Identification of a Retinal Aldosterone System and the Protective Effects of Mineralocorticoid Receptor Antagonism on Retinal Vascular Pathology

Jennifer L. Wilkinson-Berka, Genevieve Tan, Kassie Jaworski, Antonia G. Miller

Abstract—Blockade of the renin–angiotensin–aldosterone system (RAAS) is being evaluated as a treatment for diabetic retinopathy; however, whether the mineralocorticoid receptor (MR) and aldosterone influence retinal vascular pathology is unknown. We examined the effect of MR antagonism on pathological angiogenesis in rats with oxygen-induced retinopathy (OIR). To determine the mechanisms by which the MR and aldosterone may influence retinal angiogenesis; inflammation and glucose-6-phosphate dehydrogenase (G6PD) were evaluated in OIR and cultured bovine retinal endothelial cells (BRECs) and bovine retinal pericytes (BRPs). In OIR, MR antagonism (spironolactone) was antiangiogenic. Aldosterone may mediate the pathogenic actions of MR in the retina, with 11β-hydroxysoy steroid dehydrogenase type 2 mRNA being detected and with aldosterone stimulating proliferation and tubulogenesis in BRECs and exacerbating angiogenesis in OIR, which was attenuated with spironolactone. The MR and aldosterone modulated retinal inflammation, with leukostasis and monocyte chemotactant protein-1 mRNA and protein in OIR being reduced by spironolactone and increased by aldosterone. A reduction in G6PD may be an early response to aldosterone. In BRECs, BRPs, and early OIR, aldosterone reduced G6PD mRNA, and in late OIR, aldosterone increased mRNA for the NAD(P)H oxidase subunit Nox4. A functional retinal MR–aldosterone system was evident with MR expression, translocation of nuclear MR, and aldosterone synthase expression, which was modulated by RAAS blockade. We make the first report that MR and aldosterone influence retinal vasculopathy, which may involve inflammatory and G6PD mechanisms. MR antagonism may be relevant when developing treatments for retinopathies that target the RAAS. (Circ Res. 2009;104:124-133.)

Key Words: aldosterone • mineralocorticoid receptor • retina • angiogenesis • inflammation

Vision loss and blindness are consequences of the pathological angiogenesis that occurs in retinal diseases such as retinopathy of prematurity and diabetic retinopathy.1 The main treatment is laser photoocoagulation, which ablates new blood vessels and the surrounding ischemic tissue.2 However, although this procedure provides benefits, it does not always retard the disease and is damaging to the retina.2 There is considerable interest in developing treatments that target specific angiogenic and inflammatory pathways with the aim of preventing disease progression.

Angiotensin II blockade is a therapeutic candidate for retinopathy, with DIRECT (DIabetic REtinopathy Candesartan Trials) evaluating the efficacy of angiotensin II type 1 receptor (AT1R) blockade (AT1RB) in diabetic patients.3,4 The impetus for such trials is based on experimental studies in which we and others have shown that a renin–angiotensin system exists in the retina5–7 and is upregulated in experimental retinopathy of prematurity and diabetic retinopathy.5–7,8 Furthermore, angiotensin II blockade is antiangiogenic7–9 and antiinflammatory10 and improves retinal function.11 However, angiotensin II blockade alone may not be sufficient to confer complete retinoprotection in a clinical setting, with angiotensin-converting enzyme inhibition slowing but not preventing diabetic retinopathy.12

Aldosterone is a steroid hormone that elicits its effects by binding to the mineralocorticoid receptor (MR) and is released in response to a variety of stimuli including angiotensin II and changes in salt balance. Aldosterone is a potent stimulator of fibrovascular injury in cardiovascular tissues13,14; however, the mechanisms by which aldosterone adversely affects the vasculature are not fully defined. There is evidence that inflammatory and oxidative stress pathways are involved.13,14 Recently, the enzyme glucose-6-phosphate dehydrogenase (G6PD) has also been implicated.15 G6PD is a major source of reduced NAD(P)H and serves to maintain redox balance. In terms of inhibiting the actions of aldosterone, it was previously viewed that angiotensin II blockade may be sufficient; however, aldosterone can still be present because of the phenomenon of “aldosterone escape.”16 Aldosterone may also influence pathology independently of angiotensin II17 and potentiate the actions of angiotensin II via activation of the AT1R and angiotensin-converting enzyme.18,19 In support of these findings are trials such as

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Our aim was to determine whether the MR and aldosterone influence retinal vascular pathology in a rat model of oxygen-induced retinopathy (OIR) that has similarities to retinopathy of prematurity in humans. We evaluated whether the actions of aldosterone in the retina involve G6PD and the nicotinamide adenine dinucleotide phosphate [NAD(P)H oxidase] subunit Nox4. Finally, we examined whether a functional MR–aldosterone system exists in the retina, which can be modulated by the renin–angiotensin–aldosterone system (RAAS).

