setting a threshold that defined positive labeling in each section, the mean fluorescence was calculated from five separate high-power fields per eye.

All data are presented as the mean $\pm$ SEM. Data were analyzed using an independent Student's *t*-test, Dunnett's multiple comparison test, or Tukey's honestly significant difference test, as appropriate. The glutamate and PO₂ data were analyzed by one-way ANOVA and an independent Student's *t*-test was used to assess significance between the control and candesartan group. A value of $P < 0.05$ was considered statistically significant.

RESULTS

Effect of Candesartan on Extracellular Glutamate by Ischemia

The glutamate efflux time course in the vitreous body during the 45-minute ischemia and reperfusion periods in the control and candesartan groups is shown in Figure 1. Glutamate levels tended to increase after the first 5 minutes of ischemia, with the glutamate levels from the retina exhibiting a 1.5-fold increase during this time period. Subsequently, a remarkable increase in glutamate was observed after the reperfusion. Candesartan administration suppressed the ischemia-induced increase of the extracellular glutamate ($n = 4$ in each group).

Effect of Candesartan on PO₂ in the Vitreous Body after Ischemia

The PO₂ time course in the vitreous body during the 45-minute ischemia and reperfusion period in the control and candesartan groups is shown in Figure 2. After recirculation, the PO₂ levels increased, with a maximum level attained within approximately 15 minutes. The candesartan-enhanced PO₂ levels reached a maximum at approximately 10 minutes after the start of the reperfusion ($n = 4$ in each group).

AT1-R Expression in the Retina after Ischemia

Ischemia/reperfusion injury caused an upregulation of the protein levels of AT1-R in the retina (Fig. 3). AT1-R expression was low at 15 minutes (0.076 ± 0.020 ng/mL; $P = 0.997$), 1 hour (0.074 ± 0.005 ng/mL; $P = 0.996$), and 3 hours (0.452 ± 0.152 ng/mL; $P = 0.063$) after the reperfusion. At 12 hours after

