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The Relationship Between the Renin-Angiotensin–Aldosterone System and NMDA Receptor-Mediated Signal and the Prevention of Retinal Ganglion Cell Death

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Purpose. Excitotoxicity, which is due to glutamate-induced toxic effects on the retinal ganglion cell (RGC), is one of several mechanisms of RGC loss. The renin-angiotensin–aldosterone system (RAAS) has also been implicated in RGC death. Therefore, it is important to determine the exact relationship between the RAAS and N-methyl-D-aspartate (NMDA) receptor-mediated signal in order to prevent RGC death.

Methods. N-methyl-D-aspartate or aldosterone was injected into the vitreous body. After intravitreal injection of NMDA or aldosterone, animals were treated with spironolactone or memantine. Retinal damage was evaluated by measuring the number of RGCs at 4 weeks after local administration of aldosterone or at 2 weeks after local administration of NMDA. Vitreous humor levels of aldosterone were measured using enzyme immunoassay kits.

Results. A significantly decreased number of RGCs were observed after intravitreal injection of NMDA. Although spironolactone did not show any neuroprotective effects, memantine significantly reduced NMDA-induced degeneration in the retina. Furthermore, a significant decrease in the number of RGCs was observed after an intravitreal injection of aldosterone. While memantine did not exhibit any neuroprotective effects, spironolactone caused a significant reduction in the aldosterone-induced degeneration in the retina. There was no change in the aldosterone concentration in the vitreous humor after an NMDA injection.

Conclusion. Our findings indirectly show that there is no relationship between the RAAS and NMDA receptor-mediated signal with regard to RGC death.

Keywords: aldosterone, retinal ganglion cell, NMDA, glutamate

Within the central nervous system, including the retina, glutamate functions as a major excitatory neurotransmitter.1–5 Various diseases of the eye, which include glaucoma6,7 and retinal ischemia,8,9 have been shown to be associated with the glutamate receptor-mediated excitotoxicity. Glutamate excitotoxicity triggered by overactivation of the N-methyl-D-aspartate (NMDA) receptors may be a potential risk factor for retinal ganglion cell (RGC) death.10 Indeed, it has been reported in an animal model that there is a decrease in the RGCs after an intravitreal injection of NMDA.11 One of the RAAS antagonists that has been demonstrated to have therapeutic potential against several central nervous system disorders is memantine (N,N,N-trimethyl-D,L-alaninate).10 Previous studies examined the use of memantine in several animal models of glaucoma and reported finding that this antagonist might possibly provide neuroprotection, although it was not successful in recent clinical trials.12–14 Since multiple mechanisms can lead to RGC death, this suggests that a neuroprotectant with a single mode of action, such as memantine, would have only a limited positive effect in slowing down the death of the RGCs.

The major controller of the systemic blood pressure is the renin-angiotensin–aldosterone system (RAAS), with several prior studies demonstrating that RAAS components are found within the human eye.15–17 In our previous study, we reported finding that after ischemia-reperfusion there was an increase in the expression of angiotensin II type 1 receptor (AT1-R) in the retina.18,19 In addition, we also found there were reductions in retinal ischemia-reperfusion injuries after administering treatments that included the mineralocorticoid receptor antagonist, spironolactone, an angiotensin-converting enzyme inhibitor, and an AT1-R blocker.20,21 Furthermore, we also recently reported that the RGC loss associated with the thinning of the retinal nerve fiber layer without elevated IOP that occurs after the systemic administration of aldosterone could be prevented by the administration of spironolactone.22 Thus, the RAAS appears to play an important role with regard to the neuronal degeneration that occurs in the retina.

The NMDA receptor-mediated signal is one of the most important pathways involved in the death of RGCs. Therefore, it is important to understand the specific relationship between RAAS and the NMDA receptor-mediated signal. Thus, in order to definitively clarify this, therapies that are directed at the IOP-independent mechanisms of RGC loss will need to be developed. Therefore, the purpose of our current study was to investigate the relationship between the RAAS and NMDA receptor-mediated signals in the prevention of RGC death.
The Relationship Between Aldosterone and NMDA