Materials and Methods

Oxygen-Induced Retinopathy

Procedures complied with the Australian National Health and Medical Research Council (NHMRC) Code of Practice for the Care and Use of Animals for Scientific Purposes. OIR was induced in Sprague–Dawley (SD) rats by exposure between postnatal day (P)0 and P11 to 80% O2 cycled with 20% O2 for 3 hours/day. Rats were then in room air until P18 (angiogenesis period). Shams were in room air from P0 to P18. Groups comprised the following: (1) sham control; (2) OIR control; (3) OIR plus aldosterone–salt; (4) OIR plus aldosterone–salt, Sp; (5) OIR plus aldosterone–salt, Val; (6) OIR plus aldosterone–salt, Sp; (7) OIR plus aldosterone–salt, Val; (8) OIR plus aldosterone–salt, Sp and Val; (9) OIR plus aldosterone–salt, Sp; (10) OIR plus aldosterone–salt, Val; and (11) OIR plus aldosterone–salt, Sp and Val. Treatments were given to P12 and P18. Blood vessel profiles (BVPs) in the inner retina were counted using our previous method. The MR antagonist Sp (25 mg/kg per day, 10% DMSO/90% olive oil; Sigma-Aldrich) and the AT1RB valsartan (10 mg/kg per day, 0.1 mol/L Tris buffer; Novartis Pharma) were administered by subcutaneous injection. Salt is 1% NaCl given to mothers in drinking water. Aldosterone (0.75 μg/hour, 2% DMSO/0.9% saline, Sigma-Aldrich) was administered to pups by miniosmotic pump (100TD, Alzet) inserted in the flank. The dose of aldosterone is based on previous studies, and we found similar increases in plasma aldosterone (OIR control, 39.71 ± 15.61 pmol/L; OIR plus aldosterone–salt, 3280 ± 535.86 pmol/L; P < 0.005).

Real-Time PCR

See the online data supplement and supplemental Table I, available at http://circres.ahajournals.org. Genes of interest were studied in bovine retinal endothelial cells (BRECs), bovine retinal pericytes (BRPs), and OIR at P18. One exception was G6PD in OIR, which was also studied at P12 plus 8 hours (P12.8). This is because the initial stages of OIR are associated with increased growth and inflammatory mediators in response to retinal hypoxia and later subside by P18 when vascular pathology is established. Whether there are temporal changes in G6PD is unknown. On P12, aldosterone was given at 0 and 4 hours at 1 mg/kg body weight (subcutaneous), which is similar to the dose used in miniosmotic pumps in OIR studied at P18. Groups were: (1) sham control (room air only); (2) OIR control; (3) OIR plus salt; and (4) OIR plus aldosterone–salt.

The online data supplement contains information about isolation of BRECs and BRPs, BREC proliferation and tubulogenesis; translocation of MR in BRECs; leukostasis in OIR; MR and monocyte chemotactic protein (MCP)-1 immunohistochemistry; and Western blotting.

Statistics

BVP data were subjected to 1-way ANOVA with a Bonferroni post hoc test. All other data were analyzed by a Kruskal–Wallis test followed by
individual Mann–Whitney U tests between groups. Values are means±SEM. Significance was P<0.05. Investigators were blinded to the groups.

Results

In OIR, Angiogenesis Is Reduced With MR Antagonism and Increased With Low Salt and Aldosterone–Salt

OIR controls (20.09±1.83) exhibited a 1.7-fold increase in BVPs compared with shams (11.63±0.83; Figure 1A and 1B). In OIR, Sp reduced BVPs by 56% (15.0±1.00) compared with OIR control (Figure 1C). In OIR, valsartan reduced BVPs (11.83±0.60) to a greater extent than Sp, lowering BVPs to sham levels. In OIR, aldosterone alone had no effect on BVPs (21.94±1.40). Previous studies indicate that low to moderate dietary salt is required for the pathological effects of aldosterone.24 In OIR, 1% NaCl increased BVPs (28.20±1.70) compared with OIR control. In OIR+salt rats treated with Sp (11.00±1.30) or valsartan (9.56±2.01), BVPs were reduced to a similar extent, and the reduction was similar to sham levels. In OIR, aldosterone–salt had the most marked effect, increasing BVPs by 2-fold (39.47±3.30) compared with OIR control. In OIR+aldosterone–salt, Sp (18.18±1.43) and valsartan (15.75±0.49) reduced BVPs to OIR control (Figure 1E).

Aldosterone Induces Proliferation and Tubule Formation in BRECs

Aldosterone at 100 pmol/L and 1 nmol/L increased BRECs proliferation compared with control (Figure 2A), and the increase was similar to that of VEGF. Aldosterone at 1, 10,
and 100 nmol/L increased tubulogenesis compared with control, and the increase was to a similar extent as VEGF (Figure 2B and 2C). BRECs proliferation and tubulogenesis was reduced with Sp.

