Aldosterone-induced mesangial cell proliferation is mediated by EGF receptor transactivation

Songming Huang,1,2 Aihua Zhang,1,2 Guixia Ding,1,2 and Ronghua Chen2

1Department of Nephrology, Nanjing Children’s Hospital, and 2Institute of Pediatrics, Nanjing Medical University, Nanjing, China

Submitted 22 July 2008; accepted in final form 28 March 2009

Huang S, Zhang A, Ding G, Chen R. Aldosterone-induced mesangial cell proliferation is mediated by EGF receptor transactivation. Am J Physiol Renal Physiol 296: F1323–F1333, 2009.—Aldosterone (Aldo) stimulates glomerular mesangial cell (MC) proliferation, in part, through an ERK1/2-dependent pathway. In this study, we examined whether Aldo activation of ERK1/2 in MC is mediated through redox-dependent EGF receptor (EGFR) transactivation, as well as the involvement of other signaling mechanisms in Aldo-induced MC proliferation. Aldo increased human MC proliferation, as determined by [3H]thymidine incorporation and cell counts. This increase in proliferation was blocked by inhibition of the mineralocorticoid receptor (MR). Continuing our observations downstream in the signaling pathway, we examined the ability of Aldo to activate both the Ras/MAPK and the PI3K signaling pathways. Aldo increased Ki-RasA and Ki-RasA:GTP levels, and sequentially phosphorylated c-Raf, MAPK kinase (MEK1/2), and ERK1/2. Ki-RasA small interfering RNA (siRNA), the c-Raf inhibitor GW5074, and the MEK1/2 inhibitor PD98059 reduced Aldo-induced cell proliferation by ~65%. Aldo also increased phosphorylation of PI3K, Akt, the mammalian target of rapamycin (mTOR), and the 70-kDa ribosomal S6 kinase (p70S6K1). Inhibition of the PI3K pathways by the selective PI3K inhibitor LY 294002, an Akt inhibitor, or the mTOR inhibitor rapamycin reduced cell proliferation by 51%. Combining LY 294002 and PD98059 completely blocked Aldo-induced MC proliferation. Next, we confirmed that Aldo exerts its effect on MAPK and PI3K activation, as well as on cell proliferation, by activating the EGFR. Pretreatment with the EGFR antagonist AG1478 inhibited MC proliferation, as well as the activation of Ras/MAPK and PI3K/Akt, suggesting that Ras/MAPK and PI3K/Akt activation occur downstream of EGFR activation. Finally, we examined the role of reactive oxygen species (ROS) in Aldo-induced transactivation of the EGFR. Aldo-induced ROS were predominantly generated by mitochondria. Pretreatment with the antioxidant N-acetyl-L-cysteine, catalase, SOD, mitochondrial respiratory chain complex I inhibitor rotenone (Rot), NADPH oxidase inhibitor apocynin, and DPI significantly inhibited Aldo-stimulated MC proliferation as well as EGFR transactivation. However, Rot reduced MC proliferation more potently than apocynin and DPI. In conclusion, Aldo stimulated cell proliferation through MR-mediated, redox-sensitive EGFR transactivation, which was dependent on the Ki-RasA/c-Raf/MEK/ERK and PI3K/Akt/mTOR/p70S6K1 signaling pathways in human MCs.

reactive oxygen species; PI3K

A GROWING BODY OF EVIDENCE suggests that the mineralocorticoid aldosterone (Aldo), a key regulator of blood pressure and electrolyte balance, contributes to the development and progression of cardiovascular disease and chronic kidney disease (CKD), by reducing vascular compliance, as well as promoting endothelial dysfunction and myocardial and renal hypertrophy and fibrosis (1, 4, 12, 14, 48). Patients with cardiovascular disease and CKD have markedly elevated plasma Aldo concentration; exogenous infusion of Aldo reversed the renoprotective effects of angiotensin-converting enzyme (ACE) inhibitors in hypertensive remnant kidney rats (17) and stroke-prone, spontaneously hypertensive rats (SHRsp) (38). Furthermore, renal expression of the mineralocorticoid receptor (MR) in SHRsp rats was significantly elevated compared with normotensive Wistar-Kyoto rats (21). Blockade of the MR in the Nω-nitro-L-arginine methyl ester (l-NNAME) rat model protected the kidneys without altering blood pressure (39). Consistent with these data, Aldo exerts a direct detrimental effect on renal cells. In particular, Aldo stimulates proliferation of mesangial cells (MCs) (33, 34, 44), a hallmark of CKD pathological changes. However, the signaling mechanism by which Aldo promotes MC growth remains unclear.

MAPKs, a family of Ser/Thr protein kinases, are important intermediates in the intracellular signaling pathways that connect extracellular signals to intracellular regulatory proteins. This family has three primary members: ERK1/2, c-Jun NH2-terminal kinase (JNK), and p38. As in most other cell types, ERK1/2 signaling mediates proliferation of MCs through the activation of a number of tyrosine kinase-associated receptors and G protein-coupled receptors (15). Most notably, Aldo activates ERK1/2 in MCs to initiate, in part, its mitogenic and fibrotic effects (31, 44). However, the mechanism by which Aldo activates ERK1/2, and the other signaling mechanisms involved in Aldo-induced MC proliferation, are unknown.