MATERIALS AND METHODS

Animals
This study examined a total of 98 male Sprague-Dawley rats weighing 200 to 250 g. The animals were obtained from Charles River Japan (Yokohama, Japan) and CLEA Japan (Tokyo, Japan). All rats were given free access to standard rat food (Oriental Yeast Co., Ltd., Tokyo, Japan) and tap water. All of the experiments and animal care were conducted in accordance with the approved standard guidelines for animal experimentation at the Kagawa University Faculty of Medicine. This study adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Drugs
Aldosterone and spironolactone were obtained from Sigma-Aldrich Corp. (St. Louis, MO, USA). N-methylaspartate and memantine hydrochloride were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). After dissolving aldosterone in dimethyl sulfoxide (DMSO) to produce the stock solutions, these solutions were then diluted to the final required concentrations. In all cases, the final DMSO concentration never exceeded 5%. Spironolactone (10 mg/kg/d) was dissolved in carboxymethyl cellulose (CMC) to produce the stock solution. Subsequently, feeding needles that were not attached to enteral syringes were then used to orally administer the solution to each of the animals every day. In all cases, the final CMC concentration never exceeded 0.5%.

In the control group, the animals were all treated with vehicle only (0.5% CMC in PBS). N-methylaspartate was dissolved in 0.01 M PBS at pH 7.4. Memantine hydrochloride (10 mg/kg/d) was dissolved in PBS at pH 7.4 to produce the stock solution. Similar to the experimental group, feeding needles that were not attached to enteral syringes were then used to orally administer the solution daily to all of the animals.

Intravitreal Injection of NMDA or Aldosterone

The 32 rats used for this experiment were anesthetized via an intraperitoneal injection of 50 mg/kg pentobarbital sodium (Abbott Laboratories, Abbott Park, IL, USA), and 0.4% oxybuprocaine hydrochloride was administered topically to the cornea to anesthetize the rats. N-methylaspartate injury was induced as previously reported.8 Based on previously reported methodologies,19 the pupil of the right eye was dilated with tropicamide phenylephrine hydrochloride (Santen Pharmaceuticals Co. Ltd., Osaka, Japan), after which 2 μL of 20 mM NMDA or 2 μL of 80 μg/kg aldosterone was injected into the vitreous body of the right eye using a 32-gauge needle and Hamilton syringe. For the vehicle control, the same volume of PBS was then injected in the left eye. Animals were excluded from the study if a lens injury or vitreous hemorrhage was detected. Immediately after the intravitreal injection, one drop of levofloxacin ophthalmic solution (Santen Pharmaceuticals Co. Ltd., Osaka, Japan) was applied topically to the treated eye. The animals were divided into four groups that included intravitreal injection of NMDA treated with memantine, intravitreal injection of NMDA treated with spironolactone, intravitreal injection of aldosterone treated with memantine, and intravitreal injection of aldosterone treated with spironolactone. Each of the animals was given a daily oral administration of either 10 mg/kg/d memantine or 10 mg/kg/d spironolactone by feeding needles. The number of RGCs was counted at 4 weeks after the local administration of aldosterone or at 2 weeks after the local administration of NMDA.

Retrograde Labeling of RGCs

Using the stereotaxic coordinates, Fast Blue tracer (Polysciences, Inc., Warrington, PA, USA) was bilaterally injected into the superior colliculi of anesthetized rats 7 days prior to euthanasia. These injections were performed at 5 weeks after the local administration of aldosterone or 1 week after local administration of NMDA. After exposing the skull, which was kept dry and clean, the bregma was identified and marked. Subsequently, 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. All of the windows were located at 6.8 mm behind the bregma on the anteroposterior axis and 1.5 mm lateral to the midline. Using a 32-gauge needle and Hamilton syringe (Hamilton Co., Reno, NV, USA), 1.5 μL 5% Fast Blue was slowly injected into the bilateral superior colliculi. The drill holes were left open. After suturing the skin over the wound, an antibiotic ointment was applied.