**MR Antagonism Reduces Retinal Leukostasis**

In OIR, leukostasis was increased 10.3-fold (6.14 ± 0.9) compared with sham (0.59 ± 0.15; Figure 3A and 3B). In OIR, Sp (3.25 ± 0.57) and valsartan (2.56 ± 0.39) reduced leukostasis to a similar extent, although not to sham levels (Figure 3C). Because aldosterone treatment alone had no effect on retinal angiogenesis, it was not studied. Salt treatment increased leukostasis by 1.8-fold (11.55 ± 1.56) compared with OIR control. In OIR+salt, both Sp (1.97 ± 0.46) and valsartan (1.28 ± 0.26) reduced leukostasis. In OIR+aldosterone–salt, leukostasis was increased to a similar extent as salt alone (14.12 ± 1.34; Figure 3D). Sp (4.59 ± 1.46) and valsartan (1.97 ± 0.34) reduced this increase to OIR control (Figure 3E), with valsartan being more antiinflammatory than Sp.

**Inflammatory Mediators**

MCP-1 mRNA and protein followed a similar pattern as leukostasis. MCP-1 mRNA was increased 7-fold in OIR (7.58 ± 1.26) compared with sham (1.07 ± 0.17; Figure 4A). In OIR, the reduction in MCP-1 mRNA with Sp (4.56 ± 0.90) was not statistically significant compared with OIR control. However, valsartan (0.79 ± 0.32) reduced MCP-1 mRNA to sham (Figure 4A). Salt alone (28.15 ± 5.04) had a marked effect, increasing MCP-1 mRNA 3.7-fold compared with OIR control. Sp (4.05 ± 2.88) and valsartan (3.48 ± 1.87) treatment to OIR+salt animals reduced MCP-1 mRNA to OIR control but not to sham levels. Aldosterone–salt increased MCP-1 mRNA to a similar extent as salt (20.11 ± 3.89), and both Sp (7.13 ± 1.22) and valsartan (4.96 ± 0.64) reduced mRNA to OIR control but not to sham levels (Figure 4A). MCP-1 was detected in blood vessels in the inner retina, ganglion cells, and the inner nuclear layer (Figure 4B and 4C). In general, MCP-1 immunolabeling followed a similar pattern to mRNA. Immunolabeling was weak in shams and increased in OIR and OIR+ALDO–salt; however, immunolabeling tended to be increased in OIR+salt, but this was not statistically significant. In all situations, Sp and valsartan reduced immunolabeling to a similar extent. In OIR+ALDO–salt, this reduction appeared to be largely attributable to a reduction in the number of BVPs. In BRECs and BRPs, aldosterone increased mRNA for intracellular adhesion molecule-1 and cyclooxygenase-2 mRNA (supplemental Figure III).

**G6PD mRNA Is Reduced by Aldosterone**

In BRECs and BRPs, G6PD mRNA was reduced with 100 nmol/L aldosterone (supplemental Figure III). In OIR at P12.8 hours, G6PD mRNA was increased compared with sham control (Figure 5A), and reduced with both salt and aldosterone–salt to below sham levels. In OIR at P18, there was no difference in G6PD mRNA among all groups (Figure 5B).

**Nox4 mRNA Is Increased by Aldosterone**

In OIR at P18, Nox4 mRNA was increased (3.4 ± 0.58) compared with sham (0.98 ± 0.09; Figure 6). In OIR, salt increased Nox4 mRNA (6.08 ± 1.85), but this did not reach statistical significance. In OIR, aldosterone–salt increased Nox4 mRNA (8.06 ± 1.40) compared with OIR control.

**MR Is Expressed in Retina, and in BRECs Is Translocated to the Nucleus by Aldosterone**

MR immunolabeling was localized to ganglion cells, the inner nuclear layer, retinal pigment epithelium, and the vasculature (Figure 7A). MR in vascular cells was confirmed in BRECs and BRPs with immunolabeling (Figure 7B and 7C) and real-time PCR (data not shown). BRECs were
cultured in concentrations of aldosterone used to evaluate cell function and similar to that in plasma of patients with congestive heart failure (Figure 7C and 7D). In BRECs incubated with 100 nmol/L aldosterone, MR immunolabeling was reduced in the cytoplasm (67.41 ± 9.69 control; 57.39 ± 2.71 aldosterone) and increased in the nucleus (6.92 ± 2.76 control; 15.83 ± 2.49 aldosterone) compared with control (Figure 7D).

11β-Hydroxysteroid Dehydrogenase Type 1 and 11β-Hydroxysteroid Dehydrogenase Type 2 Are Present in Retina
Gene expression for the enzymes 11β-hydroxysteroid dehydrogenase type (11β-HSD)1 and 11β-HSD2 were detected in retina from SD rats and in BRECs and BRPs (Figure 8A). In rat retina, 11β-HSD2 was approximately 24-fold lower than rat kidney and adrenal (Figure 8B).

