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Elevated plasma aldosterone levels are associated with a reduction in retinal ganglion cell survival

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Abstract

Objective: The purpose of this article is to investigate the relationship between the plasma concentration of aldosterone and changes in the number of retinal ganglion cells (RGCs) after systemic administration of aldosterone.

Methods: An osmotic minipump that was subcutaneously implanted into the midscapular region of rats administered 40, 80 or 160 μg/kg/day aldosterone or vehicle. Enzyme immunoassay kits were used to measure the plasma aldosterone concentrations two weeks after the systemic administration of aldosterone or vehicle. Six weeks after these systemic administrations, the number of RGCs was measured.

Results: The plasma aldosterone concentrations at two weeks after systemic administration of vehicle or 160 μg/kg/day aldosterone were 238 ± 17 pg/ml and 1750 ± 151 pg/ml (748.5% ± 183.2%), respectively. There was a significant decrease in the number of RGCs in the central retina of the rats after the administration of either 80 or 160 μg/kg/day aldosterone. In the peripheral retina, however, there was a significant decrease in the number of RGCs in 40, 80 or 160 μg/kg/day aldosterone. There was a significant correlation between the number of RGCs and plasma aldosterone concentration.

Conclusions: After systemic administration of aldosterone, there was a negative correlation between the plasma aldosterone concentration and the number of RGCs.

Keywords

Aldosterone, retinal ganglion cell, glaucoma, rat, plasma aldosterone concentration

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Introduction

The mineralocorticoid hormone aldosterone, which regulates electrolytes, fluid volume and blood pressure, is synthesized from cholesterol in the zona glomerulosa of the adrenal gland and extra-adrenal tissue. Aldosterone is inversely regulated in conjunction with sodium chloride (NaCl) status under normal physiological situations. Thus, when plasma levels of aldosterone are higher in accordance with NaCl status, aldosterone binds to the mineralocorticoid receptor, thereby causing endothelial dysfunction, vasculopathy, vascular and ventricular remodeling, and renal injury.[1] Previous studies have additionally suggested that aldosterone could be involved in the pathogenesis of cardiovascular diseases via a pathway that is independent of angiotensin II (Ang II). Patients with primary aldosteronism normally exhibit very low levels of Ang II. Furthermore, there is substantial evidence that these patients have a much higher incidence of left ventricular hypertrophy,[2] albuminuria[3] and stroke[4,5] as compared patients with essential hypertension. In addition, higher circulating concentrations of aldosterone in chronic heart failure patients has been shown to be associated with an increased mortality.[6] In addition, it has been suggested that the plasma aldosterone level might be an independent predictor of coronary in-stent restenosis.[7]

Several studies have reported finding components of the renin-angiotensin-aldosterone system (RAAS) within the

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human eye.[8–11] In our previous studies, we also found that the administration of the mineralocorticoid receptor antagonist, spironolactone, an angiotensin-converting enzyme (ACE) inhibitor, and an AT1-receptor blocker (ARB) all led to reductions in retinal ischemia-reperfusion injuries.[12,13] Furthermore, we also demonstrated that after the systemic administration of aldosterone, the subsequent administration of spironolactone prevented the retinal ganglion cell (RGC) loss that is associated with the aldosterone-caused thinning of the retinal nerve fiber layer without elevated intraocular pressure.[14] Presently, however, it is still unknown whether there is a relationship between aldosterone blood concentration and the number of RGCs that are actually lost. Therefore, the aim of this study was to determine whether there is a relationship between plasma aldosterone concentrations and the change in the number of RGCs in the rat retina.

Materials and Methods

Animals

Male Sprague–Dawley rats weighing 200 to 250 g were obtained from Charles River Japan (Yokohama, Japan) and CLEA Japan (Tokyo, Japan). All animals had free access to standard rat food (Oriental Yeast Co Ltd, Tokyo, Japan) and tap water. The experimental protocols and procedures for animal care were performed in accordance with the standard guidelines for animal experimentation approved by the Kagawa University Faculty of Medicine. This study adhered to the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research.