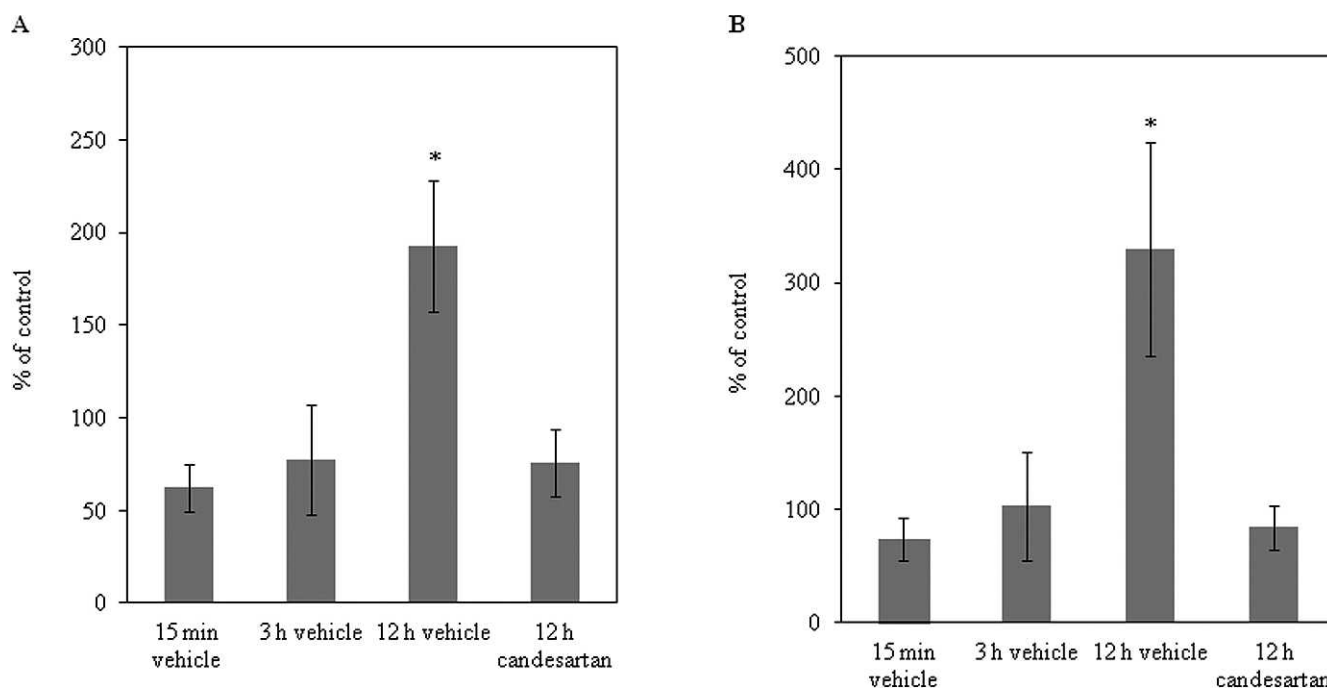


FIGURE 5. p47phox (A) and p67phox (B) mRNA expression in the rat retina. Increases in the mRNA levels of NADPH oxidase membrane components, p47phox and p64phox, were observed in the retina at 12 hours after the reperfusion. However, p47phox and p64phox levels decreased after pretreatment with 1 mg/kg candesartan. Data express the mean $\pm$ SEM. * $P < 0.05$ vs. control (Dunnett's multiple comparison test).

the reperfusion, a remarkable increase in the levels was noted (1.264 ± 0.103 ng/mL; $P < 0.001$) ($n = 4$ in each group).

ROS Production after Ischemia

At 1 hour after reperfusion, ROS production in the retina remained low (8.34 ± 0.35 ; $P = 0.457$) (Fig. 4). At 12 hours after reperfusion of the retina, an upregulation of the DHE fluorescence was noted in the retinal neurons (60.20 ± 1.99 ; $P < 0.001$). After administration of candesartan or apocynin, a significant suppression of this upregulation was observed (18.14 ± 0.93 or 13.99 ± 1.33 , respectively; $P < 0.001$, as

compared with 12 hours after reperfusion with vehicle) (Fig. 4, $n = 4$ in each group).

mRNA Expression of NADPH Oxidase Components after Ischemia

Expression of p47phox and p67phox mRNA in the retina was similar among samples for the controls, 15 minutes after (62.3 ± 25.5 ; $P = 0.605$, 73.8 ± 18.3 ; $P = 0.986$) and 3 hours after the reperfusion (77.4 ± 29.5 ; $P = 0.891$, 102.8 ± 48.3 ; $P > 0.999$) (Figs. 5A, 5B). However, an increased expression of p47phox and p67phox mRNA in the retina was observed at 12