Retinal Aldosterone Synthase mRNA Is Modulated by Low Salt, the MR, and AT1R
Aldosterone synthase mRNA was detected in retina but not increased with OIR (Figure 8C). In OIR, both Sp (0.71 ± 0.09) and valsartan (0.54 ± 0.07) reduced aldosterone synthase mRNA. Compared to controls, in OIR, salt (1.36 ± 0.09) and salt+Sp increased aldosterone mRNA to the same extent, with a further increase in OIR+valsartan (1.88 ± 0.17). To determine whether retinal renin is affected by salt, renin protein was measured. No significant changes in renin protein were observed between OIR (0.82 ± 0.08) and OIR+salt (0.58 ± 0.14) groups. Aldosterone–salt (0.79 ± 0.13) decreased aldosterone synthase mRNA compared with OIR control. In OIR+aldosterone–salt, Sp (0.85 ± 0.05) had no effect compared with OIR+aldosterone–salt, whereas valsartan (0.57 ± 0.08) reduced aldosterone synthase mRNA.
by guest on October 16, 2017

Cardiorenal tissues; however, whether this occurs in established that MR antagonism can reduce vascular injury in aldosterone. It is angiogenesis in retina. An elegant study by Ishida et al has shown that inflammation is a causative event in retinal stasis and MCP-1 mRNA and protein in OIR suggests that the angiogenic effects of aldosterone are accompanied by a reduction in G6PD mRNA and an increase in Nox4 mRNA, a NAD(P)H oxidase subunit that has recently been implicated in angiogenesis. Finally, the demonstration in retina of MR, which is active in vascular cells, and aldosterone synthase expression, which is modulated by Sp and AT\textsubscript{RB}, may indicate that a functional local MR–aldosterone system influences retinal vascular disease.

In general, little is known about the involvement of MR and aldosterone in angiogenesis. In aldosterone-producing adenomas, vascularization is positively associated with aldosterone, and in a corneal micropocket assay and a model of hindlimb ischemia, Sp inhibits angiogenesis. We make the first report that Sp reduces pathological angiogenesis in OIR. Aldosterone is likely to mediate the pathogenic actions of MR in OIR. We found that aldosterone stimulated proliferation and tubulogenesis in BREC\textsubscript{s} and exacerbated angiogenesis in OIR and that these events could be attenuated by Sp. In OIR, the effects of Sp on angiogenesis were compared with AT,RB, which we have reported previously to reduce angiogenesis to sham levels. In the OIR+Sp group, Sp was not as effective as valsartan, although in OIR+aldosterone, Sp was equally antiangiogenic as valsartan. The reasons for these differences are not known but may relate to the dose of Sp. Overall, these findings suggest that MR and aldosterone influence pathological angiogenesis in retina and that MR antagonism may have a potential therapeutic application in retinopathy.

Inflammation is an important mechanism that contributes to retinal vascular remodeling and, most notably, OIR. Given that aldosterone is a potent stimulator of inflammation, and recent evidence indicating that MR modulates the adhesion of leukocytes to endothelial cells, we speculated that MR antagonism might influence retinal inflammation. We identify that in OIR, Sp reduced retinal leukostasis to an extent similar to the comparator valsartan. Consistent with this finding was that aldosterone increased the expression of the inflammatory mediators, intercellular adhesion molecule-1, and cyclooxygenase-2 in BREC\textsubscript{s} and BRPs. A proinflammatory role for aldosterone also occurred in OIR, with aldosterone–salt increasing leukostasis, which was reduced with Sp. Evidence that the chemokine MCP-1 is increased in the ischemic retina and leads to macrophage recruitment and subsequent retinal angiogenesis led us to study aldosterone and the effects of Sp on retinal MCP-1 expression. We found retinal MCP-1 mRNA and protein to be modulated by aldosterone, with aldosterone–salt increasing MCP-1, which was reduced by Sp. Together, these results
suggest a pathogenic role for MR-aldosterone in retinal inflammation.

Leopold et al.15 have identified that aldosterone impairs vascular function by reducing G6PD. Consistent with these findings, aldosterone reduced G6PD mRNA in BRECs and BRPs. The early stages of OIR features a dramatic increase in angiogenic and inflammatory factors as an initial response to retinal hypoxia.23 Here, in the early stages of OIR at P12.8, G6PD mRNA was also increased, and consistent with the findings in retinal vascular cells, G6PD mRNA was reduced by aldosterone–salt. In the later stages of OIR at P18, when retinal angiogenesis and inflammation have peaked, G6PD mRNA returned to OIR levels following aldosterone–salt treatment, suggesting that the aldosterone–G6PD axis is most active in the initial stages of OIR.

NAD(P)H oxidase is a multicomponent enzyme that, in vascular tissues, is a major source of reactive oxygen species.36 Separate lines of evidence indicate that the Nox4 subunit of NAD(P)H oxidase is stimulated by hypoxia37 and the RAAS38 and is involved in angiogenesis, with increased expression on newly formed blood vessels.28 For these reasons, we evaluated Nox4 expression in response to aldosterone in OIR. We report that Nox4 mRNA is increased in OIR and that the enhanced pathological angiogenesis that occurs with aldosterone–salt treatment is accompanied by a further increase in Nox4 mRNA. In OIR, salt treatment alone did not increase Nox4 mRNA or angiogenesis to the same extent as aldosterone–salt, suggesting that the combination of aldosterone and salt is required to exacerbate new blood vessel growth in OIR. These findings indicate that Nox4 may participate in aldosterone-related pathological angiogenesis in the ischemic retina.

