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学　位　論　文

Gene expression changes in the retina after systemic administration of aldosterone

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Japanese Journal of Ophthalmology
https://doi.org/10.1007/s10384-018-0595-4

THE OFFICIAL INTERNATIONAL JOURNAL OF THE JAPANESE OPHTHALMOLOGICAL SOCIETY

LABORATORY INVESTIGATION

Gene expression changes in the retina after systemic administration of aldosterone

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Received: 1 May 2017 / Accepted: 2 April 2018
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Abstract

Purpose Retinal ganglion cell (RGC) loss associated with thinning of the retinal nerve fiber layer without elevated intraocular pressure (IOP) occurs after the systemic administration of aldosterone. Since it is important to determine the mechanism of cell death independent of the IOP, we examined gene expression changes in the retina after the systemic administration of aldosterone.

Methods Following subcutaneous implantation of an osmotic minipump into the mid-scapular region of rats, we administered an 80 μg/kg/day dose of aldosterone. Differences in the gene expression in the retina between normal rats and aldosterone-treated rats were investigated using microarrays. Real-time PCR was used to confirm the differential expression.

Results Analysis of the microarray data sets revealed the upregulation of 24 genes and the downregulation of 24 genes of key apoptosis-specific genes. Real-time PCR revealed 4 genes (Cdkn1a, Tbox5, Pf4, Vdr) were upregulated while 12 genes (Acvr1c, Asns, Bard1, Card9, Crh, Fcgr1a, Inhba, Kcnh8, Lck, Phlda1, Ptprc, Sh3rf1) were downregulated.

Conclusions Significant increases and decreases were noted in several genes after the systemic administration of aldosterone. Further studies will need to be undertaken in order to definitively clarify the role of these genes in the eyes of animals with normal-tension glaucoma.

Keywords Aldosterone · Retinal ganglion cell · Microarray · Retina · Glaucoma

Introduction

In normal-tension glaucoma (NTG), patients exhibit glaucomatous cupping of the optic nerve head with visual field damage even though there is an absence of elevated intraocular pressure (IOP) [1, 2]. In most patients with all other types of glaucoma, however, the IOP is reported to be a risk factor [3–6]. Although reduction of IOP prevents disease progression in most patients with NTG [7], in some there is still disease progression in spite of the reduction in IOP [8]. It is suggested that factors other than an elevated IOP might be involved in the progression of glaucoma [9]. Therefore, detailed evaluation needs to be conducted of the new therapeutic approaches designed to treat this debilitating disease.

The systemic renin-angiotensin-aldosterone system (RAAS) plays an important role in both blood pressure and electrolyte homeostasis. Aldosterone, a steroid hormone, exerts its effects after it binds to a mineralocorticoid receptor (MR). Aldosterone causes an increase in reactive oxygen species (ROS) that subsequently activates NADPH oxidase and promotes inflammation [10, 11]. Compared to patients with essential hypertension, patients with primary aldosteronism have been shown to have a higher incidence of left ventricular hypertrophy [12], albuminuria [13], and stroke [14, 15]. Data from experimental animal studies demonstrate that aldosterone may play a role in mediating cardiovascular injury in the kidney and brain [14, 15]. Beneficial effects in the retina against ischemia-reperfusion injury are also reported after blockade of the angiotensin II type 1 receptor (AT1-R) and MR [16–18]. Moreover, within the retina there is considerable evidence that shows that all the components of the RAAS are expressed [19, 20]. In our previous experiments, we demonstrated that intravitreal injection of

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Published online: 30 April 2018

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aldosterone reduced the number of RGCs [18], and more recently, we reported that, following the systemic administration of aldosterone there was a decrease in the number of RGCs without an elevation in the IOP and that, in addition, the administration of an MR blocker prevented RGC loss [20]. At the same time, the other cell layers appeared to be unaffected [18, 21].

At present the mechanism of cell death in this particular animal model remains unknown. The purpose of our current study was to investigate gene expression changes in the retina after the systemic administration of aldosterone.