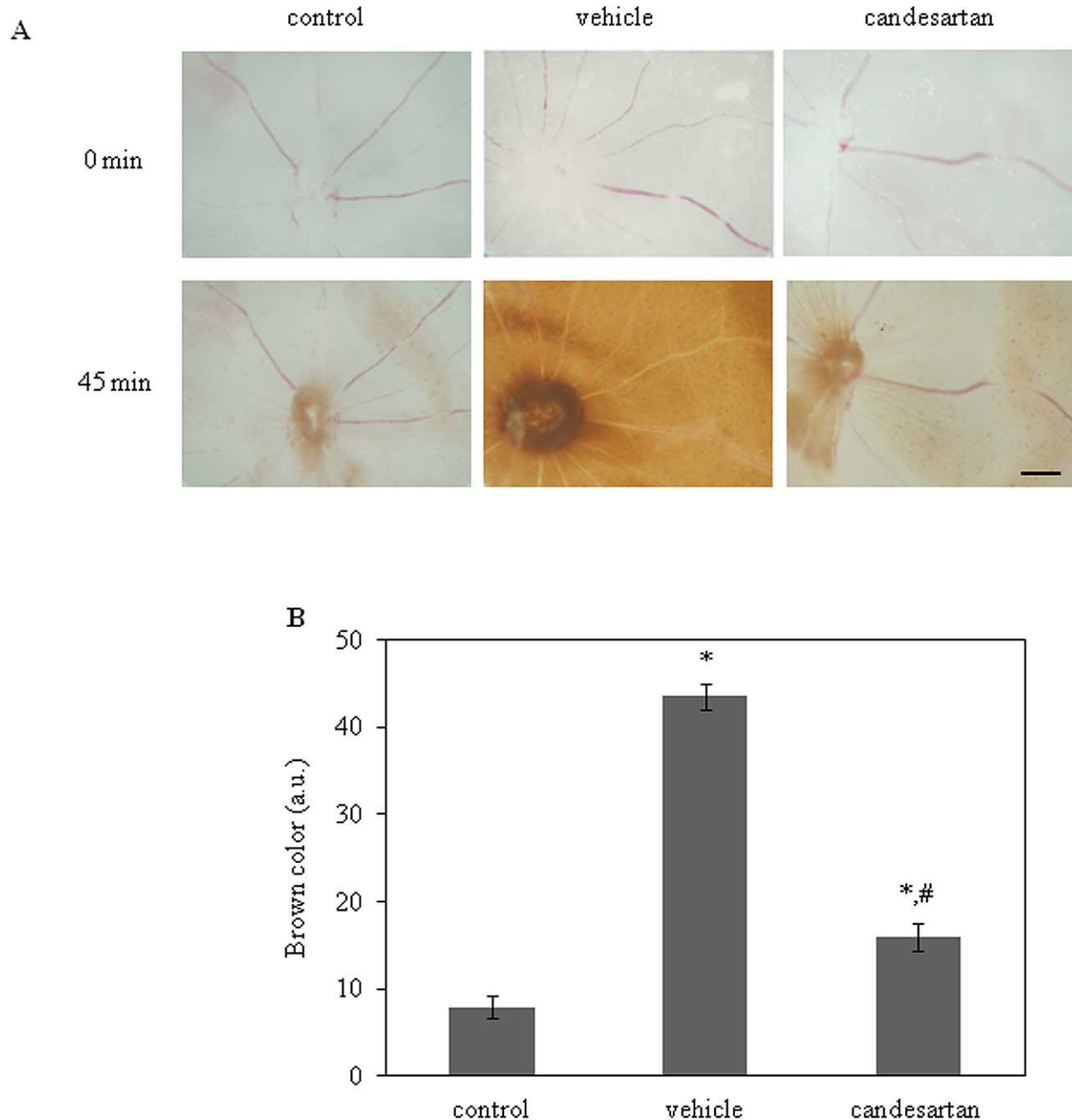


FIGURE 6. Effect of candesartan on the release of H_2O_2 . The brown color indicates the release of H_2O_2 . Color microphotographs were taken before induction of ischemia and at 45 minutes after starting ischemia induction (**A**). There was no brown staining in the control flat-mounted retina. (**B**) Quantified retina-specific brown color is expressed in AU for each of the groups. Data represent the mean $\pm$ SEM. * $P < 0.05$ vs. control (Dunnett's multiple comparison test). Scale bar, 200 μ m.

hours after the reperfusion (193.0 ± 35.3 ; $P = 0.038$, 329.6 ± 94.6 ; $P = 0.016$). Administration of candesartan suppressed the increased expression of both p47phox and p67phox (75.6 ± 18.0 ; $P = 0.863$, 84.0 ± 19.2 ; $P = 0.998$) (Figs. 5A, 5B, $n = 4$ in each group).

Effect of Candesartan on H_2O_2 Release in Vitro

Figure 6A shows a flat-mounted retina. Light microscopic photographs were taken of the treatments with and without candesartan. Without candesartan treatment, the brown color was significantly increased 45 minutes after ischemia (92.9 ± 1.7 ; $P < 0.01$) (Fig. 6B). However, in the presence of candesartan, the brown color was suppressed 45 minutes after ischemia (57.5 ± 5.5 ; $P < 0.01$, as compared with vehicle) (Fig. 6B, $n = 4$ in each group).

Effect of Candesartan at 21 Days after Ischemia

Figure 7A shows representative results for the RGC labeling in vehicle- and candesartan-treated rats. Compared with the vehicle-treated rat, RGC death seemed to be mild in the candesartan-treated rat. RGC survival rates were 52.6 ± 2.0 in the vehicle-treated group and 69.7 ± 1.6 in the candesartan-treated group ($P < 0.001$; Fig. 7C, $n = 4$ in each group).

In animals pretreated with distilled water, IPL and INL thicknesses were reduced to $64.2 \pm 1.3\%$ and $61.8 \pm 4.8\%$ ($n = 4$) of the control, respectively (Figs. 7D, 7E). When animals were pretreated with 1 mg/kg candesartan, the IPL and INL

thicknesses were $83.8 \pm 2.0\%$ ($P < 0.001$) and $88.0 \pm 3.6\%$ ($P = 0.005$; $n = 4$) of the control, respectively.

Effect of Candesartan on Retinal Function at 21 Days after Ischemia

Scotopic ERG was recorded to evaluate candesartan effects on retinal function. Scotopic ERG was measured 21 days after the IP injection of candesartan. A representative example of function is seen in Figure 8A. Mean amplitudes of the a- and b-waves are shown in Figure 8B. There was a statistically significant difference between the three groups ($n = 8$ in each group).

AT1-R Tissue Localization in the Retina at 21 Days after Ischemia

The localization of the AT1-R in the normal retina is shown in Figure 9A. AT1-R expression was detected in some cells of the ganglion cell layer (GCL). In the postischemic retina, however, immunostaining for AT1-R was detected in the GCL and INL, either with or without candesartan (Figs. 9B, 9C).