Tissue Preparation and Assessment of Labeled RGC

At 1 week after fluorescent dye application, all rats were killed using an overdose of pentobarbital. Retinal ganglion cell density was assessed using whole, flat-mounted retinas. Rat eyes were enucleated and fixed in 4% paraformaldehyde for 10 hours at room temperature. The anterior segments were then removed, with the resultant posterior eyecups left in place. After making four radial cuts in the periphery of each eyecup, the retina was then carefully separated from the retinal pigment epithelium. The flat mounts were prepared by first dissociating the retina from the underlying structures. After making four radial cuts to flatten the retinas, they were then spread on a gelatin-coated glass slide. A fluorescence microscope (Olympus BX51/DP72, Olympus, Tokyo, Japan) with an ultraviolet filter (excitation filter, 330–385 nm; barrier filter, 420 nm) was used to visualize the labeled RGCs. Fluorescent retrogradely labeled RGCs were counted in 12 different microscopic fields of retinal tissue located 1 mm (central) and 4 mm (peripheral) away from the optic disc. Counts of the total number of RGCs in each eye were performed using software (Image-Pro Plus Version 4.0; Media Cybernetics, Bethesda, MD, USA). Cell counts were conducted by the same investigator in a masked fashion; the identity of the retinas that led to the micrographs was unknown until cell counts from different groups were complete.

Measurement of Aldosterone Concentration in the Vitreous Humor

A total of 40 rats were used for this experiment. Rats were killed using an overdose of pentobarbital at 1 day after the intravitreal injection of vehicle, 80 μg/kg aldosterone, or 20 mM NMDA. After enucleation of the rat eyes, the vitreous body was extracted. In order to collect more than 0.2 mL (the minimum required quantities for the measurement of the aldosterone), vitreous humor was extracted from a plurality of rats. After preparation of the samples in accordance with the manufacturer’s instructions, vitreous humor levels of aldosterone were measured using enzyme immunoassay kits (Assay Designs, Ann Arbor, MI, USA).

Statistical Analysis

All data are presented as the mean ± SEM. Data were analyzed using 1-way ANOVA followed by Tukey’s honestly significant difference test or Student’s t-test, as appropriate. Statistical analyses were performed using analysis software (SPSS Version
Figure 1. Effect of aldosterone on RGC. Retrograde labeling of RGCs at 2, 3, or 4 weeks after an intravitreal injection of 80 μg/kg aldosterone or vehicle (A). Scale bar: 100 μm. Micrographs of the central area were taken approximately 1 mm (B) and 4 mm (C) from the optic nerve head. Graph depicts the mean ± SEM (n = 4 in each group). *P < 0.01 versus control (Student’s t-test). □ control, ■ aldosterone.

21.0; SPSS, Inc., Chicago, IL, USA). A P value < 0.05 was considered statistically significant.

RESULTS

Changes in the Time Course After Local Administration of Aldosterone

The number of RGCs was counted at 2, 3, or 4 weeks in order to determine if the intravitreal injection of aldosterone caused a decrease in the RGCs. The time-dependent effects of aldosterone are presented in Figure 1. After local administration of either aldosterone or vehicle, there were similar numbers of RGCs in the central retina between the two groups at 2 weeks (vehicle: 2267 ± 192 cells/mm², aldosterone: 2225 ± 104 cells/mm²; P = 0.58) or 3 weeks (vehicle: 2325 ± 109 cells/mm², aldosterone: 2339 ± 72 cells/mm²; P = 0.67). At 4 weeks after the local administration of aldosterone, there was a significant decrease in the RGCs observed (vehicle: 2517 ± 12 cells/mm², aldosterone: 1903 ± 36 cells/mm², P < 0.001) (n = 4 in each group). After local administration of either aldosterone or vehicle, there were similar numbers of RGCs in the peripheral retina between the two groups at 2 weeks (vehicle: 2510 ± 43 cells/mm², aldosterone: 2248 ± 104 cells/mm²; P = 0.34) or 3 weeks (vehicle: 2270 ± 41 cells/mm², aldosterone: 2072 ± 59 cells/mm²; P = 0.001). At 4 weeks after the local administration of aldosterone, however, there was a significant decrease in the RGCs observed (vehicle: 2553 ± 28 cells/mm², aldosterone: 1794 ± 57 cells/mm², P = 0.001) (Fig. 1C; n = 4 in each group).