Material and methods

Animals

Male Sprague-Dawley rats were obtained from Charles River Japan. The rats, which weighed 200 to 250 g, were permitted free access to standard rat food (Oriental Yeast Co., Ltd.) and tap water. All experiments were conducted in accordance with the approved animal care and standard guidelines for animal experimentation of the Kagawa University Faculty of Medicine. All the experiments adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Approval at our ethics committee was not deemed necessary.

Experimental animals

Subcutaneous osmotic minipumps (Alzet model 2006, DURECT Corporation), which were implanted subcutaneously into the mid-scapular region of the rats, were used to administer an 80 μg/kg/day dose of aldosterone (Sigma-Aldrich). At 7 days after the systemic administration with or without aldosterone, the rats were sacrificed by administering an overdose of pentobarbital sodium. After the eyes were enucleated, the retinae were carefully isolated.

Histological examination

For the histological examination, rats were anesthetized by intraperitoneal injection of pentobarbital sodium (50 mg/kg) at 6 weeks after the systemic administration of aldosterone and then perfused intracardially with phosphate-buffered saline (PBS), followed by perfusion with 4% paraformaldehyde in PBS. Subsequently, the anterior segments, including the lens, were removed. The posterior eyecups were then embedded in paraffin, and thin sections (5-μm thickness) were cut using a microtome. Each of the sections was carefully cut to include the full length from the superior to inferior along the vertical meridian through the optic nerve head. Each eye was then mounted on a silane-coated glass slide and stained with hematoxylin and eosin (HE). A microscopic image (Olympus BX-51, Olympus Inc.) of each section within 0.5 to 1 mm superior of the optic disc was scanned.

Microarray analysis

The microarray analysis examined a total of 7 controls and 7 treatment eyes. Each sample consisted of the retinal fraction from 7 eyes. The RNeasy mini Kit protocol (QIAGEN, GmBH) was used for the extraction of the total RNA. RNA sample integrity was verified through the use of a UV adsorption measurement and bioanalyzer. After using the Low Input Quick Amp Labeling Kit (Agilent Technologies) to amplify, label and purify the total RNA, the qualified total RNA was then further purified by the RNeasy mini spin column (QIAGEN). Subsequently, the qualified total RNA was further purified by Low Input Quick Amp Labeling Kit. Next, the array was washed by Agilent's Gene Expression Wash Buffer kit (Agilent Technologies). In the final step, the array slides were scanned by an Agilent Technologies Microarray Scanner (Agilent Technologies). Using the Agilent Feature Extraction 10.7.3.1, the same spot was quantified on each slide. Normalization of the raw data was performed as follows: importation of the scanned data to the GeneSpring GX 7.3.1, after which the data was processed and normalized to the 75 percentile.

Real-time PCR

Real-time PCR using a LightCycler FastStart DNA Master SYBR Green I kit and an ABI Prism 7000 Sequence Detection System (Applied Biosystems) were used to analyze the mRNA expression of GAPDH, and up- or downregulated genes in microarray analysis, as previously described [22, 23]. Briefly, after denaturation of the cDNA at 95°C for 30 s, it was then amplified by PCR for 45 cycles (95°C for 15 s followed by 60°C for 40 s). Table 1 lists the oligonucleotide primer sequences. After normalization of the GAPDH expression, all the data were expressed asrelative differences.

In situ hybridization

We investigated gene expression site of Cdkn1a, Vdr and Pf4 by in situ hybridization. In situ hybridization was performed using ViewRNA™ ISH Tissue Assay (Affymetrix) following the manufacturer's protocol. Tissues were fixed for 24 hours at 4°C with paraformaldehyde solution (4% paraformaldehyde in phosphate buffer saline). FFPE tissues were sectioned at 4 micron and mounted on silane coated slides (Muto pure chemical co, ltd). Each of the sections was carefully cut to include the full length from the superior to inferior along the vertical meridian through the optic

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Table 1 Primers for real time PCR