Drugs

Aldosterone, which was obtained from Sigma-Aldrich (St. Louis, MO, USA), was dissolved in dimethyl sulfoxide (DMSO) to produce the stock solutions. These solutions were subsequently diluted to the final required concentrations. Final concentrations of DMSO never exceeded 5% in all cases.

Experimental animals

Administrations of 40, 80 or 160 μg/kg/day aldosterone or vehicle were performed using a subcutaneous osmotic minipump (Alzet Model 2006, DURECT Corporation, Cupertino, CA, USA). Subcutaneous implantation of the minipump was performed in the midscapular region of the rats.

Measurement of plasma aldosterone concentration

Blood samples (0.5 to 1.0 ml) were collected from the tail vein of rats two weeks after the administration of aldosterone or vehicle. After centrifuging the blood samples at 3000 rpm for 10 minutes, plasma samples were collected and then stored at −80°C until analyzed. Enzyme immunoassay kits (Assay Designs, Ann Arbor, MI, USA) were used to measure plasma aldosterone concentrations, with all samples prepared in accordance with the manufacturer's instructions.

Retrograde labeling of RGCs

Fast Blue (Polysciences Inc, Warrington, PA, USA) was injected bilaterally into the superior colliculi of anesthetized rats five weeks after the systemic administration of aldosterone or vehicle. The exposed skull was kept dry and clean throughout the procedure. After first identifying and marking the bregma, we then drilled small windows in the scalp to a depth of 3.6 mm from the surface of the skull both in the right and left hemispheres. Each window was located at 6.8 mm behind the bregma on the anteroposterior axis, and 1.5 mm lateral to the midline. After preparing the skull, 1.5 μl of 3% Fast Blue was slowly injected into the bilateral superior colliculi using a Hamilton syringe (Hamilton Co, Reno, NV, USA). After the injection, the skin over the wound was sutured, followed by the application of antibiotic ointment. Fast Blue is taken up by axon terminals and transported back to the parent cell bodies, where it can remain for several weeks without fading or leakage and apparently without affecting the viability or subsequent development of the labeled neurons.[15]

Tissue preparation and assessment of RGC survival

One week after the application of the fluorescent dye, six weeks after the systemic administration of aldosterone or vehicle,[14] all animals were sacrificed using an overdose of pentobarbital. RGC density assays were performed using whole, flat-mounted retinas. After enucleation of the rat eyes, all samples were fixed in 4% paraformaldehyde for five hours at room temperature. The anterior segments were then removed, with the resultant posterior eyecups left in place. Subsequently, after making four radial cuts in the periphery of each eyecup, we carefully separated the retina from the retinal pigment epithelium. The flat mounts were prepared by first dissociating the retina from the underlying structures, followed by making four radial cuts to flatten the retina, which was then spread on a gelatin-coated glass slide. A fluorescence microscope (Olympus BX-51/DP-72, Olympus, Tokyo, Japan) with an ultraviolet filter (excitation filter, 330–385 nm; barrier filter, 420 nm) was used for visualization of the labeled RGCs. Counts of the fluorescence-labeled RGCs were performed in 12 microscopic fields of the retinal tissue that had been obtained from two regions in each quadrant at two different eccentricities that were

located 1 mm (central) and 4 mm (peripheral) away from the optic disc. The total number of RGCs in each eye was counted using Image-Pro Plus software (version 4.0, Media Cybernetics, Bethesda, MD, USA). Cell counts were conducted by the same investigator in a masked fashion.

