Neuroprotective Effects of Angiotensin II Type 1 Receptor Blocker in a Rat Model of Chronic Glaucoma

Hongwei Yang,1,2 Kazuyuki Hirooka,1 Kouki Fukuda,1 and Fumio Shiraga1

PURPOSE. To investigate the neuroprotective effect of candesartan, an angiotensin II type 1 receptor (AT1-R) blocker, against the neurotoxicity of the retinal ganglion cells (RGCs) in an animal model of glaucoma.

METHODS. Cauterization of three episcleral vessels in rats was used to create chronically elevated intraocular pressure (IOP) in one eye. Rats were then treated orally with candesartan (1 mg/kg/d). At 10 weeks, immunohistochemistry was used for quantification of RGC survival and examination of retinal localization of AT1-R.

RESULTS. Compared with the contralateral control eyes, there was a consistently elevated IOP of approximately 2.5-fold during the experimental period. At the end of the 10-week candesartan treatment, there were no changes noted for the blood pressure. Compared with the contralateral control eyes that had normal IOP, the RGC survival rate in the central retina of eyes with the chronic, elevated IOP was 46.5% ± 19.4% (mean ± SD) in the untreated animals and 84.2% ± 4.9% in the candesartan-treated animals (P < 0.05; unpaired t-test). In the retina of the normal IOP rat eyes, retinal vessels were positive for AT1-R. After 10 weeks of IOP elevation, immunohistochemical analysis of the retina indicated there were many AT1-R–positive RGCs in the candesartan-treated rat, whereas there was an apparent AT1-R decrease in the vehicle-treated rats.

CONCLUSIONS. In the rat chronic glaucoma model, continuous pharmacologic treatment using candesartan results in significant neuroprotection against RGC loss. (Invest Ophthalmol Vis Sci. 2009;50:5800–5804) DOI:10.1167/iovs.09-3678

Primary open-angle glaucoma, which is one of the leading causes of vision loss in the world, is an optic neuropathy that is associated with elevated intraocular pressure (IOP). Current glaucoma treatments are based on attempts to lower elevated IOP to slow or stop the progressive loss of the visual field that occurs as a result of optic nerve degeneration and the subsequent loss of retinal ganglion cells (RGCs). However, glaucomatous damage progresses in many of these cases despite adequate control of the IOP.1 Consequently, many investigators are trying to find a therapeutic modality that not only stabilizes the IOP at a lower level but that can prevent the death of RGCs or even lead to RGC regeneration.2–3

The renin-angiotensin system (RAS) plays an important part in the control of blood pressure and electrolyte homeostasis. Recent evidence suggests that in addition to the circulating RAS, there is tissue or local RAS in the vasculature, adrenal gland, kidney, brain, testis, and ovary that also may have a role in the overall control.4–6 Many of the known RAS components have been identified in the human eye.4,7–9 Angiotensinogen is an obligatory component for the eventual production of angiotensin II. Angiotensin I and angiotensin II are generated within the circulation by a sequential cleavage of the liver-derived angiotensinogen. Renin, which is synthesized in the kidney, cleaves this substrate, leading to the formation of angiotensin I. Angiotensin-converting enzyme (ACE) converts angiotensin I to angiotensin II. Angiotensin II is the effector molecule of the system and has two cognate receptors that are designated as angiotensin II type 1 and type 2 receptors (AT1-R and AT2-R, respectively).10,11 Because AT1-R mediates the major functions of angiotensin II, its antagonists are widely used to treat patients with hypertension and cardiovascular diseases. Emerging evidence obtained when using the experimental cerebral ischemia model has suggested that peripherally administered AT1-R blockers (ARBs) can cross the blood-brain barrier and interact with AT1-R, thereby reducing the infarct volume.12–14 Recently, ARBs have also been proven to attenuate inflammatory and oxidative stress in the brain15,16 and retina.17,18

The long-term use of ACE inhibitors, which are widely used as antihypertensive drugs, are believed to have a favorable effect on the visual fields in patients with normal-tension glaucoma.19 Angiotensin II receptor gene polymorphisms have been found in humans, and these may be associated with the risk for glaucoma.20 The purpose of the present study was to investigate the effects of candesartan along with the role of ARBs in RGC degeneration in an animal model of glaucoma.