Gene	Sequence (5′-3′)	Position	Size of production (bp)
Bcl3	Forward: CTGACAGCGGCCTCAAGAAC	1021-1040	92
	Reverse: AGAGGCCTTTCCCCTTAGGA	1112-1093	
Fcgr2b	Forward: CTGTCGTCCATGTGCTCTCA	107-126	108
	Reverse: GTTTCACCACAGCCTTCGGA	214-195	
Htatip2	Forward: ATGGCGGACAAGGAAACACT	12-31	78
	Reverse: GGCGCCCAAAATAAAGACGG	89-70	
Tbx5	Forward: TATTGTACCCGCAGACGACC	335-354	94
	Reverse: ATAAAGGCGACCCGGCATAG	428-409	
Acvr1	Forward: TGTTGGAGTGTGTCGGGAAG	776-795	149
	Reverse: ATGCCTCAGCATAACCGTGT	924-905	
Alox15	Forward: GCCATCCAGCTTGAACTTCC	952-971	87
	Reverse: GGCTAGGAGCCAGTCCATTG	1038-1019	
Birc3	Forward: GAAAAGGGGAGGGGGAAGCC	32-51	82
	Reverse: CCTACGGAACTTTGCTGACCA	113-93	
C-C motif	Forward: AGCCAACTCTCACTGAAGCC	34-53	84
	Reverse: AACTGTGAACAACAGGCCCA	117-96	
C7	Forward: CCCAAGCATGAAGGCAACAAG	134-154	113
	Reverse: AAGGGCCATAGGAGTCCCAC	246-227	
Cdkn1a	Forward: TCCGCTCGGATTGTAAACCTC	1766-1786	84
	Reverse: GCACCAGCTTTGGGATAGGG	1849-1830	
Cdkn2c	Forward: TCTGCGAGACGGATGGAAAG	443-462	71
	Reverse: ACAGTGGTGACTTGAGGCAG	513-494	
Fosl1	Forward: CCACACTCCTGGCTTTGTGA	1034-1053	113
	Reverse: TGGTTTGGGGCATGGGTATG	1146-1127	
Il1rn	Forward: GATGGAAATCTGCAGGGGACC	4-24	110
	Reverse: GCATCTTGCAGGGTCTTTTCC	113-93	
Lgals7	Forward: ATCCTCTAACGTGCGCTCAG	350-369	116
	Reverse: ACGATCTGACGAAACCCCAC	465-446	
Mael	Forward: GGCATGACCAAGCAACTGTG	648-667	140
	Reverse: TTCTGATGCCCGCTCCATAC	787-768	
Msx1	Forward: TTCCTCCTCCCTCTTCCGAC	1194-1213	123
	Reverse: TTTGCATCCCCCAGTTTCCA	1316-1297	
Myc	Forward: GGAAGGACTATCCAGCTGCC	1525-1544	84
	Reverse: TGGAGCATTTGCGGTTGTTG	1608-1589	
Pf4	Forward: TGATCAAAGCAGGACCCCAC	191-210	94
	Reverse: TACAGAGGTACTTGCCGGTC	284-265	
Snca	Forward: CAGCAGTCGCTCAGAAGACA	388-407	102
	Reverse: GTGGGTACCCTTCTTCACCC	489-470	
Terc	Forward: GTTCTTTTGTTCTCCGCCCG	32-51	70
	Reverse: GCTGCAGGTCTGAACTTTCC	101-82	
Txnip	Forward: CAAGTCTCCAGCCTCAAGGG	1517-1536	76
	Reverse: TTCCGACATTCACCCAGCAA	1592-1573	
Tnfrsf8	Forward: TGGGTCAGTGACAGATTCCAG	1122-1142	146
	Reverse: TGGGAGCAAAAGAGTTCCCAG	1267-1247	
Vegfa	Forward: ATTCAACGGACTCATCAGCCA	96-116	136
	Reverse: CCGTTGGCACGATTTAAGAGG	231-211	
Vdr	Forward: TGATCCAGAAACTGGCCGAC	1230-1249	86
	Reverse: GCTATTCTCGGGCTGGAAGG	1315-1296	
Adamtsl4	Forward: GACCCTGAGGCGAATTCCTG	83-102	88

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Table 1 (continued)