Staining by terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL)

Rats were euthanized with an overdose of sodium pentobarbital one week after systemic administration of 80 μg/kg/day aldosterone. The TUNEL staining was performed according to the manufacturer's instructions (Apoptosis in situ Detection Kit Wako, Wako Pure Chemical Industries, Ltd, Osaka, Japan) to detect the apoptotic cells. Sections were reacted with protein digestion enzyme for five minutes at 37°C. After adding 50 μl of the TUNEL reaction mixture, sections were incubated in a moist chamber for 10 minutes at 37°C. Slides were then rinsed three times in phosphate-buffered saline for five minutes and subsequently incubated with peroxidase-conjugated antibody at 37°C for 10 minutes. Then, 100 μl of diaminobenzidine was added and sections were kept at room temperature for five minutes, and finally counterstained with hematoxylin.

Statistical analysis

All data are presented as the mean ± SEM. Data were analyzed using Dunnett's multiple comparison test. Pearson's correlation coefficient was used to assess the correlation between serum aldosterone concentrations and the number of RGCs. Statistical analyses were performed using SPSS version 19.0 (SPSS Inc, Chicago, IL, USA). A p value of less than 0.05 was considered statistically significant.

Results

Plasma aldosterone concentration

Plasma aldosterone concentrations were increased at one to two weeks after systemic administration of aldosterone and then flattened until six weeks after systemic administration of aldosterone (data not shown). Because of this result, we collected blood samples two weeks after the administration of aldosterone or vehicle. The plasma aldosterone concentrations two weeks after systemic administration of vehicle or 40 μg/kg/day ($n = 6$), 80 μg/kg/day ($n = 6$), or 160 μg/kg/day ($n = 6$) aldosterone were 238 ± 17 pg/ml, 403 ± 38 pg/ml (168.8% ± 24.9%), 461 ±3 0 pg/ml (200.3% ± 53.6%) and 1750 ± 151 pg/ml (748.5% ± 183.2%), respectively (Figure 1). Animals treated with 160 μg/kg/day aldosterone exhibited a significant increase in the plasma aldosterone concentration ($p < 0.001$).

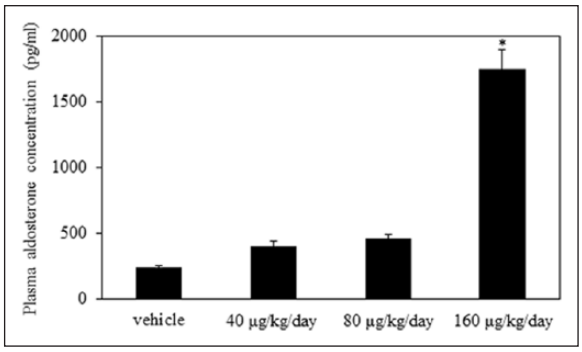

Figure 1. Plasma aldosterone concentration after being treated with vehicle or 40, 80 or 160 μg/kg/day aldosterone. Results are expressed as the mean ± SEM ($n = 6$: vehicle, $n = 6$: 40 μg/kg/day aldosterone, $n = 6$: 80 μg/kg/day, $n = 6$: 160 μg/kg/day). *$p < 0.001$ vs control (Dunnett's multiple comparison test).

Number of RGCs

Figure 2 shows representative results for the RGC labeling at six weeks in the 40, 80 and 160 μg/kg/day aldosterone-treated and vehicle-treated rats. The number of RGCs in the central retina was 2760 ± 142 cells/mm², 2252 ± 128 cells/mm², 2015 ± 405 cells/mm² and 404 ± 174 cells/mm² in the vehicle- ($n = 6$), and 40 ($n = 6$), 80 ($n = 6$) and 160 ($n = 6$) μg/kg/day aldosterone-treated rats, respectively (Figure 2(b)). There was a significant decrease in the number of RGCs in the central retina in the 80 and 160 μg/kg/day aldosterone-treated rats ($p = 0.04$, $p < 0.001$, respectively). The number of RGCs in the peripheral retina was 2627 ± 77 cells/mm², 2059 ± 69 cells/mm², 1843 ± 103 cells/mm² and 773 ± 136 cells/mm² in the vehicle-, and 40, 80 and 160 μg/kg/day aldosterone-treated rats, respectively (Figure 2(c)). There was a significant decrease in the number of RGCs in the peripheral retina in the 40, 80 and 160 μg/kg/day aldosterone-treated rats ($p = 0.032$, $p = 0.002$, $p < 0.001$, respectively).

