Aldosterone and Angiotensin II Synergistically Induce Mitogenic Response in Vascular Smooth Muscle Cells

Li-Juan Min, Masaki Mogi, Jian-Mei Li, Jun Iwanami, Masaru Iwai, Masatsugu Horiuchi

Abstract—Interaction between aldosterone (Aldo) and angiotensin II (Ang II) in the cardiovascular system has been highlighted; however, its detailed signaling mechanism is poorly understood. Here, we examined the cross-talk of growth-promoting signaling between Aldo and Ang II in vascular smooth muscle cells (VSMC). Treatment with a lower dose of Aldo (10^{-12} mol/L) and with a lower dose of Ang II (10^{-10} mol/L) significantly enhanced DNA synthesis, whereas Aldo or Ang II alone at these doses did not affect VSMC proliferation. This effect of a combination of Aldo and Ang II was markedly inhibited by a selective AT1 receptor blocker, olmesartan, a mineralocorticoid receptor antagonist, spironolactone, an MEK inhibitor, PD98059, or an EGF receptor tyrosine kinase inhibitor, AG1478. Treatment with Aldo together with Ang II, even at noneffective doses, respectively, synergistically increased extracellular signal-regulated kinase (ERK) activation, reaching 2 peaks at 10 to 15 minutes and 2 to 4 hours. The early ERK peak was effectively blocked by olmesartan or an EGF receptor kinase inhibitor, AG1478, but not by spironolactone, whereas the late ERK peak was completely inhibited by not only olmesartan, but also spironolactone. Combined treatment with Aldo and Ang II attenuated mitogen-activated protein kinase phosphatase-1 (MKP-1) expression and increased Ki-ras2A expression. The late ERK peak was not observed in VSMC treated with Ki-ras2A-siRNA. Interestingly, the decrease in MKP-1 expression and the increase in Ki-ras2A expression were restored by PD98059 or AG1478. These results suggest that Aldo exerts a synergistic mitogenic effect with Ang II and support the notion that blockade of both Aldo and Ang II could be more effective to prevent vascular remodeling. (Circ Res. 2005;97:434-442.)

Key Words: aldosterone  ■  angiotensin II  ■  vascular smooth muscle cells  ■  mitogen-activated protein kinases  ■  signal transduction

Angiotensin II (Ang II) is a critical effector of the renin-angiotensin-aldosterone system (RAAS), which plays an important role in regulation of normal cardiovascular homoeostasis and in the pathogenesis of a variety of cardiovascular diseases. The major actions of Ang II are mediated via the Ang II type 1 (AT1) receptor, and AT1 receptor blockers have been shown to have therapeutic benefit in the treatment of hypertension and cardiovascular disorders.1,2 Recently, aldosterone (Aldo), a potent mineralocorticoid and a final substance of RAAS, has attracted further interest in its role in the development and progression of cardiovascular disease, emphasized by several clinical studies examining the additional benefit of the use of an aldosterone antagonist as well as an angiotensin-converting enzyme inhibitor or AT1 receptor blockers.3

Aldo interacts with mineralocorticoid receptors (MR) to promote endothelial dysfunction, facilitate thrombosis, reduce vascular compliance, impair baroreceptor function, and cause myocardial and vascular hypertrophy and fibrosis with promotion of pathological remodeling.4 Aldo also induces growth and proliferation of vascular smooth muscle cells (VSMC),5 which causes vascular remodeling and results in atherosclerosis. A classical genomic action of Aldo has been described as binding its intracellular MR, followed by translocation of the steroid-receptor complex to the nucleus, where it acts as a transcriptional regulator to promote gene expression and protein synthesis.5,6 Aldo via MR through a genomic mechanism increases Ki-ras2A (small and monomeric GTP-binding protein) transcriptional and protein levels,7,8 and such induction of Ki-ras2A by Aldo is associated with pathological heart-remodeling, possibly by promoting fibrosis and cellular proliferation via subsequent activation of the extracellular regulated kinase (ERK1/2) cascade. Recently, in VSMC, the existence of rapid effects of Aldo to increase Na+/H+ exchanger activity,11 intracellular calcium,12 and ERK1/2 phosphorylation13 is supported by accumulating experimental evidence. Such rapid Aldo effects are characterized by their insensitivity to classical MR antagonists, such as spironolactone, as well as transcription and protein synthesis inhibitors.14,15
Ang II has also been demonstrated to be one of the most powerful promoters of cardiomyocyte hypertrophy.16 Stimulation of AT1 receptors enhances the epidermal growth factor (EGF)-induced mitogen-activated protein kinase (MAPK) pathway in NIH3T3 fibroblasts.17 In VSMC, we previously reported that Ang II treatment activated ERK, with 2 peaks at 5 to 10 minutes and around 2 to 4 hours.18 Moreover, Izumi et al reported that dominant-negative-ERK gene transfer significantly suppressed VSMC proliferation in both the intima and media after balloon injury,19 indicating that the Ang II-induced ERK-pathway could have a pivotal role in atherosclerosis formation.