Gene	Sequence (5′-3′)	Position	Size of production (bp)
	Reverse: TAGGAATCTTGGCGCAAGCC	170-151	
Bard1	Forward: TGAACACCACCGGCTATCAC	1446-1465	145
	Reverse: TCTGTGTAATCCACTGGCCG	1590-1571	
Cd3 g	Forward: TGGAGTTCGCCAGTCAAGAG	473-492	75
	Reverse: TCCTTGAGGGGCTGGTAGAC	547-528	
Fcgr1a	Forward: GCTATTTGCCACACCAGTGC	573-592	71
	Reverse: TCAGGATGACCAGACTCCCC	643-624	
Gimap5	Forward: TGTGTTCTGGCGGATGTTCA	26-45	127
	Reverse: ACTCGCAGAGCTGTAAACCC	152-133	
Ndufaf4	Forward: CTGTACCGGTGGGTTCTTGG	1361-1380	121
	Reverse: GCCTGGCCTTTTGCCATTTA	1481-1462	
Sh3rf1	Forward: TACTCGCCTCTACACCGTCA	1229-1248	124
	Reverse: GGCCGTAAATGTGCGATCTG	1352-1333	
Acvr1c	Forward: TACCTGCCAAACCGAAGGAG	260-279	140
	Reverse: CGGTCTTGGTCACGTTGTTG	399-380	
Asns	Forward: AAACCTGGAAAACTTCGGCG	71-90	119
	Reverse: TGCCACACATGCTACAGGAG	189-170	
Bdnf	Forward: CTTCGGTTGCATGAAGGCTG	108-127	135
	Reverse: GTCAGACCTCTCGAACCTGC	242-223	
Cdh1	Forward: GCCCAGGAAATACACCCCTC	3792-3811	75
	Reverse: ACTCAGGTCCAAATCAGCCG	3866-3847	
Casp7	Forward: AGGCCCTCTTCAAGTGCTTC	285-304	84
	Reverse: GCAGATCCTGCATCTTTGCG	368-349	
Card9	Forward: GGATGAGAACTACGACCTGGC	649-669	142
	Reverse: CACCTTGCAGTCATCCTCTGC	790-770	
C5ar1	Forward: TCTACTTGGCCGTGTTCCTG	174-193	89
	Reverse: GGCGTTGACAGTACGTTTGG	262-243	
C6	Forward: TCAGATGCTTACCAGACAGAACC	2053-2075	150
	Reverse: TGGGACAGGTCAGCTCAATG	2202-2183	
Crh	Forward: GCAACCTCAGCCGATTCTGA	325-344	77
	Reverse: CAGCGGGACTTCTGTTGAGG	401-382	
Cryaa	Forward: GGCTCCTGCCTGACTCATTG	7-26	71
	Reverse: CTGGATGGTGACGTCCATGT	77-58	
Inhba	Forward: CCCAGTGTCTAGCAGCATCC	833-852	71
	Reverse: CACAAGCAATCCGCACATCC	903-884	
Phlda1	Forward: GAACCGTCCCAACCTAGTGG	623-642	116
	Reverse: TATACTTGCCCTTGCGCTCC	740-721	
Kcnh8	Forward: GTACTACGGCAACAACACGC	1267-1286	128
	Reverse: TCTCTGCATCCGTGTTAGCG	1394-1375	
Ptprc	Forward: TTGCTCCCCATCCGATAAGAC	44-64	108
	Reverse: AGCTGAAGGCCAGAAGTTTGA	151-131	
Ripk3	Forward: AGTCAGGGGAATCAAGCCTTA	126-146	125
	Reverse: CCTCTTGTTGGGTCTGGATG	250-231	
Lck	Forward: CGATCTGGTCCGCCATTACA	617-636	89
	Reverse: ATGGTTTCTGGGGCTTCTGG	705-686	
ERbb3	Forward: CTGGGAGAATGCTTGGCAGA	1606-1625	111
	Reverse: TTCCCGGCTGTAGTTTCGAC	1716-1697	

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nerve head. Rat Cdkn1a-gene-specific probe (Accession No. NM_080782.3), Rat Pf4-gene-specific probe (Accession No. NM_001007729.1) and Rat Vdr-gene-specific probe (Accession No. NM_017058.1) were designed and synthesized by Affymetrix. A no-probe sample was utilized as a negative control. Nuclei were stained for 5 min with Hoechst 33342 (Sigma-Aldrich) and samples were Dako Ultramount (Dako). Hybridized target mRNAs were visualized using fluorescent microscopy (BZ-X700, KEYENCE) and observed in 4 points in each slides, 1 mm (central) and 4 mm (peripheral) away from the optic disc.