Reactive oxygen species (ROS) have been identified as a major contributor of Aldo-induced mitogenic effects in vascular smooth muscle cells (VSMCs) (28). The precise mechanism of redox-dependent regulation of cell function, such as cell growth, is not clear, given the wide range of proteins that can be targeted by ROS. However, redox-dependent activation of receptor tyrosine kinases is of particular interest (28, 35). The EGF receptor (EGFR) is a redox-sensitive tyrosine kinase that is transactivated by Aldo in VSMCs (28). EGFR activation could stimulate MC growth by activating both the Ras/MAPK and PI3K/Akt signaling pathways, thereby activating their downstream effectors, ERK1/2 and p70S6K1. In this report, we investigate whether Aldo stimulates MC proliferation by activating the EGFR, whether this activation is related to ROS production, and we attempt to elucidate the signaling steps leading from Aldo-induced stimulation of the MR to cell proliferation.

Address for reprint requests and other correspondence: S. Huang, Dept. of Nephrology, Nanjing Children’s Hospital, Nanjing Medical Univ., Nanjing, China (e-mail: smluang@njmu.edu.cn) or A. Zhang, Dept. of Nephrology, Nanjing Children’s Hospital, Nanjing Medical University (e-mail: zhaihua@njmu.edu.cn).
MATERIALS AND METHODS

Reagents and antibodies. Aldo, RU-486 (a potent glucocorticoid receptor antagonist), herbimycin A (HM-A; tyrosine kinase inhibitor), N-acetyl-l-cysteine (NAC), diphenylethionium (DPI), apocynin, rotenone (Rot), allopurinol (Allo), indomethacin (Indo), N7-nitro-l-arginine methyl ester (l-NAME), nordihydroguaiaretic acid (NDGA), ketoconazole (Keto), catalase, SOD, actinomycin D (ATD), cycloheximide (CHM), and 2',7'-dichlorofluorescein diacetate (DCFDA) were purchased from Sigma (St. Louis, MO). AG1478 (EGFR antagonist), LY294002 [phosphatidylinositol 3-kinase (PI3K) inhibitor], Akt inhibitor, rapamycin (mammalian target of rapamycin (mTOR) inhibitor), GW5074 (c-Raf inhibitor), and PD98059 (MEK inhibitor) were purchased from Calbiochem (Cambridge, MA). Antibodies against total EGFR, phospho-EGFR, total PI3K, phospho-PI3K, total Akt, phospho-Akt, total mTOR, phospho-mTOR, total p70S6K, phospho-p70S6K, total c-Raf, phospho-c-Raf, total MEK1/2, phospho-MEK1/2, and phospho-ERK1/2 were purchased from Santa Cruz Biotechnology, using Lipofectamine PLUS (Invitrogen, Carlsbad, CA). Anti-H-Ras, N-Ras and anti-K-Ras-2A antibodies, as well as all secondary horseradish peroxidase-conjugated antibodies, were from Santa Cruz Biotechnology (Beverly, MA). Anti-H-Ras, N-Ras and anti-K-Ras-2A antibodies, as well as all secondary horseradish peroxidase-conjugated antibodies, were from Santa Cruz Biotechnology (Santa Cruz, CA). The antibody against H-Ras recognizes all isoforms of Ras protein, including Ha-Ras, Ki-RasA, Ki-RasB, and N-Ras, while the antibody against K-Ras-2A recognizes only the Ki-RasA isoform of Ras proteins. All other reagents were from Sigma.

DNA synthesis and cell count. To estimate DNA synthesis, MCs were stimulated by indicated agents for 19 h and pulsed with 1 μCi/ml [3H]thymidine for 5 h. Cells then were washed twice with ice-cold PBS, incubated for 5 min in 5% TCA, washed with methanol, and dissolved in 99% formic acid. The incorporation of [3H]thymidine into TCA-insoluble material was measured by a liquid scintillation spectrophotometer. To assess cell growth, MCs in six-well plates were stimulated by indicated agents, and the cell number was counted with Z1-Coulter Counter (Luton, UK).

DCFDA fluorescence measurement of ROS. The fluorogenic substrate DCFDA is a cell-permeable dye that is oxidized to highly fluorescent 2',7'-dichlorofluorescein (DCF) by H2O2 and, therefore, is fluorescent. DCFDA fluorescence measurement of ROS.

Western blot analysis. Western blot analysis was performed using 1 g/ml leupeptin, 2 mM DTT, and 1 mM PMSF. Lysates were cleared by centrifugation at 14,000 g for 10 min at 4°C. Total protein was quantified by the Bradford assay. Equal amounts of lysates were separated on a 10% polyacrylamide gel and electrotransferred to Bio-Blot nitrocellulose membranes (Bio-Rad). The membranes were blocked in TBST (20 mM Tris-base, pH 7.6, 150 mM NaCl, 0.1% Tween 20) containing 5% bovine serum albumin for 1 h at room temperature and incubated with primary antibody in the blocking solution at 4°C overnight. The membranes were incubated with 1:1,000 diluted horseradish peroxidase-conjugated secondary antibody at room temperature for 1 h and visualized by an enhanced chemiluminescence kit (Amersham). The chemiluminescent signal was quantified using UVP software (UVP, Upland, CA).