Effect of Spironolactone or Memantine After Local Administration of Aldosterone

At 4 weeks after intravitreal injection of aldosterone, we observed a significant decrease in the RGCs in both the central and peripheral retina. Therefore, we decided to further investigate the effect of spironolactone or memantine on the aldosterone-induced toxicity that occurred at 4 weeks after local administration of aldosterone. If the RAAS exists upstream of the NMDA receptor-mediated signal, then it might be possible that memantine would be able to reduce the aldosterone-induced neurotoxicity. Figure 2A shows representative results for the RGC labeling observed at 4 weeks after local administration of aldosterone in the vehicle-, memantine-, or spironolactone-treated rats. At 4 weeks after local administration of aldosterone, there was a significant decrease in the number of RGCs in the central retina as compared to the
controls (control: 2554 ± 42 cells/mm²; vehicle: 1903 ± 36 cells/mm²). Results showed that the RGC death in the central retina was similar between the vehicle-treated and memantine-treated rats (memantine: 2049 ± 81 mm²; P = 0.08). However, when the spironolactone-treated and vehicle-treated rats were compared, the RGC death in the central retina appeared to be mild (spironolactone: 2490 ± 88 cells/mm²; P = 0.65) (Fig. 2B; n = 4 in each group). In the peripheral retina, RGC death was similar between the vehicle-treated and memantine-treated rats (vehicle: 1794 ± 49 cells/mm²; memantine: 1868 ± 55 cells/mm²; P = 0.40). However, when the spironolactone-treated and vehicle-treated rats were compared, the RGC death in the peripheral retina appeared to be mild (spironolactone: 2413 ± 61 cells/mm²; P < 0.001) (Fig. 2C; n = 4 in each group).

Effect of Spironolactone or Memantine After Local Administration of NMDA

If the NMDA receptor-mediated signal exists upstream of the RAAS, we hypothesized that spironolactone administration might be able to reduce the NMDA-induced neurotoxicity. Figure 3A shows representative results for the RGC labeling in the vehicle, memantine-, or spironolactone-treated rats. At 2 weeks after local administration of NMDA, there was a significant decrease in the number of RGCs in the central retina as compared to control (control: 2423 ± 73 cells/mm²; NMDA: 612 ± 112 cells/mm²; P < 0.001). In contrast, when vehicle-treated and spironolactone-treated rats were compared, there was similar RGC death in the central retina (718 ± 52 cells/mm²; P = 0.59). However, the RGC death appeared to be milder in the central retina for the memantine-treated versus the vehicle-treated rats (1234 ± 44 cells/mm²; P < 0.001 compared with vehicle-treated rats) (Fig. 3B; n = 4 in each group). In the peripheral retina, the number of RGCs was significantly decreased compared with control (control: 2402 ± 69 cells/mm²; NMDA: 585 ± 122 cells/mm²; P < 0.001). When vehicle-treated and spironolactone-treated rats were compared, there was a similar RGC death in the central retina (655 ± 92 cells/mm²; P = 0.76). However, the RGC death appeared to be milder in the peripheral retina for the memantine-treated versus the vehicle-treated rats (1147 ± 53 cells/mm²; P < 0.001 compared with vehicle-treated rats).
Aldosterone Concentration in the Vitreous Humor

We subsequently examined the effect of NMDA on the concentration of aldosterone. After an intravitreal injection of NMDA in a normal eye, the concentration of aldosterone in the vitreous humor was 859 pg/ml. At 1 day after the intravitreal injection of vehicle, aldosterone, or NMDA, the aldosterone concentration in the vitreous humor was 980 pg/ml, 579 × 10⁵ pg/ml and 722 pg/ml, respectively (Table). These results show that the aldosterone concentration was not influenced by NMDA. Thus, this finding demonstrates that the RAAS is not activated by the NMDA receptor-mediated signal.

Table. The Concentration of Aldosterone in the Vitreous Humor

<table>
<thead>
<tr>
<th></th>
<th>Concentration, pg/ml</th>
</tr>
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<tbody>
<tr>
<td>Normal</td>
<td>858</td>
</tr>
<tr>
<td>Vehicle*</td>
<td>980</td>
</tr>
<tr>
<td>NMDA*</td>
<td>722</td>
</tr>
<tr>
<td>Aldosterone*</td>
<td>579 × 10⁵</td>
</tr>
</tbody>
</table>

* Intravitreal injection.

**DISCUSSION**

We previously investigated RAAS and reported that it could play an important role in preventing retinal ischemia-reperfusion injuries.¹⁸⁻²⁰,²² There have been a substantial number of studies that have examined the mechanisms responsible for retinal ischemia-reperfusion injury. The findings of these studies have demonstrated the involvement of a variety of factors including glutamate,⁶,²³ free radicals,²⁴ inflammatory adhesion molecules,²⁵ nitric oxide,²⁶ and cytokines.²⁷ In addition, it has also been reported that the neuronal damage that develops after ischemia-reperfusion injury is primarily caused by glutamate-mediated signalling that occurs especially via the NMDA receptor.²⁸ In our current study, however, we showed that there was no relationship between the RAAS and the NMDA receptor-mediated signal.