Chronic Administration of Candesartan

Figure 10A shows representative results for the RGC labeling in vehicle- and candesartan-treated rats. Compared with the vehicle-treated rat, RGC death seemed to be mild in the candesartan-treated rat. RGC survival rates were 60.0 ± 3.2 in

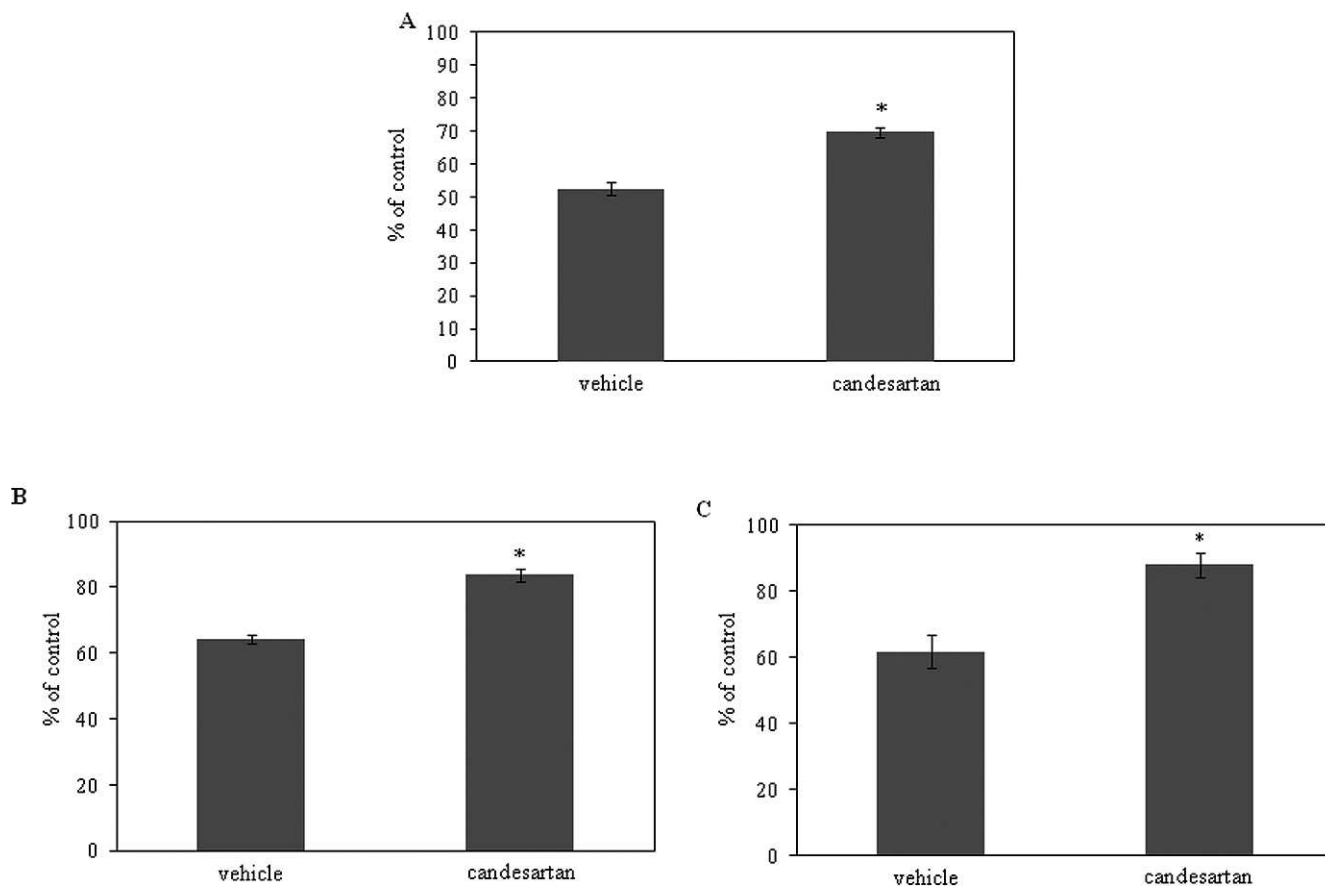


FIGURE 7. Effect of candesartan at 21 days after ischemic injury after the administration of vehicle or candesartan. Retinal ganglion cells were counted at approximately 1 mm from the optic nerve head (A). Change in mean thickness of the inner plexiform layer (IPL) (B) and inner nuclear layer (INL) (C) at 21 days after ischemia in the presence of vehicle or candesartan. Data represent the mean $\pm$ SEM.