DNA synthesis and cell count. To estimate DNA synthesis, MCs were stimulated by indicated agents for 19 h and pulsed with 1 μCi/ml [3H]thymidine for 5 h. Cells then were washed twice with ice-cold PBS, incubated for 5 min in 5% TCA, washed with methanol, and dissolved in 99% formic acid. The incorporation of [3H]thymidine into TCA-insoluble material was measured by a liquid scintillation spectrophotometer. To assess cell growth, MCs in six-well plates were stimulated by indicated agents, and the cell number was counted with Z1-Coulter Counter (Luton, UK).

DCFDA fluorescence measurement of ROS. The fluorogenic substrate DCFDA is a cell-permeable dye that is oxidized to highly fluorescent 2',7'-dichlorofluorescein (DCF) by H2O2 and, therefore, is used to monitor intracellular generation of ROS (47). To quantitate ROS levels, MCs were seeded to a 96-well plate. When the cells reached confluence, they were washed twice with PBS and incubated for 30 min with 10 μM DCFDA, then treated as indicated in the text. At the end of the incubation period, the cells were washed twice with PBS. Relative fluorescence was measured using a fluorescence plate reader (FLUOStar OPTIMA) at excitation and emission wavelengths of 485 and 528 nm, respectively, three times at 90-s intervals.
**Ras:GTP assay.** c-Raf-1 Ras binding domain (RBD) agarose was from Upstate Biotech (Lake Placid, NY). This immobilized protein corresponds to the human c-Raf-1 RBD (residues 1–149), and binds Ras complexed with GTP. Pulldown experiments were performed in 400 μl (0.4 mg total protein) of whole cell lysate isolated with GLB. Lysates were incubated with 30 μl c-Raf-1 RBD agarose overnight (at 4°C and with constant agitation); pellets were washed five times with 2 volumes of fresh glibenclamide each time, for a total wash time of

**Fig. 2. Aldo-induced reactive oxygen species (ROS) generation in human MCs.** A: time course of Aldo-stimulated ROS generation in human MCs. Confluent human MCs were treated with 100 nM Aldo for the indicated time periods (0–120 min) in the presence of 2′,7′-dichlorofluorescin (DCF; n = 8). B: concentration response of Aldo-induced ROS production. Human MCs were treated with the indicated concentration Aldo for 60 min (n = 8). C: effect of eplerenone on Aldo-induced ROS production. Human MCs were pretreated with eplerenone (10 μM) for 30 min in the presence or absence of Aldo (100 nM) for a further 60 min (n = 8). D: enzymatic sources of ROS production in response to Aldo treatment. Confluent HMCs were treated for 60 min with 500 μmol/l apocynin, 10 μmol/l diphenyleneiodonium (DPI), 10 μmol/l rotenone (Rot), 30 μmol/l allopurinol (Allo), 100 μmol/l indomethacin (Indo), 10 μmol/l nordihydroguaretic acid (NDGA), 10 μmol/l ketoconazole (Keto), or 100 μmol/l N^{G}-nitro-l-arginine methyl ester (l-NAME) and, for a further 60 min, with 100 nM Aldo in the presence of DCF. Fluorescence was quantified as described in MATERIALS AND METHODS. Values are means ± SE. *P < 0.01 vs. control. #P < 0.05, ##P < 0.01 vs. Aldo-treated group by ANOVA.

**Fig. 3. ROS-mediated Aldo-induced MC proliferation.** Human MCs were pretreated with N-acetyl-l-cysteine (NAC; 5 mM), catalase (25 μg/ml), SOD (250 U/ml), Rot (10 μmol/l), apocynin (Apo; 500 μmol/l), or DPI (10 μmol/l) for 30 min in the presence or absence of Aldo (100 nM) for a further 24 h. [3H]thymidine incorporation (A) and cell number (B) were detected as described in MATERIALS AND METHODS (n = 6). Values are means ± SE. ΔP < 0.01 vs. Aldo-treated group by ANOVA.
2 h. After being resuspended in sample buffer and heated, c-Raf-1 RBD agarose-precipitated proteins were separated by SDS-PAGE, and Ki-RasA:GTP was identified by immunoblotting.

Statistical analysis. Values shown represent means ± SE. Statistical analysis was performed by one way-ANOVA and Bonferroni tests, with a P value of <0.05 considered statistically significant.

RESULTS

Effect of Aldo on human MC proliferation. The effects of Aldo on human MC proliferation were determined by [3H]thymidine incorporation and cell count analysis. MCs were incubated with Aldo (1–400 nM) for 24 h. As shown in Fig. 1, A and B, Aldo induced human MC proliferation in a concentration-dependent manner. To address the functional role of the MR, we examined the effect of the MR antagonist eplerenone and the glucocorticoid receptor blocker RU-486 on Aldo-induced MC proliferation. As shown in Fig. 1, C and D, eplerenone and RU-486 alone were without effect on MC proliferation. However, Aldo-induced MC proliferation was reduced significantly in the presence of eplerenone, but not RU-486.