Accumulating evidence indicates that in addition to some epithelial tissues, nonepithelial tissues also express MR and respond to aldosterone.15,34 Few studies have evaluated the existence of a retinal MR–aldosterone system. We confirm a previous report19 that the MR is present in retina and extend this to reveal that retinal MR is active, with translocation of this nuclear receptor from the cytoplasm to the nucleus in response to aldosterone.40 Aldosterone synthase is the rate-limiting enzyme in aldosterone production. In the adrenal gland and cardiovascular tissues, angiotensin II stimulates aldosterone synthase via the AT1R41 and AT1RB reduces aldosterone-related pathology.18,19 We make the first report

Figure 7. A through C, MR immunolabeling in SD rat retina (A), BRPs (B), and BRECs (C). D, Nuclear translocation of MR in BRECs in response to aldosterone (ALDO). A, Paraffin section (3 μm) shows MR immunolabeling (Alexa red) in the ganglion cell layer (GCL), inner nuclear layer (INL), and retinal pigment epithelium (RPE). Scale bar=90 μm. B, a, In BRPs, MR immunolabeling is detected (Texas red). b, Overlayed with DAPI nuclear counterstain (blue) in merged image. Magnification, ×40. C, In BRECs, fluorescein isothiocyanate–lectin identifies endothelial cells (green) (a and c), and MR immunolabeling is shown (Texas red) with DAPI nuclear staining (blue) in merged images (b and d). b, BRECs incubated in control media show MR in both the cytoplasm and nucleus. d, BRECs incubated with 100 nmol/L ALDO show increased MR immunolabeling giving nuclei a purple appearance. Magnification, ×40. D, BRECs cultured in control media, 10 nmol/L ALDO, or 100 nmol/L ALDO for 1 hour. Reduced MR immunolabeling in the cytoplasm with 10 nmol/L ALDO compared with control (aP<0.05, bP<0.01).

(©P<0.05). MR immunolabeling is increased in nuclei and decreased in cytoplasm with 100 nmol/L ALDO compared with control (©P<0.05, ©P<0.01).
that a similar situation occurs in OIR, with AT1RB reducing both aldosterone synthase mRNA– and aldosterone–salt–associated inflammation and angiogenesis. Sp had a similar effect, indicating a role for the MR in the regulation of retinal aldosterone. Given these findings, it is possible that aldosterone may be produced within the retina. However, because exogenous aldosterone also influenced retinal pathology, the contribution of circulating aldosterone cannot be overlooked.

Sodium chloride is the best-known adjunct to the actions of aldosterone stimulating angiotensin II and thereby increasing aldosterone production. Without ample salt, aldosterone has minimal effects on organ pathology. We found a similar situation in OIR, with aldosterone alone having no effect, whereas aldosterone–salt potentiated OIR. Previous studies indicate that a salt diet itself can induce pathology with 1% NaCl treatment for 8 weeks in rats causing cardiac hypertrophy.26 In OIR, salt slightly increased angiogenesis but had a dramatic effect on inflammation, increasing leukostasis and MCP-1 mRNA to a similar extent as aldosterone–salt. These findings may indicate that because OIR normally features an intense inflammatory reaction,27 the retina is particularly sensitive to subsequent salt-induced inflammatory pathways, which once reaching a certain level cannot be further stimulated by aldosterone. Our data would also indicate that salt-induced retinal pathology involves the MR and AT1R, with Sp and valsartan reducing inflammation and angiogenesis. Previous studies suggest that salt may induce pathology via local increases in tissue aldosterone.24 This may also occur in OIR, with salt increasing retinal aldosterone synthase but not altering renin protein. However, the interaction between the AT1R and retinal aldosterone synthase in response to salt in OIR is less clear, with valsartan increasing aldosterone synthase mRNA yet improving OIR. On the other hand, aldosterone–salt in OIR reduced aldosterone synthase mRNA despite increasing pathology. The reasons for these differential responses are unclear and likely to be complex given that a number of factors influence aldosterone synthase expression including blood pressure and cortisol42 and, perhaps, non-RAAS pathways. Furthermore, it is possible that in the circumstance of elevated aldosterone caused by coadministration of aldosterone and salt, retinal angiotensin II down-regulates aldosterone synthase production to curtail further pathology. This could be determined by measuring tissue levels of angiotensin II and aldosterone; however, because of the small sample size of rat pup retina, this was not feasible.

In conclusion, we make the major finding that MR antagonism improves pathological angiogenesis in OIR and that this may involve the suppression of aldosterone-induced inflammatory pathways and modulation of factors such as G6PD and Nox4. We also report that a local MR-aldosterone system exists in retina, which can be modulated by MR antagonism and AT1RB. Of possible importance with respect to treatment strategies for retinopathies, including diabetic
retinopathy, that is MR antagonism and AT,RB have similar vasculoprotective effects in retina. This may indicate the potential for combination therapy in patients, a strategy that has proven successful for cardiovascular disease.20,21

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Disclosures
None.