Statistical analysis

All data were analyzed using Wilcoxon signed-rank test, with the data then presented as the mean ± SD. Statistical analyses were performed using SPSS version 19.0 (SPSS Inc., Chicago, IL). A P value of less than 0.05 was considered statistically significant.

Results

Histological examination of RGC loss

Aldosteron-treated rats showed a neuronal loss in the ganglion cell layer (Fig. 1).

Microarray analysis of gene expression

After systemic administration of aldosterone, we used microarray analysis to determine the gene expression changes in the retina. The changes in the level of expression of the genes (upregulated or downregulated by > 2.0-fold versus baseline) were then compared between the naïve rats (baseline: day 0) and the rats on day 7 after the systemic administration. The gene expression changes' observed in the retina at 1 week after systemic administration of aldosterone are summarized in Table 2, with 24 genes found to be either up- or downregulated in each cluster.

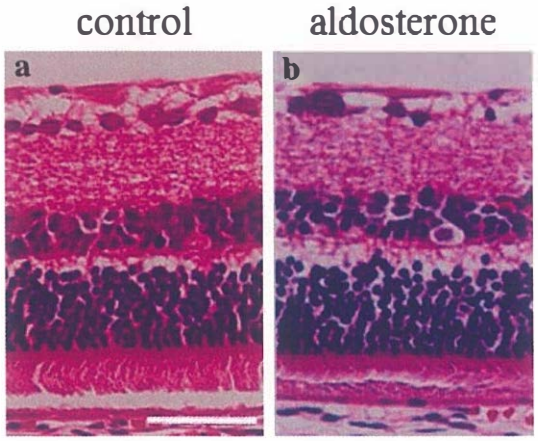

Fig. 1 Light micrographs of the retina of an eye treated with 80 μg/kg/day aldosterone for 2 weeks and a normal control eye. Scale bar, 50 μm

Ratio of RNA expression

Table 3 shows the ratio of RNA expression of protein specific RGC and other retinal neurons based on the microarray analysis. There was no significant change in either gene.

mRNA levels after systemic administration of aldosterone

Real-time PCR technique was used to measure the mRNA levels of the 48 genes that had been detected by

Table 2 Results of microarray assay

Up regulation	Ratio	Down regulation	Ratio
Acvr1	2.028	Acvr1c	0.384
Alox15	11.059	Adamtsl4	0.489
Birc3	13.423	Asns	0.224
Bcl3	9.804	Bard1	0.386
C7	2.454	Bdnf	0.364
C-C motif	7.972	Card9	0.199
Cdkn1a	3.071	C5ar1	0.168
Cdkn2c	2.031	C6	0.424
Fcgr2b	3.092	Casp7	0.385
Fosl1	13.808	Cd3g	0.443
Htaitp2	6.056	Cdh1	0.273
Il1rn	2.527	Crh	0.374
Tnfrsf8	17.7	Cryaa	0.473
Txnip	3.764	Erbb3	0.298
Vegfa	2.315	Fcgr1a	0.482
Vdr	2.003	Gimap5	0.372
Lgals7	2.973	Inhba	0.488
Mae1	5.791	Kcnh8	0.12
Msx1	2.034	Lck	0.206
Myc	2.234	Ndufaf4	0.141
Pf4	2.545	Phlda1	0.444
Snca	48.514	Ptprc	0.466
Tbx5	14.656	Ripk3	0.443
Terc	2.766	Sh3rf1	0.048

Table 3 Ratio of RNA

Gene	Ratio
Pax6	1.189
Thy1	1.051
Rho	1.024

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the microarray. Although the microarray analysis showed there was upregulation of Acvrl, Alox15, Cdkn2c, Il1rn, Snca, Terc and Vegfa, RT-PCR showed that these genes were downregulated. When compared to the normal retina, there were 4 genes (Cdkn1a, Pf4, Tbx5, and Vdr) (Table 4) that exhibited upregulated mRNA levels after the systemic administration of aldosterone, while 12 gene expressions exhibited downregulated levels (Acvr1c, Asns, Bard1, Card9, Crh, Fcgr1a, Inhba, Kcnh8, Lck, Phlda1, Ptprc, and Sh3rf1) (Table 5).