Relationship between plasma aldosterone concentration and RGC survival

There was a linear relationship between serum aldosterone concentration and the number of RGCs in the central retina (six weeks: $r = -0.886$, $p < 0.001$; four weeks: $r = -0.658$, $p = 0.07$) (Figure 3(a)). Similarly, there was also a linear relationship between the serum aldosterone concentration and the number of RGCs in the peripheral retina (six weeks: $r = -0.856$, $p < 0.001$; four weeks: $r = -0.583$, $p = 0.13$) (Figure 3(b)).

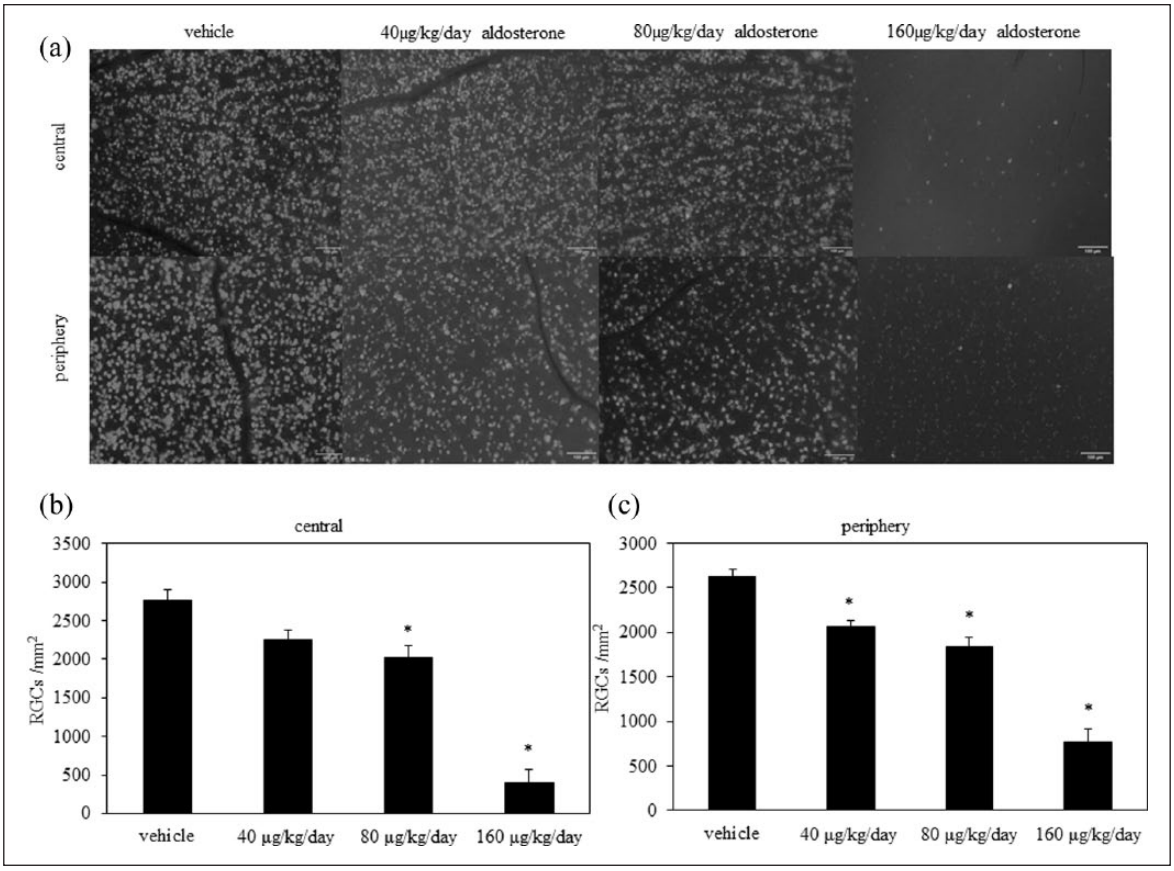

Figure 2. Effect of aldosterone on retinal ganglion cell (RGC) death. (a) Retrograde labeling of RGCs treated with vehicle or 40, 80 and 160 μg/kg/day aldosterone for six weeks. Micrographs of the central and peripheral areas were taken approximately 1 and 4 mm away from the optic nerve head. Scale bar, 100 μm. RGCs were counted in the central (b) and peripheral (c) areas. Results are expressed as the mean ± SEM ($n = 6$: vehicle, $n = 6$: 40 μg/kg/day aldosterone, $n = 6$: 80 μg/kg/day, $n = 6$: 160 μg/kg/day). *$p < 0.001$ vs control (Dunnett's multiple comparison test).

Aldosterone-induced apoptosis in rat retina

To further elucidate whether the loss of RGCs is due to cell death from apoptosis or necrosis, we performed TUNEL staining on retina sections one week after systemic administration of aldosterone. The TUNEL-positive cells in the ganglion cell layer were observed (Figure 4).

Discussion

Systemic administration of aldosterone resulted in a dose-dependent progressive loss of RGCs. Results of our analysis indicated there was a strong correlation between the plasma aldosterone concentration and the number of RGCs.