MATERIALS AND METHODS

Animals

Female Sprague-Dawley rats (Charles River Japan, Yokohama, Japan), weighing 180 to 210 g each, were housed in a standard animal room under a 12-hour light/12-hour dark cycle with free access to food and water. All surgical procedures were performed under general anesthesia that used intraperitoneal injections of pentobarbital (40–50 mg/kg; Nembutal; Abbott, Abbott Park, IL). Animal care and all experiments were conducted under 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. Two groups of rats that had unilateral elevated IOP were followed up for 10 weeks. The rats in the first group did not receive any treatments for their elevated IOP, whereas rats in the second group underwent 10 weeks of candesartan treatment (1 mg/kg/d; a gift from Takeda Pharmaceutical Co., Ltd., Osaka, Japan), with the initial dose administered on the first day of IOP elevation. Candesartan or water was orally administered to animals on a daily basis via the use of feeding needles.

From the 1Department of Ophthalmology, Kagawa University Faculty of Medicine, Kagawa, Japan; and the 2Department of Ophthalmology, Shengjing Affiliated Hospital, China Medical University, Shenyang, China.

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Corresponding author: Kazuyuki Hirooka, Department of Ophthalmology, Kagawa University Faculty of Medicine, 1750-1 Ikenobe, Miki, Kagawa 761-0793, Japan; kazu@med.kagawa-u.ac.jp.

Chronic Elevated IOP Rat Model

We performed cautery of three episcleral vessels in six rats to unilaterally create the chronic elevated IOP conditions. The contralateral eye served as the comparative control. Incision of the conjunctiva at the equator exposed 3 of the 4 to 5 major trunks that are formed by the limbal-derived veins. Each vessel was then lifted with a small muscle hook and cauterized by direct application of ophthalmic cautery (Tagawa, Tokyo, Japan) against the muscle hook. Successful cautery was defined as an immediate retraction and subsequent absence of bleeding at the cauterized end of the vessels. The eyes were dressed with 0.3% ointment. (Santen, Osaka, Japan).

IOP and BP Measurements

IOP was measured three times in the anesthetized rat using a handheld electronic tonometer (TonoPen XL, Bio-Rad, Glendale, CA), with the mean value used for the analysis. Bilateral IOP measurements were performed within 3 hours and 1 week of the surgery to confirm the unilateral, moderately elevated IOP. Thereafter, IOP was measured weekly.

Systolic blood pressure (SBP) and diastolic blood pressure (DBP) were measured weekly in unanesthetized rats using the tail-cuff method (BP-98A; Softron Co., Tokyo, Japan).

Retrograde Labeling of Retinal Ganglion Cells

At 7 days before kill, fluorescent dye (Fast Blue; Polysciences Inc., Warrington, PA) 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 in both the right and the left hemispheres. The windows were drilled to a depth of 3.6 mm from the surface of the skull, located 6.8 mm behind the bregma on the anterior-posterior axis and 1.5 mm lateral to the midline. Using a Hamilton syringe, 1.5 mL of 3% fluorescent dye (Fast Blue; Polysciences Inc.) was slowly injected into the superior colliculi. After suturing the skin over the wound, antibiotic ointment was applied.

Tissue Preparation and Assessment of RGC Survival

Animals were killed by an overdose of pentobarbital (Nembutal) at 1 week after the fluorescent dye (Fast Blue; Polysciences Inc.) 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 epithelium. To prepare the flat mounts, the retina was dissociated from the epithelium. Whole flat-mounted retinas were then assayed for retinal ganglion cell density. This was defined as an immediate retraction and subsequent absence of bleeding at the cauterized end of the vessels. The eyes were dressed with 0.3% ophthalmic ointment (Santen, Osaka, Japan).

Immunohistochemistry

At 3 or 10 weeks after the initial elevation of the IOP, rats were anesthetized by intraperitoneal injection of pentobarbital and then perfused intracardially with phosphate-buffered saline (PBS). Animals were subsequently perfused with 4% paraformaldehyde in PBS. Retinal sections were rinsed in 100% ethanol twice for 5 minutes each, followed by a separate 95% ethanol and 90% ethanol rinse for 3 minutes. The sections were then washed using PBS, pH 7.4, three times for 10 minutes each, followed by treatment with 0.3% Triton-X-100 in PBS, at pH 7.4, for 1 hour. After further washing three times for 10 minutes each with PBS at pH 7.4, sections were blocked in 3% normal horse serum and 1% bovine serum albumin (BSA) in PBS for 1 hour to reduce the nonspecific labeling. Tissues were incubated overnight at 4°C with primary antibodies in PBS containing 0.5% Triton X-100, 5% normal horse serum, and 1% BSA. Primary antibody was diluted as follows: rabbit polyclonal antibody against human AT1-R (Santa Cruz Biotechnology, Santa Cruz, CA) 1:100. Control sections were prepared by omitting both the primary antibody and the rabbit IgG (1:1000; Vector Laboratories Inc., Burlingame, CA) 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, tissues were immersed in the 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-Reagent; Vectastain ABC-AP Kit; Vector Laboratories Inc.) for 1 hour at room temperature. Images were acquired using 40× objective lenses (DXM 1200; Nikon, Tokyo, Japan). Graphics editing software (Photoshop version 5.0; Adobe, San Jose, CA) was used to adjust the brightness and contrast of the images.