Recently, it has been shown that Aldo interacts with the EGF receptor signaling in CHO cells.20 Transactivation of the EGF receptor has also been described as a crucial step in the Ang II-induced MAPK signaling cascade.21 A reciprocal interaction has also been reported between Ang II and Aldo in vivo and in vitro.3,22,23 In addition, Ang II and Aldo have a reciprocal interaction has also been reported between Ang II and Aldo in vivo and in vitro.3,22,23 In addition, Ang II, suggesting a synergistic mitogenic interaction of aldosterone with angiotensin II in the signaling pathway elicited by mitogenic interaction between Aldo and Ang II receptors could accelerate the development of cardiovascular tissue injury and subsequent remodeling.

Therefore, the present study was undertaken to examine the potential interactions of signaling between aldosterone and angiotensin II in adult rat VSMC resulting in mitogenic responses. We also investigated the crosstalk between the rapid and genomic mechanisms involved in EGF receptor transactivation by Aldo in Ang II-mediated VSMC proliferation, and explored the signaling pathway elicited by mitogenic interaction between Aldo and Ang II, with focusing on the ERK signaling cascade including mitogen-activated protein kinase phosphatase-1 (MKP-1) and Ki-ras2A.

Materials and Methods

Cell Culture

Vascular smooth muscle cells (VSMC) were isolated from adult Sprague-Dawley rat thoracic aorta (Clea Japan Inc, Tokyo, Japan) as previously described, which exclusively express the AT1 receptor but not AT2 receptor.20 The cells were cultured on 100-mm dishes in Dulbecco’s Modified Eagle Medium (DMEM) (Life Technologies, Gaithersburg, Md) supplemented with 10% fetal bovine serum (FBS) and antibiotics at 37°C in a humidified atmosphere of 5% CO2 and 95% air. Cells at passage 3 to 8 were used for the experiments. Subconfluent cells were serum-starved for 48 hours to induce a quiescent state before the experiments.

[3H]-Thymidine Incorporation

DNA synthesis was assayed by measurement of [3H]-thymidine incorporation. Subconfluent and quiescent VSMC cultured in 24-well plates were treated with various stimuli for 12 hours, and pulsed with 1 μCi/mL [3H]-thymidine specific activity (DuPont NEN Research Products, Boston, Mass) for an additional 24 hours. The radioactivity of the cell lysates was determined using a liquid scintillation β-counter.

Measurement of Aldo Concentration

Aldo concentration was determined as previously described.21 Subconfluent and quiescent VSMC in 1 mL of growth medium DMEM cultured in 24-well plates were replaced with phenol-red-free DMEM, serum-starved for 48 hours and incubated with Ang II (10^{-10} mol/L or 10^{-7} mol/L). At the end of incubation, a 200 μL aliquot of supernatant was removed for measurement of Aldo concentration by using Aldo ELISA kit (Alpha Diagnostic). The cells were isolated with trypsin-EDTA (Invitrogen Corp) for cell number count. Aldo production was expressed as pmol/L.

Immunoblot Analysis

Subconfluent and quiescent VSMC cultured in 100-mm dishes were treated under different experimental conditions. The proteins were subjected to SDS-PAGE and immunoblotted with appropriate anti-phospho-ERK1/2 antibody, anti-ERK1/2 antibody, anti-phospho-EGF-receptor (Tyr 992) antibody, anti-EGF-receptor antibody (Cell Signaling Technology, Beverly, Mass), anti-MKP-1 antibody, anti-Ki-ras2A antibody (Santa Cruz Biotechnology, Calif), or anti-α smooth muscle actin antibody (Sigma-Aldrich). The bands of proteins were visualized with an ECL system (Amersham Biosciences). Densitometric analysis was performed using NIH image software.26,28

RNA Interference (RNAi) of Ki-ras2A

For small interfering RNA (siRNA) assay, VSMC were transiently transfected with lamin A/C siRNA as a control or Ki-ras2A-specific siRNA, a cocktail of 3 siRNAs designed by B-Bridge (Sunnyvale, Calif), using Lipofectamine PLUS (Invitrogen, Carlsbad, Calif).29 Thirty-six hours after transfection, cells were treated with or without Aldo and Ang II.

