*Critical Review***Possible Underlying Mechanisms Responsible for Aldosterone and Mineralocorticoid Receptor–Dependent Renal Injury**Hideyasu Kiyomoto¹, Kazi Rafiq², Mahbub Mostofa³, and Akira Nishiyama^{2,*}¹Department of CardioRenal and Cerebrovascular Medicine, and ²Department of Pharmacology, Faculty of Medicine, Kagawa University, 1750-1 Ikenobe, Miki-cho, Kita-gun, Kagawa 761-0793, Japan³Department of Pharmacology, Bangladesh Agricultural University, Mymensingh, Bangladesh

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Abstract. There is increasing evidence indicating the roles of aldosterone and mineralocorticoid receptor (MR) in the pathogenesis of renal injury. In rats, chronic treatment with aldosterone and salt results in severe proteinuria and renal tissue injury, characterized by glomerulosclerosis and tubulointerstitial fibrosis. Aldosterone-induced renal tissue injury is associated with increases in reactive oxygen species (ROS) levels and activation of mitogen-activated protein kinases (MAPKs) or Rho-kinase. Treatment with a selective MR antagonist, eplerenone, prevents aldosterone-induced increases in ROS levels and MAPK activity and ameliorates renal injury. In vitro studies have revealed that MR is highly expressed in glomerular mesangial cells (RMCs), podocytes, and renal interstitial fibroblasts. In these renal cells, aldosterone induces cellular injury through NADPH oxidase–dependent ROS production and activation of MAPKs or Rho-kinase. Such aldosterone-induced renal cellular injury is markedly attenuated by treatment with eplerenone. These data suggest that aldosterone induces renal injury via activation of MR through mechanisms that cannot be simply explained by changes in blood pressure. In this review, we summarized recent findings on the roles of aldosterone and MR in the pathogenesis of renal injury with particular emphasis on potential underlying mechanisms.

Keywords: aldosterone, mineralocorticoid receptor, reactive oxygen species (ROS), extracellular signal–regulated kinases (ERK) 1/2, eplerenone, kidney

Introduction

Aldosterone is recognized as a steroid hormone that regulates body fluid homeostasis (1, 2); however, accumulating evidence suggests that aldosterone is a key factor in mediating cardiovascular injury (3). Patients with primary aldosteronism have a higher incidence of cardiovascular complications than do patients with essential hypertension (4). The Randomized Aldactone Evaluation Study (RALES) revealed that adding a non-specific mineralocorticoid receptor (MR) antagonist, spironolactone, to standard therapy, including

angiotensin-converting enzyme (ACE) inhibitors, significantly reduces morbidity and mortality in patients with moderate to severe heart failure (5). More recently, the Eplerenone Post-Acute Myocardial Infarction Heart Failure Efficacy and Survival Study (EPHESUS) showed that adding a selective MR antagonist, eplerenone, to optimal medical therapy reduces morbidity and mortality among patients with acute myocardial infarction complicated by left ventricular dysfunction and heart failure (6). Based on the results from these two multicenter clinical analyses and other clinical studies (7, 8), most national guideline groups now recommended MR antagonists in preference to other anti-hypertensive agents in hypertensive patients with heart diseases (9–11). However, recent studies have also indicated the potential roles of aldosterone and MR in the pathogenesis of renal injury. For example, patients with primary aldosteronism have higher incidence of

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proteinuria or albuminuria than do patients with essential hypertension (4, 12, 13). Plasma aldosterone levels are positively correlated with urinary protein excretion levels and negatively correlated with glomerular filtration rate in patients with primary aldosteronism (14), chronic kidney disease (CKD), and diabetic nephropathy (15). Furthermore, addition of MR antagonists to either ACE inhibitors or angiotensin-receptor blockers (ARBs) reduces albuminuria in patients with type 2 diabetic nephropathy (16) or CKD (17–19). In this review, we have summarized recent evidence regarding the roles of aldosterone and MR in the pathogenesis of renal injury with particular emphasis on their possible underlying mechanisms.