Involvement of MR-dependent ROS generation in Aldo-induced cell proliferation. Given the recent evidence for involvement of ROS in MC proliferation induced by ANG II (9) and PDGF (45), we tested the possibility that ROS might mediate MC proliferation in our experimental model. Using DCFDA, we validated the time course of ROS production in response to Aldo treatment in cultured human MCs. As shown in Fig. 2A, 100 nM Aldo induced ROS production as early as 3 min, which gradually increased and peaked at 60 min (2.5-fold). Therefore, the 60-min time point was used in subsequent experiments for determination of the concentration response of Aldo stimulation of ROS. Figure 2B shows that Aldo increased ROS production in a concentration-dependent manner, with maximal stimulation at 100 nM. The induction of ROS production in response to Aldo treatment was completely blocked by eplerenone, indicating that Aldo-induced ROS generation was mediated by the MR (Fig. 2C).

To determine the enzymatic sources of ROS, we measured Aldo-induced ROS production in the presence and absence of inhibitors of various oxidant-producing enzymes. As shown in

Fig. 4. Aldo-induced EGF receptor (EGFR) transactivation in human MCs. A: time course of Aldo-stimulated EGFR phosphorylation in human MCs. Confluent human MCs were treated with 100 nM Aldo for the indicated time periods (0–240 min; n = 4). B: concentration-response of Aldo-induced EGFR phosphorylation. Human MCs were treated with the indicated concentration of Aldo for 60 min (n = 4). C: effect of eplerenone, NAC, and AG1478 on Aldo-induced EGFR phosphorylation. Human MCs were pretreated with eplerenone (10 μM), NAC (5 mM), or AG1478 (250 nM) for 30 min in the presence or absence of Aldo (100 nM) for a further 60 min (n = 4). D: effect of actinomycin D (ATD) or cycloheximide (CHM) on Aldo-induced EGFR phosphorylation. Human MCs were treated with the transcription inhibitor ATD (20 μM) or translation inhibitor cycloheximide (5 μM) for 30 min before the addition of Aldo (100 nM) for 60 min. EGFR phosphorylation was examined by immunoblotting with an antibody that recognizes phosphorylated (Tyr992) EGFR, and the comparative levels of phosphorylation were determined by the ratio of phosphorylated EGFR to total EGFR. Results are expressed as fold-increase over vehicle control and are shown as means ± SE. Top: representative immunoblots. Bottom: densitometric analysis. #P < 0.05, *P < 0.01 vs. control. ΔP < 0.01 vs. Aldo-treated group by ANOVA.
Fig. 2D, ROT, inhibitor of mitochondrial respiratory chain complex I, almost completely blocked Aldo-induced ROS generation in HMCs. DPI and apocynin, two structurally distinct NADPH oxidase inhibitors, also significantly inhibited Aldo-stimulated ROS production, but were less potent than ROT. In contrast, inhibitors of other oxidant-producing enzyme systems, including Allo, Indo, NDGA, Keto, and l-NAME, were without effect, which indicated that mitochondria and NADPH oxidase are involved in Aldo-induced ROS generation in HMCs.

We subsequently examined the role of ROS in Aldo-induced MC proliferation. MCs were pretreated with NAC, catalase, SOD, ROT, apocynin, and DPI for 30 min and then incubated with 100 nM Aldo for a further 24 h. NAC, catalase, SOD, ROT, apocynin, and DPI have no effect on cell proliferation in basal conditions; however, NAC, catalase, SOD, ROT, apocynin, and DPI effectively inhibited Aldo-induced human MC proliferation. Consistent with inhibition in ROS production, ROT reduced MC proliferation more potently than apocynin and DPI. (Fig. 3, A and B).

Role of ROS in Aldo-induced EGFR transactivation. We examined the role of ROS in Aldo-induced EGFR transactivation. We first examined the transactivation kinetics of the EGFR after treatment with 100 nM Aldo. EGFR transactivation was determined by immunoblotting with an antibody that recognized phosphorylated EGFR. Aldo increased the level of phosphorylated EGFR at 5 min, with maximal stimulation at 60 min, which was sustained to 240 min (Fig. 4A). Therefore, the 60-min time point was used in subsequent experiments to determine the concentration response of EGFR phosphorylation to Aldo stimulation. As shown in Fig. 4B, Aldo increased phosphorylation of the EGFR in a concentration-dependent manner, with maximal stimulation at 100 nM.

Pretreatment with the MR antagonist eplerenone significantly blocked Aldo-induced EGFR transactivation (Fig. 4C), suggesting that the MR mediated Aldo-stimulated EGFR phosphorylation. The best-studied aspects of aldosterone action are its stimulation or suppression of gene expression through MR-mediated transcription. To exclude the possibility that a change in EGFR expression was responsible for the observed apparent increase in EGFR transactivation, stimulation of EGFR phosphorylation was examined under conditions of transcription inhibition by actinomycin D (20 nM) and translation inhibition by cycloheximide (5 μM) for 30 min before Aldo treatment. The increase in EGFR phosphorylation observed in Aldo-treated cells at 60 min was not reduced by preincubation with actinomycin D or cycloheximide (Fig. 4D). This demonstrates that phosphorylation of the EGFR is not dependent upon the initiation of MR-regulated transcriptional events.