Materials

Reagents not listed above were as follows; Aldo and spironolactone were obtained from Sigma-Aldrich, and stored frozen at 10^{-2} mol/L (in ethanol). An AT1 receptor blocker, olmesartan, was donated by Sankyo Pharmaceutical (Tokyo, Japan). PD98059 was purchased from New England Biolabs (Beverly, Mass). All other reagents were purchased from Sigma-Aldrich.

Statistical Analysis

All values are expressed as mean±SEM in the text and figures. The data were evaluated by ANOVA followed by post-hoc analysis for multiple comparisons. Differences with P<0.05 were considered to be significant.

Results

Effect of Aldo With or Without Ang II on DNA Synthesis in VSMC

To examine the interaction of Aldo and Ang II in VSMC proliferation, we investigated the effect on [3H]-thymidine incorporation as a marker of DNA synthesis in rat-cultured VSMC. As shown in online Table I (available at http://circres.ahajournals.org), a higher dose of Aldo (10^{-9} mol/L) or Ang II (10^{-7} mol/L) stimulation significantly increased DNA synthesis in VSMC by 75% or 180%, respectively and combination of Aldo with Ang II further increased DNA synthesis than Aldo or Ang II alone at these doses. Treatment with a lower dose of Aldo (10^{-12} mol/L) or Ang II (10^{-10} mol/L) stimulation alone did not affect [3H]-thymidine incorporation, whereas the combination of these lower doses of Aldo (10^{-12} mol/L) with Ang II (10^{-10} mol/L) showed significantly enhanced DNA synthesis.

A MR antagonist, spironolactone (10^{-3} mol/L), or a selective AT1 receptor blocker, olmesartan (10^{-5} mol/L), abolished the increase in [3H]-thymidine incorporation in VSMC induced by the lower dose combination of Aldo and Ang II, suggesting a synergistic mitogenic interaction of the Aldo receptor and AT1 receptor (Figure 1). To determine whether Ang II influenced Aldo production and
secretion of VSMC in our assay condition, Aldo concentration in cultured-medium was measured. We observed that Ang II at a higher dose (10⁻⁷ mol/L) increased Aldo secretion in conditioned medium, but Ang II at a lower dose (10⁻¹⁰ mol/L) did not influence Aldo production and secretion (Figure 2). To examine the signaling mechanism, by which the Aldo and Ang II receptors mediated VSMC proliferation, we focused on ERK activity and EGF receptor activation. Pretreatment with a mitogen-activated protein kinase/extracellular signal-regulated kinase kinase (MEK) inhibitor, PD98059 (25 μmol/L) or AG1478 (10⁻⁷ mol/L) markedly inhibited VSMC proliferation induced by a lower dose combination of Aldo and Ang II (Figure 1), suggesting a synergistic mitogenic interaction between ERK cascade signaling through the Aldo receptor and AT₁ receptor activation, and EGF receptor activation is required for this mitogenic interaction between Aldo and Ang II.

**Effect of Aldo With or Without Ang II on ERK Activity in VSMC**

To further examine the signaling mechanism involved in the synergistic mitogenic interaction between Aldo and Ang II, we next investigated ERK activity determined by its phosphorylation. A higher dose of Aldo (10⁻⁷ mol/L) enhanced ERK activation as early as 7 minutes, reaching a peak at 15 to 30 minutes, followed by a gradual decrease in ERK activity, which was sustained for 2 hours in VSMC (Figure 3A). Ang II (10⁻⁷ mol/L) treatment activated ERK determined by its phosphorylation, reaching a peak at 5 to 10 minutes, followed by a decrease in its activity, and then reactivation showing a second peak after around 2 to 4 hours of Ang II stimulation (Figure 3B). A lower dose of Aldo (10⁻¹² mol/L) or Ang II (10⁻¹⁰ mol/L) alone did not significantly affect ERK activation (Figure 3C), whereas a combination of Aldo and Ang II at these doses synergistically induced time-dependent ERK activation, reaching a peak at around 10 to 15 minutes followed by a decrease, and showing a second peak at around 2 to 4 hours (Figure 3D).