Although reductions in the number of RGCs occur after both the systemic administration and the intravitreal injection of aldosterone, the administration of aldosterone has not been reported to affect any of the other retinal neurons.[14,16] Therefore, our current study focused only on changes in the number of RGCs. A previous study reported that administration of 0.66 μg/hour aldosterone via subcutaneously implanted osmotic pumps resulted in an elevation of plasma aldosterone concentrations.[17] Furthermore, a 0.75 μg/hour systemic administration of aldosterone in an experimental retinopathy of prematurity model led to the exacerbation of pathological neovascularization.[18] In the current study, systemic administration of aldosterone at a dose of more than 80 μg/kg/day (0.67–0.83 μg/hour) caused a significant reduction in the number of RGCs in the central and peripheral retina. Moreover, our current study also found that a systemic administration of aldosterone at a dose of 80 μg/kg/day resulted in a plasma aldosterone concentration ranging from 368 to 527 pg/ml. There was a strong negative correlation between the plasma aldosterone concentration and the number of RGCs ($r = -0.869, -0.911$). These findings indicate that not only is the dose of the systemic administration of aldosterone important, but the resulting plasma aldosterone concentrations should also be taken into consideration.

In the initial attempt to determine patients suspected of having primary aldosteronism, a previous study measured the ratio of the plasma aldosterone concentration to the