Localization of MR in the kidney

It has been shown that chronic administration of aldosterone with salt loading to rats elicits proteinuria, glomerular mesangial injury, and tubulointerstitial fibrosis (20, 21). Since these aldosterone-induced renal injuries were prevented by treatment with MR antagonists, it is possible that aldosterone directly induces renal tissue injury via activation of locally expressed MR. Based on the observations in aldosterone-infused rats (20–24), we investigated the existence of MR in cultured rat mesangial cells (RMCs) (25) and renal fibroblasts (26) by Western blotting and real-time RT-PCR. An MR-specific antibody detected significant MR protein expression in RMCs and renal fibroblasts with a band of approximately 110–120 kDa, which corresponds to the molecular weight of rat MR in tissues (25, 26). We also investigated the subcellular localization of MR in RMCs (25). In fluorolabeling experiments using confocal microscopy, MR protein expression was observed in both the cytoplasm and nuclear fraction with barely detectable levels in the membrane fraction (Fig. 1A). These observations were consistent with those by Terada et al. (27) that MR translocation from the cytoplasm to the nucleus is induced by treatment with aldosterone in RMCs. Recently, Nishikawa et al. (28) have reported that human glomerular mesangial cells produced aldosterone via steroidogenic enzymes such as P450scc, 3 β -hydroxysteroid dehydrogenase, 21-hydroxylase, and CYP11B2. Therefore, it seems likely that locally produced aldosterone stimulates the transcription rates of mineralocorticoid-responsive genes by activating MR in mesangial cells (28). MR expression was also detected in podocytes (24, 29) and renal tubular cells (20, 30, 31). Taken together, MR is localized not only in distal tubular cells but also in other renal cells, which mediate aldosterone-induced renal tissue injury.

Renal tissue injury induced by chronic infusion of aldosterone and its possible mechanisms

Chronic administration of aldosterone with salt supplement to uninephrectomized (24, 32–35) or nonnephrectomized (23) rats caused hypertension with proteinuria and podocyte abnormality (Fig. 1B). Aldosterone treatment also induces severe glomerular injury and tubulointerstitial fibrosis (23) (Fig. 1C). Increases in multiple factors, including MAPKs (23), Rho-kinase (35), plasminogen activator inhibitor-1 (36–38), transforming growth factor- β 1 (33, 35, 36), connective tissue growth factor (33, 35), proinflammatory cytokines such as osteopontin (32, 36) and monocyte chemoattractant protein-1 (32, 35), and reactive oxygen species (ROS) (23, 24), have also been observed in renal tissues of aldosterone-infused rats. Among those factors, ROS production may be an important mediator of aldosterone-induced renal injury (23, 24). We have shown that aldosterone/salt-induced renal injury is associated with increases in thiobarbituric acid reactive substances (TBARS) contents, a marker of ROS production (Fig. 2A), and in mRNA levels of NADPH oxidase components, p22phox, Nox-4, and gp91phox in renal cortical tissue (23). Treatment with eplerenone suppressed aldosterone-induced increases in TBARS levels (Fig. 2A) and NADPH oxidase expression. Interestingly, tempol, a superoxide dismutase mimetic, normalized renal cortical TBARS levels (Fig. 2A) and prevented progression of proteinuria and renal tissue injury (Fig. 1C) in these animals (21, 23). These data suggest that ROS production is essential in the progression of renal injury induced by aldosterone.

Shibata et al. (24) showed that chronic infusion of aldosterone with high-salt treatment induced hypertension with massive proteinuria and glomerular podocyte injury in uninephrectomized rats. They also showed that glomerular expressions of nephrin and podocin, slit diaphragm-associated molecules, were markedly reduced, whereas expression of desmin, a damaged podocyte marker (39), was upregulated in uninephrectomized aldosterone-infused rats. Treatment with tempol or eplerenone significantly reduced oxidative stress markers and attenuated podocyte damage and proteinuria in aldosterone-infused rats (24). Moreover, both tempol and eplerenone have been shown to markedly attenuate salt-induced podocyte injury and proteinuria in other rodent models of hypertensive glomerulosclerosis (29, 40, 41). These findings suggest that aldosterone-induced podocyte injury underlies the pathogenesis of proteinuria, possibly through MR activation and ROS production (20, 21).