We then examined the effects of the antioxidant NAC on Aldo-induced EGFR phosphorylation. NAC completely blocked phosphorylation of the EGFR at 5 min, with maximal stimulation at 60 min, which was sustained to 240 min (Fig. 4A). Therefore, the 60-min time point was used in subsequent experiments to determine the concentration response of EGFR phosphorylation to Aldo stimulation. As shown in Fig. 4B, Aldo increased phosphorylation of the EGFR in a concentration-dependent manner, with maximal stimulation at 100 nM.

Pretreatment with the MR antagonist eplerenone significantly blocked Aldo-induced EGFR transactivation (Fig. 4C), suggesting that the MR mediated Aldo-stimulated EGFR phosphorylation. The best-studied aspects of aldosterone action are its stimulation or suppression of gene expression through MR-mediated transcription. To exclude the possibility that a change in EGFR expression was responsible for the observed apparent increase in EGFR transactivation, stimulation of EGFR phosphorylation was examined under conditions of transcription inhibition by actinomycin D (20 nM) and translation inhibition by cycloheximide (5 μM) for 30 min before Aldo treatment. The increase in EGFR phosphorylation observed in Aldo-treated cells at 60 min was not reduced by preincubation with actinomycin D or cycloheximide (Fig. 4D). This demonstrates that phosphorylation of the EGFR is not dependent upon the initiation of MR-regulated transcriptional events.

We then examined the effects of the antioxidant NAC on Aldo-induced EGFR phosphorylation. NAC completely blocked phosphorylation of the EGFR at 5 min, with maximal stimulation at 60 min, which was sustained to 240 min (Fig. 4A). Therefore, the 60-min time point was used in subsequent experiments to determine the concentration response of EGFR phosphorylation to Aldo stimulation. As shown in Fig. 4B, Aldo increased phosphorylation of the EGFR in a concentration-dependent manner, with maximal stimulation at 100 nM.

Pretreatment with the MR antagonist eplerenone significantly blocked Aldo-induced EGFR transactivation (Fig. 4C), suggesting that the MR mediated Aldo-stimulated EGFR phosphorylation. The best-studied aspects of aldosterone action are its stimulation or suppression of gene expression through MR-mediated transcription. To exclude the possibility that a change in EGFR expression was responsible for the observed apparent increase in EGFR transactivation, stimulation of EGFR phosphorylation was examined under conditions of transcription inhibition by actinomycin D (20 nM) and translation inhibition by cycloheximide (5 μM) for 30 min before Aldo treatment. The increase in EGFR phosphorylation observed in Aldo-treated cells at 60 min was not reduced by preincubation with actinomycin D or cycloheximide (Fig. 4D). This demonstrates that phosphorylation of the EGFR is not dependent upon the initiation of MR-regulated transcriptional events.

We then examined the effects of the antioxidant NAC on Aldo-induced EGFR phosphorylation. NAC completely blocked phosphorylation of the EGFR at 5 min, with maximal stimulation at 60 min, which was sustained to 240 min (Fig. 4A). Therefore, the 60-min time point was used in subsequent experiments to determine the concentration response of EGFR phosphorylation to Aldo stimulation. As shown in Fig. 4B, Aldo increased phosphorylation of the EGFR in a concentration-dependent manner, with maximal stimulation at 100 nM.
Aldo-induced phosphorylation of the EGFR (Fig. 4C), suggesting that Aldo-induced EGFR activation is mediated by MR-dependent ROS generation.

Involvement of EGFR transactivation in Aldo-induced cell proliferation. To determine whether the mitogenic effect of Aldo is mediated by EGFR activation, the cells were pretreated with AG1478 (250 nM) or HM-A (1 μM; tyrosine kinase inhibitor) for 30 min before the 100 nM Aldo treatment. As expected, AG1478 inhibited the Aldo-induced phosphorylation of the EGFR (Fig. 4C). Pretreatment with either AG1478 or herbimycin A abolished Aldo-induced cell proliferation (Fig. 5, A and B).

To classify the downstream signaling of EGFR transactivation, we tested the involvement of the PI3K/Akt/mTOR/p70S6K1 pathways in Aldo-induced cell proliferation. PI3K/Akt/mTOR/p70S6K1 signaling activation was determined by immunoblotting to detect phosphorylation of PI3K, Akt, mTOR, and p70S6K1. As shown in Fig. 6A, incubation with 100 nM Aldo for 60 min significantly increased phosphorylation of PI3K, Akt, mTOR, and p70S6K1. Pretreatment with AG1478 and the antioxidant NAC completely abolished Aldo-induced PI3K, Akt, mTOR, and p70S6K1 phosphorylation. Also, Aldo-induced phosphorylation of Akt, mTOR, and p70S6K1 was blocked by pretreatment with LY 294002 (PI3K inhibitor, 1 μM), the Akt inhibitor (10 μM), or rapamycin (mTOR inhibitor; 1 nM). In addition, LY 294002, the Akt inhibitor, or rapamycin inhibited Aldo-induced cell proliferation by 51% (Fig. 6B). These results suggest that EGFR