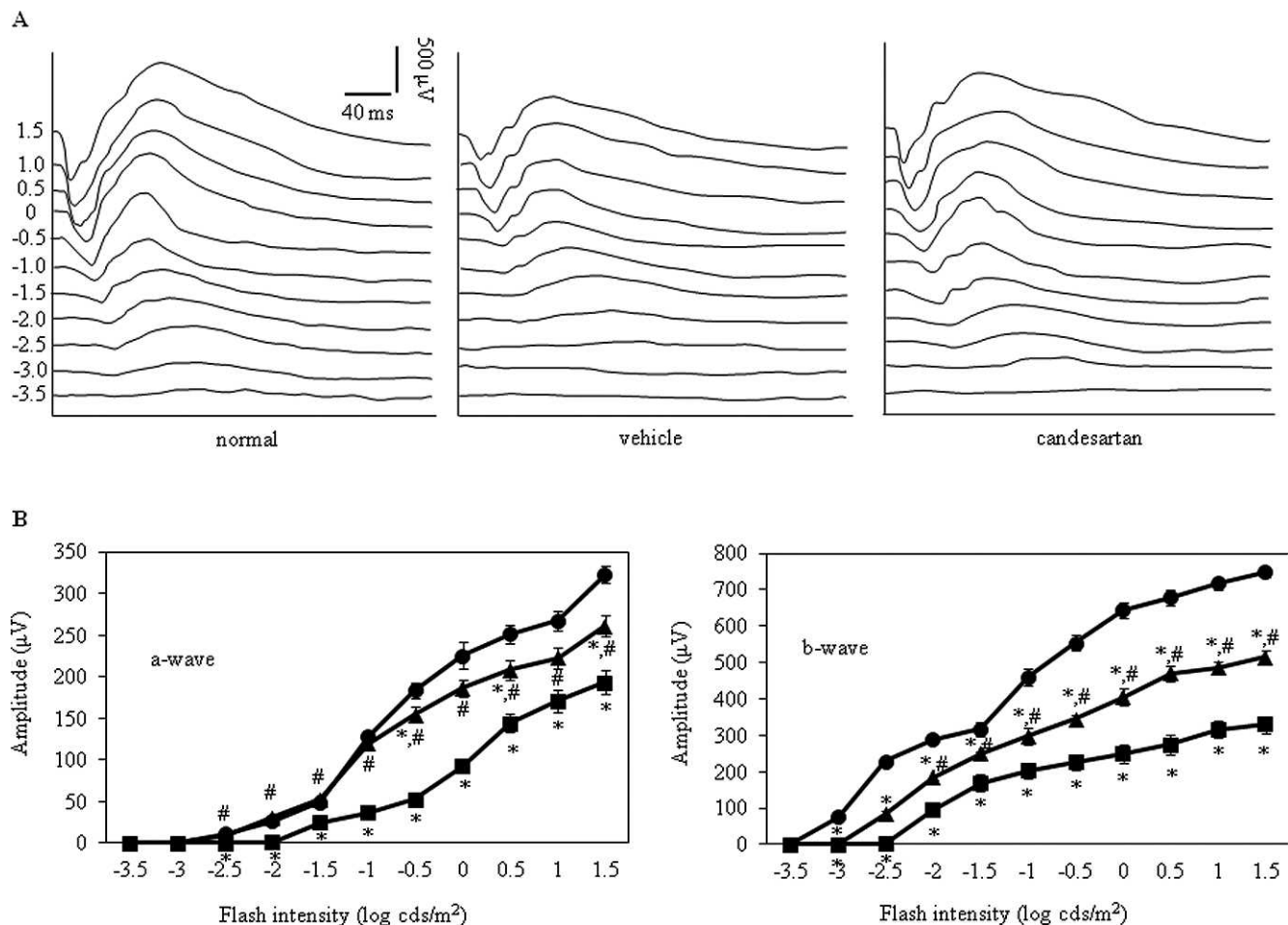


FIGURE 8. Effect of candesartan on retinal function 21 days after ischemia. Representative scotopic ERGs at baseline and at 21 days after ischemia when treated with vehicle or candesartan (A). Amplitude for a- and b-waves plotted as a function of flash intensity (B). Pretreatment with candesartan markedly suppressed the reduction of the amplitudes. Data represent the mean $\pm$ SEM. Normal (solid circle); Vehicle (solid square); Candesartan (solid triangle). * $P < 0.05$ vs. normal. # $P < 0.05$ vs. vehicle (Tukey's honestly significant difference test).

the vehicle-treated group and 71.1 ± 1.2 in the candesartan-treated group ($P = 0.03$; Fig. 10C, $n = 4$ in each group).