**Aldo Potentiates AT₁ Receptor-Mediated ERK Activity Through Genomic and Nongenomic Mechanisms in VSMC**

Our results suggested that Aldo could increase AT₁ receptor-mediated VSMC proliferation at least in part by enhancing ERK activation. To further study the signaling mechanism of potential crosstalk of Aldo and Ang II receptors and determine whether Aldo potentiates Ang II-induced ERK activity by a genomic or nongenomic mechanism, we examined the effects of spironolactone, olmesartan, actinomycin D, and cyclohexamide on ERK activation induced by Aldo and Ang II. We observed that spironolactone (10⁻⁵ mol/L) markedly blocked Aldo (10⁻⁹ mol/L)-induced 30 and 60 minutes of ERK activation, but did not affect 10 and 15 minutes of ERK activation in VSMC (Figure 3E). As shown in Figure 3F, the early phase of ERK activation in VSMC after 10 minutes of stimulation with a lower dose combination of Aldo (10⁻¹² mol/L) and Ang II (10⁻¹⁰ mol/L) was effectively blocked by olmesartan, but not by spironolactone (Figure 3F). These results suggest that the rapid effect of Aldo on ERK activation with Ang II is mainly attributable to its nongenomic effects, because spironolactone has been reported to mainly act on the classic MR but not to efficiently blunt the rapid nongenomic action. In contrast, the late phase of ERK activation after 2 hours stimulation with Aldo and Ang II was markedly attenuated by spironolactone, olmesartan, actinomycin D, and cyclohexamide (Figure 3G).

**EGF Receptor Transactivation Is Required for ERK Activation Through Interaction Between Aldo and Ang II in VSMC**

We examined EGF receptor activation determined by its phosphorylation. We observed that the combination of Aldo

![Figure 1](http://circres.ahajournals.org/)

**Figure 1.** Effect of a lower dose combination of Aldo and Ang II on DNA synthesis in VSMC. DNA synthesis was assayed by measuring [³H]-thymidine incorporation as described in Materials and Methods. PD98059 or AG1478 was added 30 minutes before Aldo and Ang II stimulation. Similar results were obtained in 4 different culture lines. Values are expressed as mean±SEM (n=4). Aldo indicates aldosterone; Ang II, angiotensin II; Spiro, spironolactone; Olm, olmesartan. *P<0.05 vs control. †P<0.05 vs Aldo+Ang II (+).

![Figure 2](http://circres.ahajournals.org/)

**Figure 2.** Effect of Ang II on Aldo secretion. Aldo ELISA assay was performed as described in Material and Methods. Similar results were obtained in 3 different culture lines. Values are expressed as mean±SEM (n=3). Aldo indicates aldosterone; Ang II, angiotensin II; *P<0.05 vs Ang II (−).
(10^{-12} \text{ mol/L}) and Ang II (10^{-10} \text{ mol/L}) significantly enhanced EGF receptor phosphorylation 10 minutes after stimulation, whereas Aldo or Ang II at these doses did not affect EGF receptor phosphorylation. Moreover, we observed a sustained increase in EGF receptor phosphorylation 2 hours after combination treatment (Figure 4A). Furthermore, we pretreated VSMC with an EGF receptor tyrosine kinase inhibitor, AG1478 (10^{-7} \text{ mol/L}), and examined the effect of AG1478 on ERK activation. Interestingly, AG1478 markedly inhibited the early and late phases of ERK activation by a combination of lower doses of Aldo (10^{-12} \text{ mol/L}) and Ang II (10^{-10} \text{ mol/L}) (Figure 4B) to the basal level, suggesting that late phase activation of ERK as well as early activation of ERK requires transactivation of the EGF receptor in response to Aldo and Ang II in VSMC.

**Downregulation of MKP-1 by Aldo Combined With Ang II in VSMC**

To further address the signaling mechanism of the late phase of ERK activation by treatment with Aldo and Ang II, we examined MKP-1 expression in VSMC. MKP-1 is a dual specific phosphatase, which downregulates ERK activity in the nucleus.\textsuperscript{32} Aldo at a lower dose (10^{-12} \text{ mol/L}) or Ang II at a lower dose (10^{-10} \text{ mol/L}) alone had no detectable effect on MKP-1 expression (data not shown), whereas a combination of Aldo and Ang II at these doses markedly decreased MKP-1 expression, starting at 1 hour (Figure 5A). This decrease in MKP-1 expression was markedly inhibited in the presence of spironolactone or olmesartan (Figure 5B).