Statistical Analysis

Statistical analysis was performed (SPSS for Windows; SPSS Inc., Chicago, IL). A paired t-test was used to determine the statistical significance of the BP measurements. An independent Student’s t-test was used to compare the RGC survival rate and IOP measurements. P < 0.05 was considered statistically significant. All statistical values are presented as the mean ± SD.

RESULTS

Intraocular Pressure and Blood Pressure

There was no significant difference noted between the baseline IOP values of vehicle- and candesartan-treated rats. Elevated IOP was observed over the 10-week experimental period in all eyes that underwent the three-vessel cauteryization (Fig. 1). Three hours after the surgery, IOP was elevated to approximately 26 mm Hg (range, 25.7–30.3 mm Hg), and it was consistently elevated approximately 2.5-fold compared with the contralateral control eyes. In both groups, the elevated IOP was significantly higher than in contralateral control eyes. The elevation in the IOP was essentially the same between the candesartan-treated and the nontreated animals. Our success rate was 6 of 8 animals. Animals in which elevated IOP were not maintained for more than 2 weeks were excluded from the subjects.

The changes in the SBP and DBP are shown in Figure 2. Treatment with vehicle did not affect either the SBP or the DBP at week 10 in the rats (118.8 ± 6.9 mm Hg and 93.1 ± 6.4 mm Hg, respectively). Furthermore, there were no changes seen in the SBP and DBP in the rats at 10 weeks after the candesartan treatment (113.1 ± 6.1 mm Hg and 87.9 ± 5.9 mm Hg, respectively).

Effect of Candesartan on RGC Survival

Figure 3A shows representative results of the RGC labeling in both the vehicle- and the candesartan-treated rat. Compared

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with the vehicle-treated rat, RGC death seemed to be mild in the candesartan-treated rat. RGC survival rates in the central retinas of the eyes with elevated IOP were 46.5% ± 19.4% (mean ± SD) in the vehicle-treated group and 84.2% ± 5.0% in the candesartan-treated group (P = 0.02; Fig. 3B). In the peripheral retina, RGC survival rates in the eyes with elevated IOP were 52.3% ± 21.8% in the vehicle-treated group and 82.5% ± 2.4% in the candesartan-treated group (P = 0.004; Fig. 3B).

**Immunohistochemistry**

In the retinas of rat eyes with normal IOP, AT1-R immunoreactivity was detected on the endothelial cells of the inner retinal vessels (Fig. 4A). In the retinas of rat eyes with chronic elevated IOP, immunohistochemical analysis indicated there were many AT1-R-positive cells within the ganglion cell layer in the candesartan-treated rat, though this number was apparently decreased in the vehicle-treated rat (Figs. 4B, C). However, immunohistochemical analysis at 3 weeks after the initial elevation of IOP showed there were many AT1-R-positive cells within the retinal ganglion cell layer in the vehicle-treated rat (Fig. 4D).

**Discussion**

The present study demonstrates for the first time that AT1-R upregulation is associated with chronic elevated IOP in the rat model of glaucoma and that the AT1-R signaling blockade of candesartan effectively prevents retinal neuronal death. Candesartan (1 mg/kg/d) has neuroprotective effects against cerebrovascular ischemia in spontaneously hypertensive rats. In animals pretreated with 0.1 or 1 mg/kg candesartan, only 1 mg/kg candesartan reduced ischemic damage to the retina against retinal ischemia-reperfusion injury in the rat retina (K.F., K.H., et al., unpublished data, 2009). Therefore, the 1-mg/kg/d dose used in this study was selected.

Oral administration of the ARB losartan reduced the IOP in human subjects with both normal tension and glaucoma. Hasizume et al. have recently shown that candesartan causes a decrease in IOP, with an IOP reduction noted for 5 hours after drug administration in healthy subjects. The total outflow facility increased significantly in all subjects, with a decrease in the SBP noted only in patients with hypertension. These results suggest that the mechanism is not mediated by a decrease in blood pressure but, rather, is more specific. These findings confirm the role of the RAS in the regulation of IOP. Because candesartan treatment was performed before elevation of the IOP, we were unable to compare the IOP before and after its oral administration in this study. However, given that all the animals had cautery-induced IOP elevation, we compared the animals that were not treated pharmacologically with those that did receive candesartan and determined there was no significant difference between the two groups. Thus, we concluded that candesartan treatment provides a neuroprotective effect that is independent of IOP reduction.