In animals pretreated with distilled water, IPL and INL thicknesses were reduced to $68.5 \pm 1.8\%$ and $62.9 \pm 4.5\%$ ($n = 4$) of the control, respectively (Figs. 10B, 10C). In animals treated with 1 mg/kg candesartan, IPL and INL thicknesses were $83.0 \pm 2.4\%$ ($P = 0.004$) and $93.4 \pm 4.2\%$ ($P = 0.003$; $n = 4$) of the control, respectively.

DISCUSSION

Observed increases in the AT1-R levels after ischemia-reperfusion injury were found to reach a maximum at 12 hours after reperfusion both in the present and in previous¹⁵ studies. Based on these findings, we selected 12 hours as a time point for performing all of our measurements. In diabetes, dysmetabolism or apoptosis of cells in the neuroglial retina has been postulated to contribute to degeneration of retinal capillaries.¹⁹ ROS mediates this change by both direct and indirect mechanisms.²⁰ Zheng et al.²¹ previously reported that retinal ischemia-reperfusion injury caused capillary degeneration similar to diabetes, with a greatly increased number of degenerated capillaries found 7 to 8 days after the injury. Thus, damage to the neural retina might contribute to capillary degeneration.

Accumulated glutamate activates NMDA receptors, thus opening calcium channels and leading to an increase in the calcium influx. This intracellular calcium overload can then trigger apoptosis of the neuronal cells in the retina.²² Cell injury during periods of ischemia, hypoxia, and reperfusion is not only caused by a loss of energy supply due to oxygen and glucose deprivation, but in addition, can also be caused by oxidative stress. We have previously reported that candesartan can prevent retinal damage.¹⁵ In the present study, we further showed that candesartan was responsible for reducing the extracellular glutamate levels.

The amount of ROS produced during reperfusion has been shown to be related to the oxygen supply and metabolism and, when these changes are exacerbated, this leads to neuronal cell damage.^{4,23} Our results demonstrated that candesartan enhanced the PO₂ levels after reperfusion, which suggests that candesartan reduces the oxygen supply after reperfusion. Therefore, the neuroprotective effect of candesartan may be related to a reduction in the extracellular glutamate levels and oxygen supply after reperfusion.

Many studies have previously demonstrated that Ang II induces cellular changes through NADPH oxidase-mediated ROS production.^{9,24,25} In the present study, we showed that there were increased levels of ROS in the retina 12 hours after reperfusion, with these levels shown to be associated with an