Fig. 7. Aldo increases active GTP-bound Ki-RasA levels. A: Western blot analysis of total Ras (top), Ki-RasA (middle), and Ki-RasA:GTP (bottom) in MCs treated with Aldo (100 nM) for 3 h. A: representative blot probed for Ki-RasA:GTP (bottom) contained the precipitate from whole cell lysate using Raf-RBD agarose, which specifically binds GTP-bound Ras and the blot probed with anti-Ki-RasA antibody to detect Ki-RasA:GTP. B: summary graph showing the relative changes in the levels of Ki-RasA and Ki-RasA:GTP in response to Aldo treatment for 3–4 h. The comparative levels of Ki-RasA and Ki-RasA:GTP were determined by the ratio of Ki-RasA and Ki-RasA:GTP to total Ras, respectively. Results are expressed as fold-increase over vehicle control and are shown as means ± SE. IB, immunoblotting; IP, immunoprecipitation.

Fig. 8. Effect of ROS and EGFR on Aldo-stimulated total and active Ki-RasA levels, c-Raf, MEK1/2, and ERK1/2 phosphorylation in MCs. Human MCs were treated for 3 h with Aldo (100 nM) in the presence or absence of NAC (5 mM) or AG 1478 (250 nM). Ki-RasA and Ki-RasA:GTP expression was examined as described in Fig. 7. c-Raf, MEK1/2, and ERK1/2 activation was determined by immunoblotting with antibodies against phospho-c-Raf, total c-Raf, phospho-MEK1/2, total MEK1/2, phospho-ERK1/2, and total ERK1/2. Each blot shown is representative of 4 independent experiments. A: representative immunoblots. B: densitometric analysis. The comparative levels of c-Raf, MEK1/2, and ERK1/2 phosphorylation were determined by the ratio of the phosphorylated kinases to the total kinases. Results are expressed as fold-increase over vehicle control and are shown as means ± SE. *P < 0.01 vs. the Aldo-treated group by ANOVA.
transactivation-dependent activation of the PI3K/Akt/mTOR/p70S6K1 signaling pathways is involved in Aldo-induced human MC proliferation.

Aldo increases absolute and active GTP-bound levels of Ki-RasA. Aldo increases Ki-RasA protein levels in MCs (44) and cardiac fibroblasts (43) through the MR. We tested the hypothesis that aldosterone in these cells also increases the amount of active Ki-RasA (Fig. 7A). Activated Ras bound by GTP interacts with the RBD of Raf (7, 13). The amount of Ki-RasA and Ki-RasA:GTP was identified by probing Western blots with anti-Ki-RasA antibody, while total Ras protein was identified with the anti-H-Ras antibody, which recognizes

Fig. 9. Effect of Ki-RasA small interfering (si) RNA on Ki-RasA expression in MCs. A: human MCs were transfected with increasing concentrations (0–1,000 nM) of Ki-RasA siRNA or control siRNA (500 nM). Ki-RasA protein expression was evaluated after 48 h by Western blotting (n = 4). B: human MCs were transfected with 500 nM control siRNA for 48 h or Ki-RasA-specific siRNA for 24 or 48 h. Ki-RasA, H-Ras, and N-Ras expression was determined by immunoblotting. C: human MCs were transfected with 500 nM Ki-RasA siRNA or control siRNA for 24 h before treatment with Aldo (100 nM; n = 4). Top: representative immunoblots. Bottom: densitometric analysis. Values are means ± SE. *P < 0.01 vs. control by ANOVA.

Fig. 10. Effect of Ki-RasA/c-Raf/MEK/ERK signaling on Aldo-induced MC proliferation. A: human MCs were transfected with 500 nM Ki-RasA siRNA or control siRNA. Twenty-four hours after transfection, they were treated for 24 h with 100 nM Aldo (n = 6). B: human MCs were pretreated with GW5074 (10 μM) or PD98059 (10 μM) for 30 min followed by incubation with Aldo (100 nM) for 24 h. [3H]thymidine incorporation and cell number were detected as described in MATERIALS AND METHODS (n = 6). Values are means ± SE. ΔP < 0.01 vs. Aldo-treated control cells. $P < 0.01$ vs. Aldo-treated group by ANOVA.
Ki-RasA and B, Ha-Ras, and N-Ras isoforms. The representative Western blots and the summary graph in Fig. 7 show that exposing MCs to Aldo for 3 h markedly increases Ki-RasA and Ki-RasA:GTP levels but has little effect on total Ras levels. Since Ki-RasA is expressed at much lower levels than other Ras isoforms (20, 36), the lack of a marked change in total Ras was not unexpected.