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Correction to: Identification of a Retinal Aldosterone System and the Protective Effects of Mineralocorticoid Receptor Antagonism on Retinal Vascular Pathology

In the article by Wilkinson-Berka et al, “Identification of a Retinal Aldosterone System and the Protective Effects of Mineralocorticoid Receptor Antagonism on Retinal Vascular Pathology,” which published in the January 2, 2009 issue of the journal (Circ Res. 2009;104:124–133. DOI: 10.1161/CIRCRESAHA.108.176008.), a correction was needed.

In response to an enquiry, Monash University identified an error in authorship attribution involving the above article published in Circulation Research. Following University processes, it was determined that Dr Jacqueline Harbig warrants authorship as fourth author on the article, and the lack of attribution Dr Harbig received occurred through an oversight.

The author list has been updated and corrected in the online version of the article, which is available at http://circres.ahajournals.org/content/104/1/124.
Supplemental Material

Isolation of Bovine Retinal Endothelial Cells (BRECs) and Bovine Retinal Pericytes (BRPs)

BRECs were maintained in DMEM, 10% horse serum, 0.9KU heparin, 1x insulin transferrin-selenium, 15µg/ml endothelial growth cell supplement and antibiotic/antimycotic (Sigma-Aldrich, NSW, Australia). BRECs were sorted to 95% purity by DiI-Ac-LDL uptake and FACS (FACSAria, BD) and purity further confirmed with von Willebrand factor (Dako, NSW, Australia) immunolabelling. BRPs were maintained in low glucose DMEM, 10% FBS (Invitrogen, VIC, Australia) and antibiotic/antimycotic, and purity confirmed by positive α-smooth muscle actin immunolabelling (Dako) and negative DiI-Ac-LDL uptake and von Willebrand factor immunolabelling. Passage numbers were between 3 to 6 for BREC and 3 and 7 for BRP. Unless otherwise stated, BRECs and BRPs were incubated in 0.5% and 2% serum-containing media respectively. In experiments with aldosterone (Sigma-Aldrich), the DMSO vehicle was added to controls (<5mM). All treatments were in triplicate and the experiment repeated three-times.

BREC Proliferation and Tubulogenesis

For tubulogenesis, BRECs were cultured in DMEM/1% horse serum containing either 1, 10, or 100nM aldosterone, 1 and 10nM aldosterone in the presence of 0.5µM spironolactone or vascular endothelial growth factor (VEGF, 20ng/ml) were seeded on-top of polymerised Matrigel (BD, 1:1 in DMEM) at 2 x 10^4 cells/well in a 96 well plate. Plates were incubated at 37°C/5% CO₂ for 4.5 hours. Eight fields per well were captured using a Nikon Eclipse TE2000-U microscope interfaced with NIS-Elements software (F2.20). Tubule area per well was quantitated using AIS software 6.0 (Analytical Imaging System, Ontario, Canada).
**Proliferation and Translocation of the MR in BRECs**

Proliferation was determined by MTT assay. For translocation experiments, BRECs were cultured on 0.1% fibronectin-coated coverslips in the presence or absence of aldosterone for 1 hour. Cells were fixed in 4% paraformaldehyde, permeabilized with 80% methanol/phosphate buffered saline (PBS), blocked with 3% non-fat dry milk/1% bovine serum albumin/PBS, and incubated with a mouse anti-rat antibody to MR (1:50, Abcam, NSW, Australia) and lectin-FITC (1:100, Sigma-Aldrich) in 0.1% BSA/PBS. Goat anti-mouse IgG-Texas Red (1:400, Invitrogen) was applied after washing with PBS. Cells were then incubated in 300nM DAPI (Invitrogen) and mounted onto slides. Eight random fields (200, typical cell number) were photographed per coverslip using a Leica DC300 microscope interfaced with IM50 software, and nuclear translocation quantitated.

**Leukostasis**

Leukostasis was performed using a modified method. Briefly, animals were perfused via the right atrium with 0.1M PBS (10mL) to clear blood cells. Rhodamine-coupled Concanavalin A lectin (25mg/kg, Vector Laboratories, Inc., CA, USA; 4mL) was perfused to stain adherent leukocytes and the endothelium. Eyes were fixed in 4% paraformaldehyde in PBS for 30 minutes, and retinae flat-mounted. The vasculature was observed using an Olympus BX51 fluorescent microscope fitted with a rhodamine filter and Olympus U-RFL-T light source. Non-overlapping images were captured and the number of leukocytes per retina counted.