**Induction of Ki-ras2A by Aldo Combined With Ang II in VSMC**

To examine the upstream signaling of MKP-1 regulated by Aldo combined with Ang II, we focused on Ki-ras2A, which is one of the MKP-1 upstream targets and is reported to be mediated by the MR genomic pathway.\textsuperscript{33} We observed that Ki-ras2A expression was increased by a combination of lower doses of Aldo (10^{-12} \text{ mol/L}) and Ang II (10^{-10} \text{ mol/L}) (Figure 5C), whereas Aldo or Ang II alone at these doses did not affect Ki-ras2A expression (data not shown). This increase in Ki-ras2A expression was markedly inhibited in the presence of spironolactone or olmesartan (Figure 5D).

*Figure 3. Effect of Aldo (10^{-9} \text{ mol/L}; A) or Ang II (10^{-7} \text{ mol/L}; B) on ERK phosphorylation. C, Effect of Aldo (10^{-12} \text{ mol/L}) or Ang II (10^{-10} \text{ mol/L}) on ERK phosphorylation. D, Effect of Aldo (10^{-12} \text{ mol/L}) with Ang II (10^{-10} \text{ mol/L}) on ERK phosphorylation. E, Effect of spironolactone (10^{-5} \text{ mol/L}) on Aldo (10^{-9} \text{ mol/L})-induced ERK phosphorylation. \textsuperscript{*}P<0.05 vs control. \textsuperscript{#}P<0.05 vs Aldo (+). F, Effect of spironolactone (10^{-5} \text{ mol/L}), olmesartan (10^{-5} \text{ mol/L}), actinomycin D (5 \mu\text{g/mL}), or cyclohexamide (20 \mu\text{g/mL}) on ERK phosphorylation 10 minutes after stimulation (F) or 2 hours after stimulation (G) with Aldo (10^{-12} \text{ mol/L}) and Ang II (10^{-10} \text{ mol/L}). Actinomycin D or cyclohexamide was added 30 minutes before stimulation with Aldo and Ang II. Western blot analysis was performed as described in Materials and Methods. Representative immunoblots are shown from 4 separate experiments. Values are expressed as mean±SEM of densitometric measurements (n=4). Aldo indicates aldosterone; Ang II, angiotensin II; Spiro, spironolactone; Olm, olmesartan; ACD, actinomycin D; CHX, cyclohexamide. \textsuperscript{*}P<0.05 vs control. \textsuperscript{#}P<0.05 vs Aldo+Ang II (+).*
Downregulation of Ki-ras2A Attenuated MKP-1 Decrease and Late Phase of Activation of ERK by Aldo Combined With Ang II in VSMC

To assess the effect of the upregulated Ki-ras2A expression by a combination of Aldo and Ang II on ERK activation, we analyzed ERK activation in Ki-ras2A knock-down cells treated by RNA interference. To confirm the effectiveness of Ki-ras2A-siRNA, we performed immunoblot analysis and determined Ki-ras2A expression level. Ki-ras2A was expressed in control-siRNA-treated VSMC, but its expression was significantly suppressed in Ki-ras2A-siRNA-treated VSMC. Furthermore, the reduction of MKP-1 expression after 2 hours of incubation with a combination of Aldo (10^{-12} mol/L) and Ang II (10^{-10} mol/L) was markedly restored in Ki-ras2A-siRNA-transfected VSMC (Figure 6A). In such conditions, ERK was markedly and clearly attenuated in the late phase, as shown in Figure 6B.

Rapid ERK Pathway Involved in EGF Receptor Transactivation Mediates Genomic Induction of Ki-ras2A in VSMC

Finally, to assess whether the early phase of ERK activation involved in EGF receptor transactivation could affect MKP-1 and Ki-ras2A expressions, we pretreated VSMC with PD98059 (25 μmol/L) or AG1478 (10^{-7} mol/L) and analyzed the expressions of MKP-1 and Ki-ras2A. Interestingly, both PD98059 and AG1478 markedly restored the combined effect of Aldo (10^{-12} mol/L) and Ang II (10^{-10} mol/L)-reduced MKP-1 expression (Figure 7A and 7B) and effectively suppressed the increase in Ki-ras2A expression by Aldo and Ang II at these doses (Figure 7A and 7B), suggesting that rapid ERK pathway involved in EGF receptor transactivation in response to Aldo and Ang II could modulate genomic actions including induction of Ki-ras2A and subsequent downregulation of MKP-1.