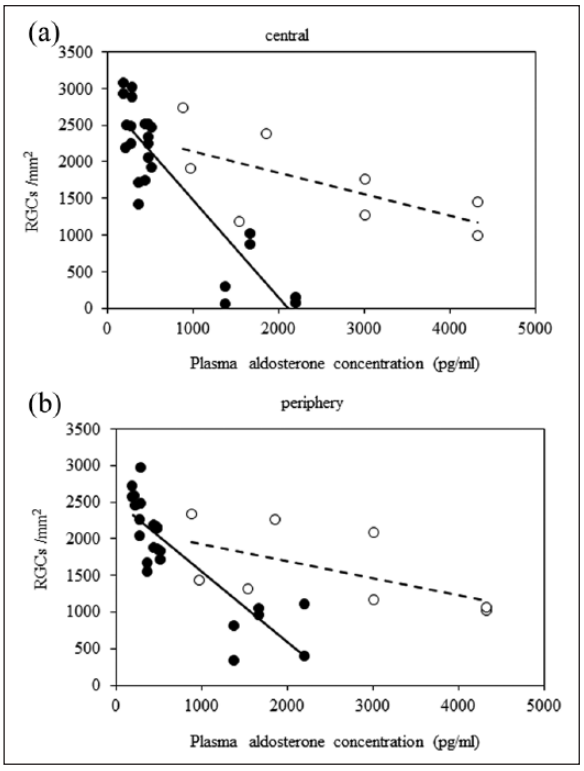

Figure 3. Scatter plots showing association between plasma aldosterone concentration and the number of retinal ganglion cells (RGCs) in the central retina (a) and peripheral retina (b). (a) Correlation coefficient = −0.911. Regression equation $y = -1.33x + 2807$. (b) Correlation coefficient = −0.869. Regression equation $y = -0.96x + 2512$.

plasma renin activity.[19] A further study additionally recommended that plasma aldosterone concentrations greater than 150 pg/ml were also required to definitively identify suspected primary aldosteronism.[20] Evidence from a subsequent study further demonstrated that aldosterone is independently involved with the development of cardiovascular injury in the kidney and brain.[21] In rats as well, it is reported that cardiovascular disorder is caused by the administration of aldosterone.[22] Overall, these findings indicate the importance of determining the plasma aldosterone concentration to help prevent organ complications, including in the retina.

Although the number of RGCs were similar between vehicle- and 8 μg/kg/day aldosterone-treated rats, RGCs were decreased in the 80 μg/kg/day aldosterone-treated rats.[14] Since 80 μg/kg/day was defined as standard concentration, we explored around 80 μg/kg/day, half and twice concentrations, in the current study. We recently reported that in aldosterone-treated rats, the tendency of neuronal degeneration in the ganglion cell layer started first from the peripheral retina, two weeks after continual administration of aldosterone, and then spread to the central retina, four weeks after continual administration of aldosterone.[14] For this reason, we separated peripheral and central data in the current study.

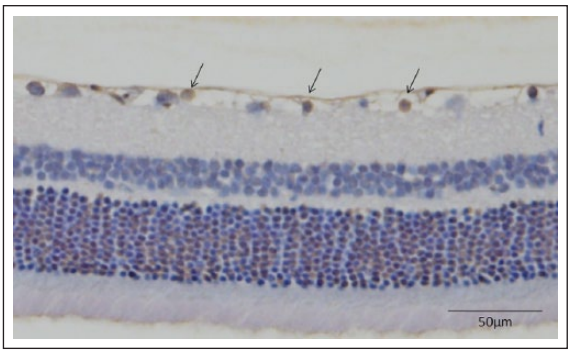

Figure 4. Aldosterone-induced apoptosis one week after systemic administration of aldosterone. The TUNEL-positive cells are indicated by arrows. Scale bar. TUNEL: terminal deoxynucleotidyl transferase dUTP nick-end labeling.

In our current study, we found there was a negative correlation between the plasma aldosterone concentration and the number of RGCs in the central and peripheral retina. Although a previous study reported finding the plasma aldosterone concentration in normal rats to be approximately 180 pg/ml,[23] our current study found that the plasma aldosterone concentration in rats treated with vehicle was 212 ± 17 pg/ml. This difference suggests that the normal value of aldosterone might depend on the species examined.

High plasma aldosterone concentration is known to cause vasculopathy.[24] We are currently performing additional studies designed to investigate the retinal blood flow after the systemic administration of aldosterone.

Presently, the relationship between normal-tension glaucoma and primary aldosteronism remains unclear. However, population-based studies have reported finding an association between hypertension and glaucoma.[25–27] Therefore, it is important that future studies additionally investigate the prevalence of glaucoma among patients with primary aldosteronism.

Conclusions

The findings of our current study demonstrate that there is a negative correlation between plasma aldosterone concentrations and the number of RGCs. We speculate that the plasma aldosterone concentration may be an important risk factor associated with RGC loss in diseases such as glaucoma.

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