IOP in the control eyes was lower than has been previously reported by other investigators (11.6–13.2 mm Hg). This might have been due to differences associated with the anesthetic used in the present study. Anesthetics can affect IOP, and this effect can change over time, depending on the level of anesthesia present. The most commonly used device for measuring IOP in rats is the TonoPen electronic tonometer (Reichert, Depew, NY). Recent reports have shown the TonoLab device (Colonial Medical Supply, Franconia, NH) to be more effective than the TonoPen when measuring IOP in rats. Moore et al. have shown that there is a high correlation between the results obtained when using a TonoPen and those obtained by directly measuring the rat eye (r = 0.94–0.98). They also have reported the repeatability and consistency of IOP measurements when using the TonoPen. In our study, the coefficient of variation in measuring IOP with TonoPen was 8.5%.

In the present study, we found that the 1 mg/kg/d candesartan treatment in normal rats did not affect either the SBP or the DBP. Although our results are similar to those of another study that found there was no SBP decrease from baseline after the same dose of candesartan was administered in adult and old rats, a second study has shown that a higher dose (10 mg/kg/d) of candesartan caused a significant decrease in the SBP. However, since a 1-mg/kg/d candesartan dose has been shown to reduce BP in spontaneously hypertensive rats, it may be that the 1-mg/kg/d candesartan dose level affects only hypertensive rats.

Kurihara et al. recently reported that intense inflammation caused local upregulation of angiotensin II expression and disturbed visual function by electroretinography. The RAS polymorphisms that exist for the angiotensin II receptor gene may be a major genetic risk factor for the development or progression of glaucoma in the Japanese population. Angiotensin II activates the NADPH-dependent oxidase complex, which serves as a major source of superoxide and is upregulated in several pathologic conditions associated with oxidative stress. Given that oxidative stress induces apoptosis in neurons, hydrostatic pressure-induced oxidative stress could
very well be the mechanism responsible for the similar pressure-induced apoptosis seen in animal models\textsuperscript{36,37} and in glaucoma patients with high IOP.\textsuperscript{38} In the present study, we demonstrated that the AT1-R level increased in the ganglion cell layer in candesartan-treated rats. In vehicle-treated rats, however, there was a decrease in the AT1-R-positive RGCs in the retina 10 weeks after the elevation of IOP compared with that seen in the candesartan-treated rat. Vehicle-treated rats were

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\caption{Survival of RGCs at 10 weeks in rat eyes with chronic elevated IOP. (A) Retrograde labeling of the RGCs in eyes with normal IOP and in eyes with elevated IOP at 10 weeks after administration of candesartan or vehicle. Micrographs in the central and peripheral areas were taken approximately 1 and 4 mm from the optic nerve head, respectively. Scale bar, 20 µm. (B) RGCs were counted in the central and peripheral areas at approximately 1 and 4 mm from the optic nerve head, respectively. The graph depicts the mean ± SD of six animals treated with candesartan and six animals treated with vehicle. A significant difference in the RGC survival rate in eyes with elevated IOP that were treated with candesartan and vehicle was evident between the central (\(P = 0.02\)) and peripheral (\(P = 0.004\)) areas. *\(P < 0.05\)}
\end{figure}

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\caption{Immunohistochemical staining of the AT1-R expression in the retina. Retinal sections from normal animals (A) and from animals with chronic elevated IOP that were administered candesartan over a 10-week period (B) or vehicle over a 10-week (C) or a 3-week period (D). An increase in the AT1-R-positive RGCs (arrow) was noted for the candesartan-treated rats at 10 weeks and vehicle-treated rats at 3 weeks, compared with the normal and vehicle-treated rats at 10 weeks. Scale bar, 25 µm.}
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also found to have many AT1-R–positive RGCs in the retina 3 weeks after IOP elevation (i.e., a few weeks before the beginning of cell death). Thus, increases in the AT1-R–positive RGCs occur when IOP is chronically elevated. Treatment with candesartan effectively prevented cell death.

In conclusion, the current data suggest that candesartan does indeed have a therapeutic effect in this animal model of glaucoma. Because ARBs are safely and widely used to treat hypertension, clinical administration of candesartan for the purpose of pharmacologic neuroprotection may be a new and beneficial therapy that can be used in patients with glaucoma.

References