**Immunohistochemistry**

After anaesthesia, eyes were enucleated and fixed in paraffin. Six sections (3-µm) per animal were randomly selected, dewaxed in histolene and hydrated in graded ethanol. Antigen retrieval was performed by microwaving in 0.1M citrate buffer for 10 minutes. After washing in
0.1M PBS, sections were incubated with 10% normal goat serum in 0.1M PBS for 1 hour, then incubated overnight with a 1:1000 mouse monoclonal antibody to either MR (Abcam) or 1:150 MCP-1 (R&D Systems). For MR immunolabelling, sections were washed in 0.1M PBS, sections were incubated with 1:200 rabbit anti-mouse IgG Alexa-Fluor 568 (Molecular Probes, OR, USA) for 1 hour, and then washed and mounted with coverslips using Dako fluorescent mounting medium. For MCP-1 immunolabelling, sections were washed in 0.1M PBS for and incubated with a biotin-conjugated goat-anti-rabbit IgG (1:200; Vector Laboratories), then washed in 0.1M PBS and incubated with Vectastain ABC Standard Kit (Vector Laboratories) and Liquid DAB+substrate chromagen system (Dakocytomation). The sections were washed in H₂O, counterstained with Harris’ Haematoxylin for 2 minutes, rinsed in H₂O, dehydrated through graded ethanols, cleared in histolene and coverslipped with DPX (VWR International Ltd., Poole, England). Negative controls were sections in which either the primary or secondary antibodies were omitted to detect non-specific staining. Immunolabelling was quantitated in 4 randomly chosen sections per eye, which were at least 60µm apart. Four non-overlapping fields from each section were captured using the Spot digital camera (SciTech) at x20 magnification, and the Analytical Imaging System software used to detect brown immunolabelling in the ganglion cell layer, inner limiting membrane and on any blood vessels protruding into the vitreous that were attached to the retinal surface. Results are expressed as immunolabelling per proportional field of inner retina.

Real-time PCR
For retina, total RNA was isolated using an RNeasy kit (Qiagen, CA, USA). One µg of RNA was subjected to DNase treatment (DNA-free kit, Applied Biosystems, CA, USA) and reverse transcription-PCR (First Strand cDNA Synthesis Kit, Roche, Basel, Switzerland). Primer design for real-time PCR was conducted using Primer Express 2.0 (Applied Biosystems). Primers, probes (Online Table I) and cDNA were mixed with Taqman Universal Master Mix (Applied
Biosystems) and real-time PCR conducted using an ABI 7900 HT Sequence Detection System (Applied Biosystems). mRNA was normalized to 18s rRNA endogenous control and the relative fold difference in expression calculated using the $2^{-\Delta\Delta CT}$ method. For cell culture, total RNA was harvested using Trizol (Invitrogen) DNA removal, reverse transcription and real-time PCR was conducted as described. For $11\beta$ HSD1 and 2 detection, SYBR green based real time PCR was conducted and the resulting products resolved on a 4% agarose gel and imaged using a 1000 Ultraviolet Documentation System (Bio-Rad, Hercules, USA). In addition, PCR products were subjected to DNA sequencing (Micromon, Monash University, Clayton, Australia).

**SDS-PAGE and Western Blotting**

Retina were homogenized according to the method of Heeneman et al. Twenty µg of protein was separated on 12% SDS-PAGE gels and transferred to nitrocellulose membranes. Membranes were blocked in 5% non-fat dried milk (NFDM)/PBS and then incubated with rabbit anti-renin diluted 1:2000 in 5% NFDM/PBS overnight at 4°C. After washing with PBS/0.05% Tween-20 (PBS/T), membranes were incubated with HRP-conjugated anti rabbit-IgG (Cell Signalling) in PBS/T, washed, incubated with West Pico SuperSignal Chemiluminescent stain (Pierce) and exposed to X-ray film (Fuji). The membrane was stripped and re-probed with the antibody against, β-actin (Novus Biologicals), and processed as described above. Bands were quantitated using ImageJ (NIH software), and normalized to b-actin levels.
Online Figure I. Three-µm paraffin sections of retina from SD rats with OIR at P18. ALDO, aldosterone. Val, valsartan. Salt is 1% NaCl. Stain, haematoxylin and eosin. Ganglion cell layer (GCL), inner plexiform layer (IPL). Scale bar=50 µm. A. In OIR+Val, BVPs (arrows) were reduced compared to OIR control. B. In OIR+salt, BVPs (arrowhead) were increased compared to OIR control. In OIR+salt, both Sp (C) and Val (D) reduced BVPs. E. In OIR+ALDO-salt, Val reduced BVPs (arrow) to OIR but not to sham control.
Online Figure II. Flatmounts of retina showing leukostasis in SD rats with OIR at P18. Salt is 1% NaCl. ALDO, aldosterone. Val, valsartan. Scale bar=80µm. A. In OIR+Val, adherent leukocytes (arrow) were reduced compared to OIR control. B. In OIR+salt, leukostasis (arrows) was increased compared to OIR control. In OIR+salt, both Sp (C) and Val (D) reduced leukostasis (arrows). E. In OIR+ALDO-salt, Val reduced leukostasis (arrows) to sham levels.
Online Figure III. Gene expression in BRECs and BRPs with real-time PCR for ICAM-1, COX-2 and G6PD. A and B. 100nM ALDO increased ICAM-1 and COX-2 mRNA compared to control (\(^aP<0.05\)). C. 100nM ALDO reduced G6PD mRNA (\(^aP<0.05\)).
### Online Table I. Real-time PCR primers and probes