Aldo stimulates MAPK1/2 signaling through EGFR transactivation. We next examined whether Aldo activates Ki-RasA and its downstream effectors by MR-mediated ROS-dependent EGFR transactivation in MCs. The representative Western blot in Fig. 8A shows the effects of Aldo in the absence and presence of EGFR antagonists (AG1478) or the antioxidant NAC on the levels of Ki-RasA, Ki-RasA:GTP, phosphorylated and total c-Raf, MEK1/2, and ERK1/2. As shown in the summary graph (Fig. 8B), Aldo significantly increased the relative levels of Ki-RasA, Ki-RasA:GTP, phospho-c-Raf, phospho-MEK1/2, and phospho-ERK1/2, whereas the total Ras, c-Raf, MEK1/2, and ERK1/2 levels were not affected. NAC and AG1478 markedly attenuated the effect of Aldo (100 nM) on Ki-RasA, Ki-RasA:GTP, and activated (phosphorylated) c-Raf, MEK1/2, and ERK1/2.

Involvement of EGFR transactivation-dependent Ras/Raf/MEK/ERK signaling in Aldo-induced cell proliferation. Aldo-induced rat MC proliferation is mediated by MEK/ERK signaling (34, 44). Since Aldo increased active and absolute GTP-bound levels of Ki-RasA, we used specific siRNAs designed against the human Ki-Ras sequence to determine whether Ki-RasA mediates the effects of Aldo on MC proliferation. In preliminary experiments, cells were transfected with 500 nM Ki-RasA siRNA or control siRNA. Twenty-four hours after transfection, they were treated for 1 h with 100 nM Aldo. PI3K phosphorylation was determined by immunoblotting using an anti-phospho-PI3K antibody. Left: representative immunoblots. Right: densitometric analysis.

Aldo induced PI3K/Akt activation through a Ki-RasA-independent mechanism. We next tested whether Aldo induced PI3K/Akt activation in a Ki-RasA-dependent manner. Human MCs were transfected with 500 nM Ki-RasA siRNA for 24 h followed by Aldo treatment for 60 min; PI3K phosphorylation was determined by immunoblotting using anti-phospho-PI3K. As shown in Fig. 11, Ki-RasA siRNA has no effect on Aldo-induced PI3K phosphorylation, suggesting that there is no cross talk between Aldo-induced PI3K/Akt signaling and Ras/Raf signaling. To confirm that no cross talk occurs between Aldo-induced PI3K/Akt and Ras/Raf signaling, we examined the effect of combining the PI3K and MEK1/2 inhibitors on Aldo-induced cell proliferation. Human MCs were pretreated with both the PI3K inhibitor LY 294002 and the MEK1/2 inhibitor PD 98059 for 30 min followed by Aldo exposure for 24 h. As shown in Fig. 12, the combination of LY 294002 and PD 98059 completely abolished Aldo-induced human MC proliferation.

DISCUSSION

Several groups of investigators have reported that Aldo is a strong mitogen for MCs (33, 34, 44). Earlier studies showed that Aldo stimulates MC proliferation via MAPK activation (34, 44). In VSMCs, Aldo-induced MAPK activation was dependent on ROS generation and EGFR phosphorylation (28). MCs are derived from mesenchymal cells, and have similar properties to VSMCs (46). Therefore, in this study, we extend these observations and show that Aldo treatment of MCs stimulates proliferation by inducing PI3K/Akt and Ras/MAPK activation through a novel mechanism involving MR-mediated ROS generation and EGFR activation. The proposed pathway is presented in Fig. 13. To our knowledge, this is the first time that Aldo has been shown to stimulate EGFR and PI3K activation in MCs.

The role of ERK1/2 in the transduction of proliferative signals to the nucleus in MCs and cardiac fibroblasts is well known (22, 41, 43). In accordance with previously published data (34, 44), our results demonstrate that Aldo-induced MC proliferation depends, in part, on ERK1/2 phosphorylation, because blocking ERK1/2 activation by PD98059 inhibited Aldo-induced cell proliferation. ERK1/2 is activated by receptor tyrosine kinases and by stimulation of G protein-coupled
receptors (27). When the ligand binds to the receptor, proliferation or differentiation signals are transmitted to the nucleus, activating cyclin-dependent kinases and thereby promoting cell cycle progression (16). The small GTPase Ras has a prominent role in the signaling pathways leading from activated growth factor receptors to ERKs (10, 24, 43). Aldo increases Ki-RasA protein levels in MCs and cardiac fibroblasts through induction of the MR (43, 44). However, the molecular basis whereby Aldo induces Ki-RasA is unknown. It also is unclear whether merely increasing absolute Ki-RasA levels in response to Aldo is sufficient to activate Ki-RasA, leading to dependent stimulation of its effector MAPK cascades. The current study demonstrates that Aldo increases active, GTP-bound Ki-RasA levels proportionately with absolute Ki-RasA levels. This is consistent with a mechanism where, through mass action, Aldo-induced Ki-RasA leads to concomitant increases in the active pool of Ki-RasA (19, 43). The ability of Ki-RasA siRNA to block Aldo-induced MC proliferation is consistent with this Aldo-increased active pool of Ki-RasA, the subsequently stimulating effector of MAPK signaling.