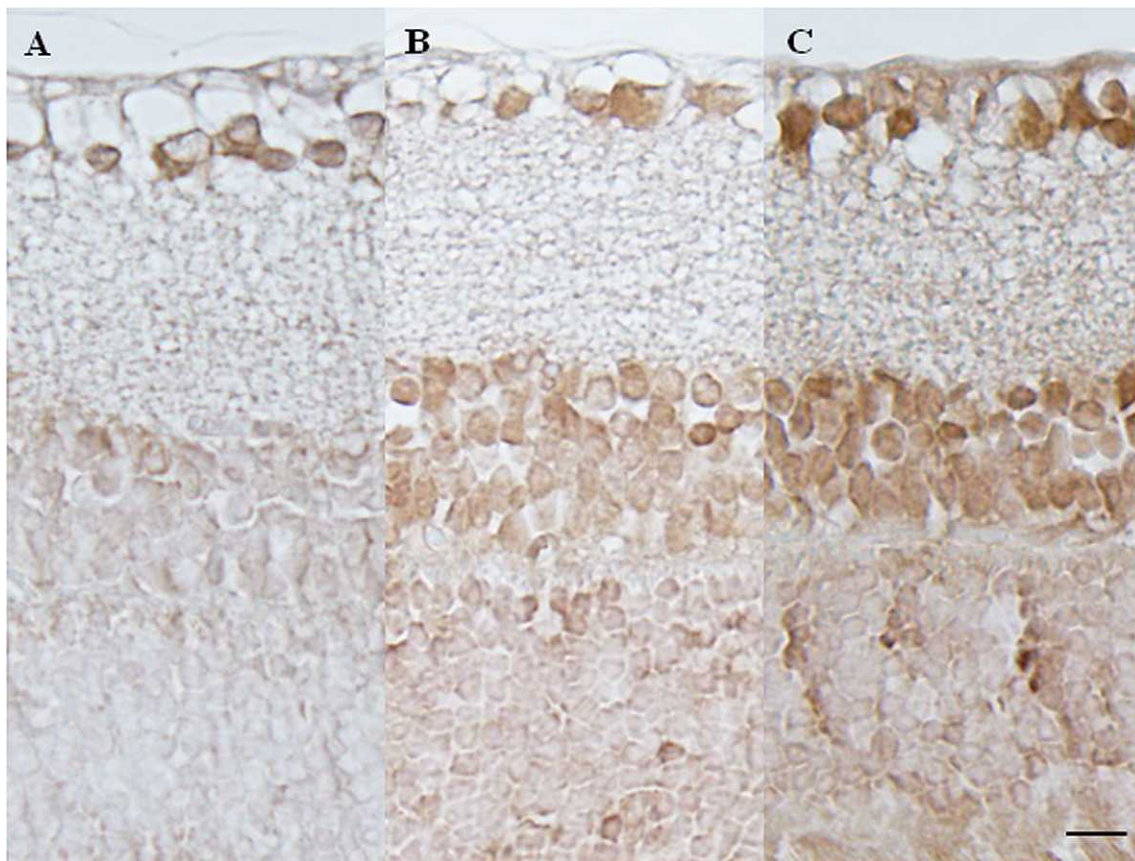


FIGURE 9. Immunohistochemical staining of AT1-R expression in the retina. Retinal sections from normal rats (A) or at 21 days after ischemia when treated without (B) or with (C) candesartan. AT1-R expression was found in the GCL and INL of the ischemic retina in the presence and absence of candesartan. Scale bar, 10 μ m.

increased p47phox and p67phox mRNA expression. ROS production was suppressed after administration of apocynin in this study. These findings suggest that ROS production in the retina at 12 hours after reperfusion is mediated via a NADPH oxidase pathway. The present study also showed that administration of candesartan prevented the ischemia-reperfusion injury-induced increase of retinal ROS levels and NADPH oxidase expression. These results are consistent with previous studies that also reported finding candesartan treatments were able to decrease the increase in NADPH oxidase activity and the ROS production that occurs during pathologic conditions.^{26,27} These results additionally suggest the possibility that the neuroprotective effects of candesartan are due to a reduction of the production of ROS via modulation of the AT1-R signaling.

We recently reported finding an increase in the ROS production at 15 minutes after reperfusion.¹⁵ However, the expression of p47phox and p67phox mRNA in the retina was the same as that observed in the control retinas. Thus, these data suggest that the production of ROS in the retina at 15 minutes after reperfusion is independent of the NADPH oxidase pathway. It has been demonstrated that there are three distinct mechanisms involved with the generation of ROS and oxidative stress that contribute to neuronal injury, each of which will operate at a different stage of the ischemia and reperfusion.²⁸ Mitochondria generate an initial burst of ROS, an action that is curtailed once the mitochondria depolarize or are prevented from further generation by previous depolarizations with an uncoupler. After a slight delay, the second phase of the ROS generation begins. This phase involves xanthine oxidase

activation, which is caused by accumulating products of the ATP consumption. The third phase of the ROS generation was found to be attributable to a calcium-dependent activation of NADPH oxidase. Thus, this mitochondrial ROS generation is believed to be potentially dependent, at least in situations in which a reversed electron flow is possible,^{29,30} such as might occur during the early stages of limiting oxygen delivery.

Accumulations of glutamate activate the NMDA receptors, resulting in an opening of the calcium channels. This intracellular calcium overload can then trigger apoptosis of the neuronal cells in the retina.²² Excessive accumulation of intracellular free Ca^{2+} is known to have a wide range of detrimental effects, including the inhibition of mitochondrial function, reduction of cellular ATP levels, enhancement of ROS production, and activation of cellular proteases and nitric oxide (NO) synthase.³¹ When these effects occur at the same time, this can lead to delayed neuronal death. It has also been previously reported that the histopathologic changes that result after retinal ischemia involve two different phases.^{32,33} In the first or early phase of the reperfusion (posts ischemic day 1), edema is observed in the inner nuclear layer. In the second or late phase of the reperfusion (posts ischemic days 7–14), a prominent neuronal loss and thinning of the inner nuclear layer develop. Thus, to develop effective treatments that will help prevent delayed neuronal death, it is important that the changes that occur during the early phases of reperfusion be further investigated in detail.