<table>
<thead>
<tr>
<th>Gene</th>
<th>Species</th>
<th>Accession number</th>
<th>Primers and Taqman probe (MGB, FAM label)</th>
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<tbody>
<tr>
<td>MCP-1</td>
<td>rat</td>
<td>M57441</td>
<td>Forward: 5’GCTGTAGTATTTGGTCACCAGCTCAA3’&lt;br&gt;Reverse: 5’GTACCTCTGGGACCATTCTTTATTG3’&lt;br&gt;Probe: AGAGAGATCTGTGCTGACC 3’</td>
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<td>Aldosterone synthase</td>
<td>rat</td>
<td>NM_012538</td>
<td>Forward: 5’CCCAGAAGGGCCATGTCAGA3’&lt;br&gt;Reverse: 5’CAGGGTAGAGTCATCAAGGCTCTTTAAA3’&lt;br&gt;Probe: 5’CCCTTGCTGCGGGC 3’</td>
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<tr>
<td>MR</td>
<td>rat</td>
<td>M36074</td>
<td>Forward: 5’AAACGTGTCAGCTCTACTTACGAA3’&lt;br&gt;Reverse: 5’GTGACACCAGAAGGCTCTAC3’&lt;br&gt;Probe: 5’AGACCTTTCAAGATCTGTTT 3’</td>
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<td>MR</td>
<td>bovine</td>
<td>XM_617787</td>
<td>Forward: 5’CCGGGATTTTTACCATCTGACAT3’&lt;br&gt;Reverse: 5’CCGTGGCCAAAGTTCCCT3’&lt;br&gt;Probe: 5’ACCGAGCTGGAATC3’</td>
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<td>COX-2</td>
<td>bovine</td>
<td>AF031698</td>
<td>Forward: 5’TCAGATGATTAATGGGAGAGATGTAC3’&lt;br&gt;Reverse: 5’AGGCAGGTTGAGATCTTCCGA3’&lt;br&gt;Probe: 5’CCACAGTCAAAGATACTCA3’</td>
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<td>ICAM-1</td>
<td>bovine</td>
<td>U65789</td>
<td>Forward: 5’ GACATCGAGCTGTTTTGCTACTCA3’&lt;br&gt;Probe: 5’ACAATAGCTTCAATGAAC3’&lt;br&gt;Reverse: 5’CATGCTCCGGGAACCAGTATA3’</td>
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<td>Nox4</td>
<td>rat</td>
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<td>Forward primer: 5’CTGGACAGAAGAGATTTCCGAGATTT3’&lt;br&gt;Reverse primer: 5’GAATGAGGGGCAGAATCTACGAA3’&lt;br&gt;Probe: 5’CTACTGCTCCCATCAAGAA3’</td>
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<td>18s</td>
<td>eukaryote</td>
<td>X03205.1</td>
<td>Applied Biosystems gene expression assay – endogenous control – VIC label</td>
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<td>Forward: 5’CCTCCGTGAGTTCGTTGCT’&lt;br&gt;Reverse: 5’ACTTCAGACCTGACCTTCA3’</td>
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<td>11β−HSD1</td>
<td>rat</td>
<td>NM_017080</td>
<td>Forward: 5’TTCAGAGCATGTTGTTG’&lt;br&gt;Reverse: 5’TGATGAGTTGAGATGATGCTG3’</td>
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<td>11β−HSD1</td>
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<td>AF176811</td>
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</table>

MCP-1, monocyte chemoattractant protein. MR, mineralocorticoid receptor. COX-2, cyclooxygenase-2. ICAM-1, intracellular adhesion molecule-1. G6PDH, glucose-6-phosphate dehydrogenase. 11β−HSD1, 11β−hydroxysteroid dehydrogenase 1. 11β−HSD2, 11β hydroxysteroid dehydrogenase 2.
References


Correction

Correction to: Identification of a Retinal Aldosterone System and the Protective Effects of Mineralocorticoid Receptor Antagonism on Retinal Vascular Pathology

In the article by Wilkinson-Berka et al, “Identification of a Retinal Aldosterone System and the Protective Effects of Mineralocorticoid Receptor Antagonism on Retinal Vascular Pathology,” which published in the January 2, 2009 issue of the journal (Circ Res. 2009;104:124-133. DOI: 10.1161/CIRCRESAHA.108.176008.), a correction was needed.

In response to an enquiry, Monash University identified an error in authorship attribution involving the above article published in Circulation Research. Following University processes, it was determined that Dr Jacqueline Harbig warrants authorship as fourth author on the article, and the lack of attribution Dr Harbig received occurred through an oversight.

The author list has been updated and corrected in the online version of the article, which is available at http://circres.ahajournals.org/content/104/1/124.