The activation of NMDA receptors that subsequently induce cell death is believed to be primarily caused by an influx of Ca²⁺ into the cells, which leads to the generation of free radicals.²⁹ In addition, it has also been reported that an enhancement of the reactive oxygen species (ROS) production occurs after excessive increases in the intracellular free Ca²⁺ concentration.²⁹ The predominant form of glutamate neurotox-
The Relationship Between Aldosterone and NMDA

icinity that occurs in retinal tissues has been shown to be mediated by an overstimulation of the NMDA subtype of glutamate receptors. As a result, this causes an increase of the Ca2+ influx, which is then followed by cell death.6,20,23

Furthermore, in various eye diseases, such as retinal ischemia, glaucoma, diabetic retinopathy, and age-related macular degeneration, it has been proposed that glutamate excitotoxicity and oxidative stress contribute to the retinal damage that occurs in these disorders.22,23

An increase in the retinal angiogenin mRNA has also been found after ischemic injury to the retina of rats.22 We previously examined ischemia-reperfusion in rats and found that there were increases in the AT1-R expression starting at 3 hours, with the peak expression occurring at 12 hours after the ischemia-reperfusion.29 In a subsequent study, we found the ROS production that occurred during a 12-hour ischemia-reperfusion was mediated by a nitrosoanamide adenine dinucleotide phosphate oxidative pathway.29 Overall, these results indicate that the ROS production that causes the retinal injury occurs via the local RAAS. Thus, these findings demonstrate that not only the NMDA receptor-mediated signal but also the RAAS are important pathways that are involved in retinal neuronal degeneration. Our current study showed that while spinonealactone protected against RGC death after the local administration of aldosterone, memantine did not. In contrast, our study also found that memantine but not spinonealactone protected against RGC death after local administration of NMDA. Thus, these results indirectly show that RAAS does not exist downstream of the NMDA receptor-mediated signal and that the NMDA receptor-mediated signal does not exist downstream of the RAAS.

In a previous study, we found that there was no effect on the number of RGCs after the systemic administration of 8 µg/kg aldosterone.22 In addition, at 2 weeks after the systemic administration of either 8 µg/kg or 80 µg/kg d aldosterone, the concentration of aldosterone in the vitreous humor was 708 pg/mL and 4200 pg/mL, respectively (data not shown). Since we previously found that the decrease in the RGCs after the systemic administration of 80 µg/kg d aldosterone started at 2 weeks,21 we measured the aldosterone concentration in the vitreous humor in the current study at 2 weeks after a systemic administration of aldosterone. Thus, after an intravitreal injection of vehicle or NMDA, we found the concentration of aldosterone in the vitreous humor to be 980 pg/mL and 722 pg/mL, respectively. Although we have yet to determine the maximum aldosterone concentration that exhibits neurotoxicity, the present finding does indicate that there is no neurotoxicity in the retina for aldosterone concentrations that are below 1000 pg/mL in the vitreous humor. Furthermore, our results also showed that there were no increases in the concentration of aldosterone after intravital injection of NMDA, which indicates that the NMDA receptor-mediated signal does not exist upstream of the RAAS.

While we have previously demonstrated that systemic administration of aldosterone caused an RGC loss that was associated with thinning of the retinal nerve fiber layer without elevated IOP, it appears that the other cell layers are unaffected.20 Furthermore, although the number of RGCs were reduced after an intravitreal injection of aldosterone, other retinal neurons were not affected.22 Thus, based on the results of these previous studies, our current study evaluated only the number of RGCs. In conclusion, even though both the RAAS and NMDA receptor-mediated signals are important pathways that are associated with RGC death, we found that there was no relationship in the rat retina between the RAAS and NMDA receptor-mediated signal. Therefore, careful attention should be paid to both the NMDA receptor-mediated signal and the

RAAS when considering the use of neuroprotections for the management of glaucoma.

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References

13. Osborne NN. Recent clinical findings with memantine should not mean that the idea of neuroprotection in glaucoma is abandoned. Acta Ophthalmol. 2009;87:450-454.