We have shown that in aldosterone-infused rats,

Fig. 1. Localization of mineralocorticoid receptor (MR) and aldosterone-induced renal injury in rats. A: Distribution of mineralocorticoid receptor (green) and $\alpha 1$ -integrin (red) staining in cultured rat mesangial cells (RMCs). Superimposition of the image does not reveal any areas of co-localization of MR and $\alpha 1$ -integrin (known as a membrane marker), indicating a lack of expression of MR in the cell membrane of RMC. Data are modified from Ref. 25 with permission. B: Glomerular histological findings for podocyte ultrastructure (original magnification, $\times 3000$). Rats treated with aldosterone/1% NaCl exhibit podocyte abnormality (arrow). C: Photomicrographs of glomeruli (periodic acid-Schiff stain, original magnification $\times 400$). Rats treated with aldosterone/1% NaCl exhibit glomerular sclerosis. Treatment with eplerenone or tempol markedly ameliorates the changes induced by aldosterone/1% NaCl. Data are from Ref. 23 with permission.

Fig. 2. Effects of aldosterone on reactive oxygen species production in rats and RMCs. A: Thiobarbituric acid reactive substances (TBARS) contents in renal cortical tissues. Rats treated with aldosterone/1% NaCl show increases in TBARS levels in renal cortical tissues. Treatment with eplerenone or tempol markedly prevents aldosterone-induced increases in TBARS levels. $*P < 0.05$ vs vehicle-treated rats. Data are from Ref. 23 with permission. B: Effects of aldosterone on dihydroethidium staining in RMCs. Eplerenone (10 μ mol/L) significantly attenuated aldosterone-induced (100 nmol/L for 3 h) increases in dihydroethidium staining. Original magnification, $\times 1600$. Data are modified from Ref. 43 with permission.

Fig. 3. Effects of aldosterone and eplerenone on NADPH oxidase activity and membranous translocation of p47phox and p67phox in RMCs. **A:** Effects of aldosterone on NADPH oxidase activity in RMCs. Eplerenone (10 μ mol/L) significantly attenuated aldosterone (100 nmol/L)-induced increases in NADPH oxidase activity. **B:** Effects of aldosterone on membranous translocation of p47phox and p67phox in RMC. Eplerenone significantly attenuated aldosterone-induced membranous translocation of p47phox and p67phox. V = vehicle. Data are modified from Ref. 43 with permission.

renal injury is associated with activation of MAPKs: extracellular signal-regulated kinase (ERK) 1/2, c-Jun N-terminal, and big MAPK-1 in renal cortical tissues, but not p38 MAPK (23). These data suggest the MAPKs are involved in aldosterone-dependent renal injury. However, effects of specific inhibition of MAPKs have not been examined in aldosterone-infused rats. The small GTP-binding protein RhoA has recently been proposed as another candidate for mediating hypertensive glomerulosclerosis (35, 42). We demonstrated that a specific Rho-kinase inhibitor, fasudil, attenuated aldosterone-induced TGF- β activation and ameliorated the progression of renal injury in rats (35). Further studies are needed to clarify the precise molecular mechanisms of aldosterone/MR-induced renal injury.

Renal cell injury induced by aldosterone and its possible mechanisms

As mentioned above, renal injury in aldosterone-infused rats is associated with increases in NADPH expression and ROS levels (23). The finding that aldosterone-induced renal injury was attenuated by treatment with tempol indicates that ROS are an important mediator of aldosterone-dependent renal injury (23, 24). These observations prompted us to investigate whether aldosterone and MR have direct

effects on NADPH oxidase activation and superoxide anion (O_2^-) generation in cultured RMCs. We found that aldosterone directly stimulates O_2^- generation in RMCs (Fig. 2B). Aldosterone-induced O_2^- production was accompanied by an increase in NADPH oxidase activity (Fig. 3A) and translocation of p47phox and p67phox, cytosol components of NADPH oxidase, to the RMC membrane (Fig. 3B) (43). These findings are consistent with the hypothesis that aldosterone stimulates ROS generation through NADPH oxidase-dependent mechanisms (21). Recent studies show that aldosterone induces mesangial cell apoptosis and that administration of antioxidants, free radical scavengers, or MR blockers partially attenuates the proapoptotic effects of aldosterone (22).