Expression of mRNA in the retina

Expression of Cdkn1a, Vdr and Pf4 was examined using in situ hybridization (Fig. 2). Cdkn1a was widely observed in the retina. In particular, strong Cdkn1a expression was observed in the ganglion cell layer (GCL). Vdr and Pf4 expression were observed in the outer plexiform layer (OPL) and in the outer nuclear layer (ONL). In addition, weak expression of Vdr was observed in the inner plexiform layer (IPL) and in the inner nuclear layer (INL).

Table 4 Results of real time PCR for upregulated genes

Gene	Control (n=10) Mean±SD	Aldosterone (n=10) Mean±SD	*P*-value
Acvrl	0.732±0.089	0.598±0.059	0.028*
Alox15	0.755±0.260	0.486±0.363	0.028*
Bcl3	0.779±0.121	0.963±0.449	0.386
Birc3	0.609±0.074	0.655±0.113	0.284
C7	1.237±0.276	1.046±0.125	0.169
C-C motif	0.936±0.208	0.896±0.179	0.959
Cdkn1a	0.958±0.183	1.661±0.474	0.012*
Cdkn2c	0.790±0.161	0.603±0.082	0.028*
Fcgr2b	1.098±0.190	1.141±0.266	0.575
Fosl1	0.740±0.231	0.728±0.143	0.721
Htatip2	0.452±0.089	0.497±0.227	0.959
Illrn	3.615±1.765	1.473±0.900	0.005*
Lgals7	1.409±0.693	0.948±0.130	0.092
Mael	0.551±0.343	0.552±0.119	0.799
Msx1	0.586±0.185	0.662±0.278	0.386
Myc	0.794±0.104	0.756±0.161	0.444
Pf4	0.589±0.108	0.990±0.247	0.005*
Snca	1.052±0.097	0.751±0.066	0.005*
Tbx5	0.688±0.363	0.862±0.080	0.005*
Terc	1.073±0.050	0.871±0.165	0.004*
Tnfrsf8	0.624±0.318	0.869±0.247	0.070
Txnip	0.788±0.098	0.682±0.351	0.382
Vegfa	0.988±0.127	0.646±0.097	<0.001*
Vdr	0.950±0.183	1.130±0.193	0.046*

SD standard deviation, *$P<0.05$, Wilcoxon signed-rank test

Table 5 Results of real time PCR for downregulated genes

Gene	Control (n=10) Mean±SD	Aldosterone (n=10) Mean±SD	*P*-value
Acvr1c	2.591±4.875	0.653±0.162	0.005*
Adamtsl4	0.810±0.177	1.012±0.263	0.092
Asns	0.925±0.159	0.201±0.043	0.005*
Bard1	0.625±0.157	0.469±0.058	0.028*
Bdnf	1.004±0.098	1.006±0.124	0.959
Card9	0.862±0.112	0.771±0.038	0.012*
C5ar1	0.872±0.160	0.973±0.270	0.114
C6	0.461±0.182	0.611±0.217	0.114
Casp7	0.887±0.037	0.869±0.187	0.444
Cd3 g	0.481±0.129	0.389±0.152	0.241
Cdh1	0.512±0.244	0.464±0.070	0.878
Crh	2.482±3.993	0.784±0.102	0.005*
Cryaa	0.521±0.295	0.498±0.214	0.721
Erbb3	0.528±0.369	0.350±0.181	0.284
Fcgr1a	0.520±0.201	0.323±0.095	0.005*
Gimap5	0.588±0.289	0.504±0.060	0.444
Inhba	0.749±0.131	0.644±0.045	0.007*
Kcnh8	0.794±0.205	0.504±0.084	0.005*
Lck	0.785±0.168	0.636±0.094	0.028*
Ndufaf4	0.572±0.273	0.432±0.057	0.114
Phlda1	0.956±0.218	0.644±0.198	0.037*
Ptprc	0.794±0.164	0.494±0.103	0.007*
Ripk3	0.621±0.174	0.793±0.367	0.241
Sh3rf1	0.788±0.257	0.575±0.104	0.012*