To determine thresholds for irreversible cellular injury in the rat retina, Hughes³⁴ showed that there were histologic changes after 30–180 minutes of ischemia. However, signifi-

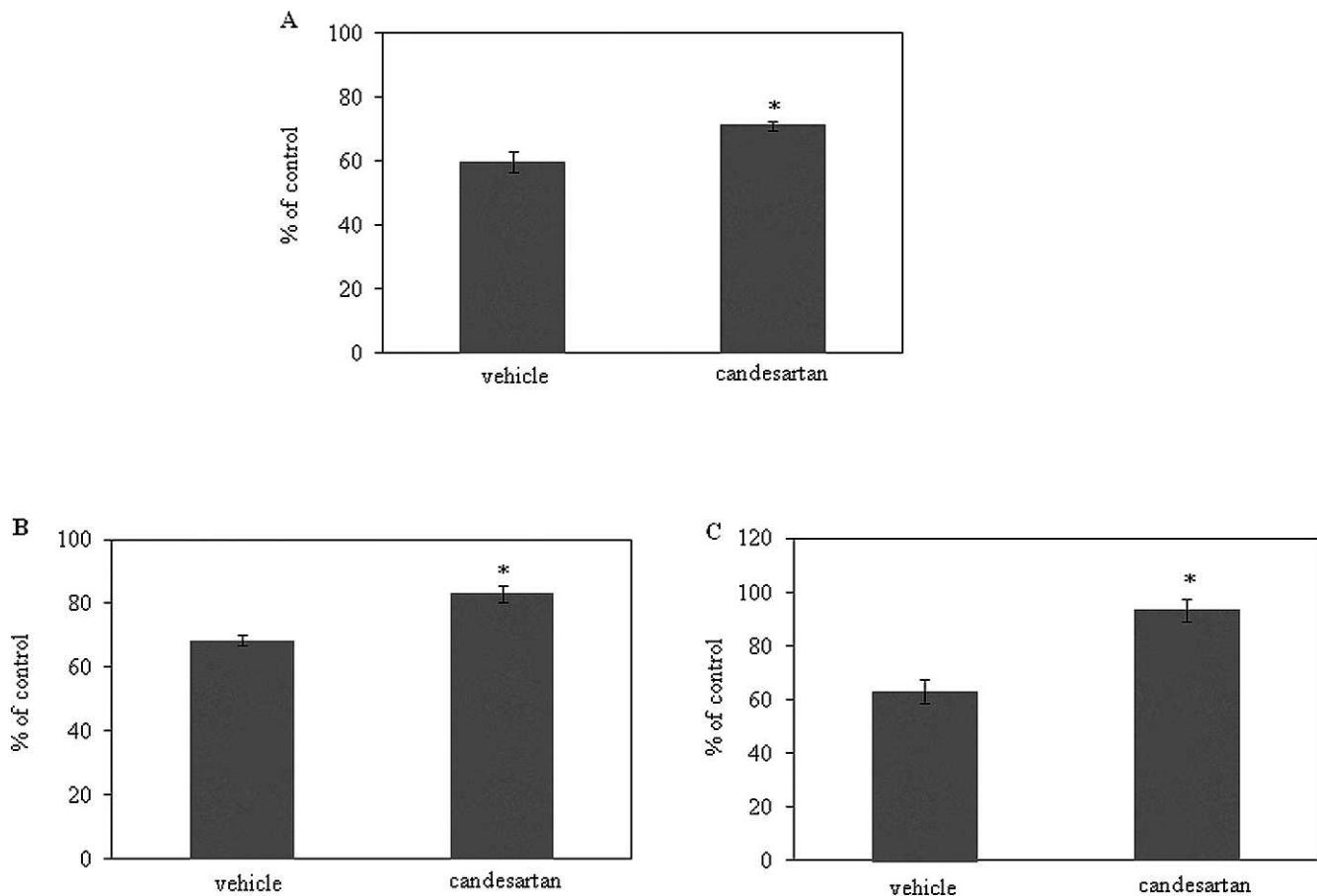


FIGURE 10. Effect of chronic administration of candesartan 7 days after ischemia. Retinal ganglion cells were counted at approximately 1 mm from the optic nerve head (A). Change in mean thickness of the inner plexiform layer (IPL) (B) and inner nuclear layer (INL) (C) at 7 days after ischemia in the presence of vehicle or candesartan.

cant photoreceptor loss occurred after only about 90 minutes of ischemia. Thus, the optimum time point for maximal damage of both the inner and outer retinal neurons was at 120 minutes of ischemia.

In conclusion, two distinct mechanisms are responsible for the neuroprotective effects of candesartan against retinal ischemia-reperfusion injury. Both of these lead to a suppression of the production of the ROS, with the first mechanism acting by decreasing glutamate release during the first 15 minutes after reperfusion, whereas the second mechanism modulates the AT1-R signaling at 12 hours after the reperfusion.

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