The present results show that Aldo increases cell proliferation in activated human MCs by a mechanism involving activation of Ras/MAPK signaling. Moreover, both the c-Raf inhibitor GW5074 and the MEK inhibitor PD98059 inhibited Aldo-stimulated cell proliferation by ~65%. Although these data suggest that Aldo stimulates MC proliferation through the Ras/MAPK pathway, the incomplete inhibition of Aldo-enhanced cell proliferation by c-Raf and MEK inhibitors suggests the existence of an alternate intracellular signaling pathway for Aldo mitogenic effects on human MCs. PI3K signaling through p70S6 kinase acts synergistically with Ras/MAPK to stimulate the G1-to-S phase transition of the cell cycle (5, 23). These observations prompted us to conduct the present study to elucidate this signaling pathway for the Aldo mitogenic effect on human MCs. Our data show, for the first time in MCs, that the inhibition of both Ras/MAPK and PI3K/Akt completely blocks Aldo-induced cell proliferation.

PI3K mediates various cellular processes such as proliferation and survival (23). In epithelial cells, PI3K mediates Aldo stimulation of the epithelial sodium channel (ENaC) in mouse cortical collecting duct cells (18). PI3K also mediates the effect of Aldo on rat vascular endothelial and smooth muscle cell function (26). In the present study, we observed that Aldo activates PI3K signaling through the MR in human MCs. These results suggest that MR-activated PI3K is highly conserved among different species and various cell types and might have a crucial role in the proliferation of human MC cells. Our data show that blocking the activation and expression of Ki-RasA by siRNA had no effect on Aldo-induced PI3K phosphorylation, which suggests that Aldo-induced PI3K/Akt pathways are independent of Ki-RasA. Furthermore, we found that Akt and mTOR are the predominant downstream effectors of PI3K in MCs, because the PI3K inhibitor LY 294002 and the Akt inhibitor inhibited Akt and mTOR phosphorylation, respectively. mTOR controls protein synthesis, in part by phosphorylating downstream substrates, including p70S6K kinase (3), consistent with our result that the mTOR inhibitor rapamycin decreased p70S6K1 phosphorylation.

Cell proliferation requires the coordinate activation of p70S6 kinase. This kinase participates in the translation of mRNAs that encode many of the components of the translational apparatus, including ribosomal proteins and elongation factors. Thus activation of p70S6 kinase is a prerequisite for protein synthesis.
synthesis (11). The present study demonstrated that p70S6 kinase is activated in MCs by sequential phosphorylation of PI3K, Akt, and mTOR. Rapamycin inhibits mTOR and prevents it from activating p70S6 kinase by forming a stable complex with FK506 binding protein, which binds mTOR (2). Results presented in this study show that LY 294002, the Akt inhibitor, and rapamycin potently inhibited Aldo-stimulated MC proliferation, indicating the importance of PI3K/Akt/mTOR/p70S6 pathway activation in MC proliferation.

PI3K is composed of two subunits, the regulatory p85 subunit and the catalytic p110 subunit, that possess both lipid kinase and protein kinase activities (8). PI3K activation occurs after binding of p85, through its SH2 domain, to the cytoplasmic region of receptor tyrosine kinases, which recruit p110 to the plasma membrane where the lipid substrates are localized. Also, GTP-bound Ras can bind and activate PI3K (40).

The mechanism by which Aldo activates MEK/ERK and PI3K/Akt signaling cascades, inducing MC proliferation, was not known previously. Aldo enhanced EGFR phosphorylation, resulting in potentiated ERK1/2 phosphorylation in VSMCs (28). Further evidence about the role of the EGFR in Aldo signaling comes from studies with Chinese hamster ovary (CHO) cells that lack the EGFR and do not respond to Aldo (25). Our results demonstrate, for the first time, that the EGFR is potently activated by Aldo stimulation in MCs. This activation is inhibited by the EGFR kinase inhibitor AG1478. As further proof of the involvement of the EGFR in Aldo-induced cell signaling, AG1478 inhibited Aldo-induced cell proliferation, as well as MEK/ERK and PI3K/Akt activation.

The relevance of Aldo-induced ROS production has been demonstrated in vivo and in vitro. Nishiyama and coworkers (31, 32) showed that Aldo/salt treatment induced glomerular injury associated with increased production of ROS in renal cortical tissues and that this injury was significantly attenuated by treatment with tempol, a membrane-permeable radical scavenger. These findings establish an important role of ROS in Aldo-induced renal injury (31, 32). A separate study from the same group of investigators provides further evidence for a direct stimulatory effect of Aldo on ROS production in cultured rat MCs (30). However, the source of Aldo-induced ROS generation in human MCs is unclear. There is a growing list of oxidant-generating systems that contribute to ROS production within 3 min of Aldo treatment, ROS production is increased, resulting in activation of the EGFR by 5 min, which leads to activation of the Ras/MAPK and PI3K/Akt pathways and increased cell proliferation (Fig. 13). These studies may be important for the development of therapies for treatment of glomeruloproliferative diseases.

REFERENCES

ALDOSTERONE INDUCES MC PROLIFERATION VIA EGFR


26. Lopez-Itasaca M. Signaling from G-protein-coupled receptors to mito-


45. Zhang A, Jia Z, Guo X, Yang T. Aldosterone induces epithelial-