SD standard deviation, *$P<0.05$, Wilcoxon signed-rank test

Discussion

The current study showed that apoptosis was associated with the systemic administration of aldosterone, with 4 genes exhibiting upregulation and 12 genes showing downregulation. Since our previous study demonstrated there was a significant decrease in RGCs at 2 weeks after the continual administration of aldosterone [21], the present study investigated the changes in the gene expression in the retina at 1 week after administration, at a point prior to the death of the RGCs.

In our previous work, we showed that the local aldosterone/MR system that exists in the retina can be modulated by the RAAS both dependently and independently [18]. Moreover, we also demonstrated that there was an increase in the expression of AT1-R at 12 hours after reperfusion [16, 17] and that the ROS production after 12 hours of ischemia-reperfusion was mediated via the NADPH oxidase pathway [17]. Thus, these results suggest that the ROS production via the local RAAS might be responsible for the retinal ischemic injury. Furthermore, our findings also suggested that the RGC death observed in aldosterone-treated rats might have

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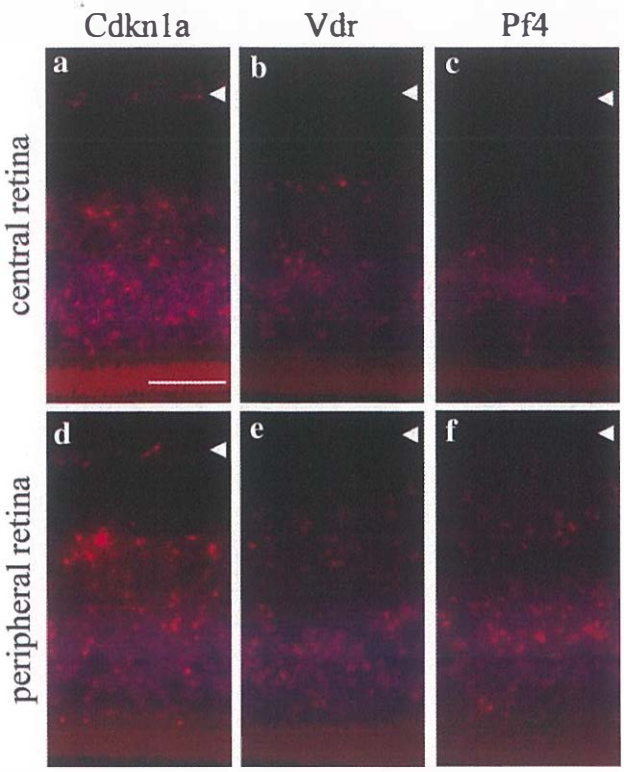

Fig. 2 Expression of Cdkn1a, Vdr or Pf4 in the retina. Fluorescent micrographs of in situ hybridization. Cdkn1a, (a) central and (d) peripheral retina. Vdr, (b) central and (e) peripheral retina. Pf4, (c) central and (f) peripheral retina. Micrographs of the central and peripheral areas were taken approximately 1 and 4 mm away from the optic nerve head. Scale bar, 50 μm. Arrow head shows ganglion cell layer (GCL)

been induced by aldosterone in a ROS-dependent manner via a NADPH oxidase pathway. Based on these findings, we further explored the relationship between 15 genes and the NADPH oxidase pathway. Our results indicate that Cdkn1a, Pf4 and Vdr are associated with cell death via a NADPH oxidase pathway. However, RT-PCR showed that Acvr1, Alox15, Cdkn2c, Il1rn, Snca, Terc and Vegfa were downregulated while microarray analysis indicated that these genes were upregulated. Since microarray is a global gene analysis, false positive genes are sometimes observed. Another possible explanation of this discrepancy is that it is impossible to deny a cross reaction. Based on these findings, we decided not to further pursue the analysis of these 6 genes.