In rats, aldosterone-induced renal injury is associated with increased activity of ERK1/2 (23) and Rho-kinase (35). In cultured mesangial cells (25, 27, 44) and renal fibroblasts (26), aldosterone activates ERK1/2, and its activation is reversed by treatment with spironolactone or eplerenone. We confirmed that eplerenone or the inhibition of the ERK1/2 cascade with PD98059 abolishes aldosterone-induced cell proliferation and deformability in RMCs (25) and collagen synthesis in renal fibroblasts (26). These data support the possible contributions of MAPKs to aldosterone-induced renal cell injury. However, the specific relationship between

Fig. 4. Effects of aldosterone and eplerenone on extracellular signal-regulated kinase (ERK) 1/2 phosphorylation in RMCs. **A:** Effects of actinomycin D (AD) and cycloheximide (CH) on aldosterone (100 nmol/L)-induced ERK 1/2 phosphorylation in RMCs. Aldosterone-induced ERK1/2 phosphorylation is not altered by pretreatment with AD (5 $\mu\text{g}/\text{mL}$) or CH (10 $\mu\text{g}/\text{mL}$). V = vehicle. Data are modified from Ref. 25 with permission. **B:** Effects of eplerenone on aldosterone-induced ERK 1/2 phosphorylation in RMCs. Results were normalized by arbitrarily setting the densitometry of vehicle-treated cells to 1.0. Aldosterone-induced ERK1/2 phosphorylation is dose-dependently inhibited by treatment with eplerenone. * $P < 0.05$ vs control (0 nmol/L of aldosterone). Data are from Ref. 25 with permission.

ROS and MAPKs in mediating renal cell injury remains unknown. Furthermore, the role of Rho-kinase in aldosterone-induced renal cell injury is under investigation. The roles of other factors including transforming growth factor- β , plasminogen activator inhibitor type 1, and osteopontin in mediating renal fibroblast proliferation, collagen synthesis, or matrix degradation have also been indicated by recent studies (45, 46).

In addition to its classical genomic actions mediated through the regulation of nuclear gene transcription and protein synthesis, aldosterone elicits rapid responses probably via the non-genomic pathways in a variety of cells (47–50). Rapid activation of ERK1/2 was observed with a peak at 5–10 min after stimulation of aldosterone in RMCs (25) and renal fibroblasts (26). We also found that aldosterone activated ERK1/2 in both RMCs and renal fibroblasts under the condition where *de novo* synthesis of proteins was inhibited by treatment with actinomycin D and/or cycloheximide (Fig. 4A) (25, 26). These data suggest the involvement of non-genomic mechanisms in aldosterone-induced activation of signal transduction pathways in renal cells. The rapid effect of aldosterone on ERK1/2 activation was attenuated by treatment with eplerenone in a dose-dependent manner (Fig. 4B). MR antagonists also block aldosterone-induced activation of ERK1/2 in vascular smooth muscle cells (51) and Chinese hamster ovary cells transfected with human MR (52). On the other

hand, Terada et al. (27) showed that aldosterone-induced ERK1/2 activation in RMCs was attenuated by inhibition of genomic pathways. It has also been shown that spironolactone had no inhibitory effects on rapid activation of ERK1/2 induced by aldosterone in cortical collecting duct cells (50). There is no satisfactory explanation for the discrepancies between these observations. However, these findings combined with the data obtained from animal experiments (23) indicate that ERK1/2 is an important mediator of aldosterone-induced renal cellular injury.

Conclusions

In this review, we discussed the potential roles of aldosterone and MR in the pathogenesis of renal injury. Both in vitro and in vivo experiments indicate that aldosterone and MR contribute to the progression of renal injury via direct actions on tubulointerstitial fibroblasts, glomerular mesangial cells, and podocytes. Molecular mechanisms including those involving ROS, MAPKs, and Rho-kinase may be involved in aldosterone/MR-induced renal injury. However, further studies are needed to determine the precise mechanisms of aldosterone- and MR-mediated renal cell injury, including the contributions of genomic and non-genomic pathways.

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