Platelet factor 4 (Pf4) activated monocytes are responsible for a long-lasting release of ROS that can selectively induce apoptosis in the endothelial cells [24]. This causes programmed cell death in endothelial cells, as inhibitors of the NADPH oxidase effectively blocked Pf4-induced monocyte oxidative burst and protected endothelial cells from undergoing apoptosis [24]. There are a number of soluble factors released by endothelial cells that can regulate vascular tone and blood flow, including nitric oxide [25, 26]. Previous studies in animals and humans show that the inhibition of nitric oxide synthase reduces the blood flow [27, 28]. If there is an upregulation of Pf4, it is expected to reduce the blood flow. Thus, although in our current study we did not investigate the blood flow in the aldosterone-treated rats, these previous findings suggest that a reduced blood flow could have contributed to the RGC death in our animals. In fact, other studies that examined the retina and optic nerve head of glaucoma patients report finding reduced blood flow in these subjects [29, 30].

One of the important cyclin-dependent kinase inhibitors that induce cell cycle arrest is the cyclin-dependent kinase inhibitor 1A (CDKN1A), which is also referred to as p21. Since this kinase inhibitor can inhibit cell proliferation, it was initially thought that it could be used as a tumor suppressor [31, 32]. After damage to a cell, p53 will directly bind to the CDKN1A locus. Subsequently, it then activates the transcription of CDKN1A, PANDA and LincRNA-p21. p21 is able to mediate gene silencing by recruiting hnRPK, which then promotes apoptosis. Previous studies have examined p53 and demonstrate its ability to promote apoptosis. This is accomplished by transcriptionally activating or by repressing the expression of a panel of pro- and anti-apoptotic proteins [33]. Shi et al. [34] examined aldosterone-induced mesangial cell apoptosis and report that it caused the apoptosis via p53 both *in vitro* and *in vivo*.

Several studies report that depending upon the cell type and context, both the vitamin D receptor (VDR) and p53-signaling can regulate a variety of cellular functions involved in the development of cancer, including proliferation, differentiation, apoptosis and cell survival [35–37]. In addition, activators of the VDR have been shown to exhibit suppressant effects on the RAAS [38]. For example, activation of the VDR and the administration of losartan to block Ang II result in the inhibition of ROS generation [39]. However, none of the previous findings can explain why we found there was an upregulation of Vdr after the systemic administration of aldosterone.

Since our results indicate that Cdkn1a, Pf4 and Vdr were associated with cell death via a NADPH oxidase pathway, we investigated gene expression of Cdkn1a, Pf4 and Vdr using in site hybridization. Cdkn1a, but not Vdr or Pf4, signals were observed in GCL. This finding suggests that Cdkn1a may be associated with RGC death via a NADPH oxidase pathway.

MR is expressed in RGCs and in cells of the INL in the normal retina [19, 40]. So far, it is not clear why systemic administration of aldosterone causes only RGC loss, and not a loss of INL cells. Therefore, further investigation is needed to reveal why aldosterone causes only RGC loss.

The findings of our current study suggest there might be two possible mechanisms associated with the RGC death

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that occurs after systemic administration of aldosterone. First, it is possible that ocular blood abnormalities due to the upregulation of PF4 could be involved in the death of the RGCs. Second, increases in the level of ROS might induce p53 activation as an upstream signal, thereby triggering the apoptosis. Further investigations are needed to clarify the mechanisms of RGC death after the systemic administration of aldosterone. We are currently performing additional studies designed to investigate the retinal blood flow after the systemic administration of aldosterone.

In conclusion, the systemic administration of aldosterone can lead to significant increases and decreases in various genes. Further functional studies on the effects of these genes are needed in order to definitively clarify the molecular mechanisms in the animal NTG model.

Acknowledgements This work was supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science, and Technology of Japan (26462689).

Conflicts of interest A. Ono, None; K. Hirooka, None; Y. Nakano, None; E. Nitta, None; A. Nishiyama, None; A. Tsujikawa, Grant (Alcon, AMO Japan, Bayer, HOYA, Kowa, Novartis, Pfizer, Santen, Senju), Lecture fees (Alcon, AMO Japan, Bayer, Chugai, Kowa, Nidek, Novartis, Pfizer, Santen, Sanwa Kagaku, Senju).

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