Discussion

In this study, we demonstrated that treatment with lower doses of Aldo (10^{-12} mol/L) and Ang II (10^{-10} mol/L) significantly enhanced VSMC proliferation with an increase in ERK activation, whereas Aldo or Ang II alone at these doses did not affect VSMC proliferation. These results suggest that a lower dose combination induces a synergetic rather than an additive effect on VSMC proliferation. In cultured VSMC, Ang II at a higher dose (10^{-7} mol/L) increased Aldo concentration in conditioned medium, but Ang II at a lower dose (10^{-10} mol/L) did not influence Aldo production and secretion. Moreover, we demonstrated that treatment with Aldo (10^{-9} or 10^{-12} mol/L) did not change AT_{1} and AT_{2} receptor binding in VSMC (data not shown). Various in vivo and in vitro data have shown that Aldo increased Ang II receptor binding in rat VSMC, smooth muscle and vessels. This apparent discrepancy might be attributable to the experimental conditions. These findings suggest that the synergistic mitogenic response of VSMC to lower doses of Aldo (10^{-12} mol/L) and Ang II (10^{-10} mol/L) could be mediated via direct signaling cross-talk between the Aldo receptor and AT_{1} receptor.

Indeed, consisted with previous study that the synergism between Aldo and Ang II in terms of intracellular calcium via nongenomic signaling in VSMC, here we demonstrated the synergistic interaction of signaling between Aldo and Ang II receptor via nongenomic and genomic signaling, resulting in mitogenic response of VSMC. Our results indicated that a combination of Aldo and Ang II at lower doses synergistically induced time-dependent ERK activation, reaching a peak at around 10 to 15 minutes, followed by a second peak at around 2 to 4 hours. Early phase ERK activation was markedly inhibited by olmesartan, but not by spironolactone, whereas late phase ERK activation was attenuated by both, as
well as by actinomycin D or cyclohexamide. These results provide us with the new concept that Ang II signaling interacts with Aldo signaling mediated via both rapid and genomic mechanisms, resulting in the potentiation of ERK activity in VSMC. The classical genomic mechanism of aldosterone characterized by several hours of stimulation has been well described to involve binding to intracellular receptors, transcription and protein synthesis.4,5 Recently, rapid Aldo effects have been extensively characterized in vitro as interfering with the mechanisms of pH-regulation, calcium homeostasis, generation of inositol-1, 4, 5-trisphosphate, and protein kinase C.35–38 However, there is still considerable controversy on the identity of the receptors that mediate Aldo-effects on ERK activation. Some recent studies reported that Aldo rapidly interacts with EGF receptor-induced ERK1/2 signaling though non-genomic Aldo receptor.20 In contrast, Stockand et al demonstrated that Aldo via classical MR activates the ERK1/2 cascade in rat cardiac fibroblasts.10 Mazak et al also suggested that the potentiation of Ang II-induced ERK1/2 and JNK phosphorylation by Aldo is dependent on MR.21 Here, we clearly showed that there are two different pathways via rapid Aldo signaling and genomic classical MR binding, which interact with AT1 receptor-mediated signaling of the ERK-activation cascade and exerts growth-promoting effects in VSMC.

Spindler et al8 and Stockand et al9,10 showed that Ki-rasA gene expression and its protein level are increased by Aldo binding to MR. Moreover, it is demonstrated that induction of Ki-rasA is associated with a stimulation of cardiac fibroblast proliferation.10 Lin et al demonstrated that activated ERK can trigger MKP-1 degradation.39 These findings lead to the speculation that Aldo- and Ang II-induced Ki-ras2A participates in proteolysis, contributing to downregulation of MKP-1, thereby facilitating the late phase of ERK activation during VSMC proliferation. Indeed, we demonstrated that a combination of Aldo and Ang II at lower doses caused a significant increase in Ki-ras2A expression. Using RNA interference of Ki-ras2A, we further linked the induction of Ki-ras2A with downregulation of MKP-1 and consequent activation of ERK. Therefore, our findings indicate that genomic de novo protein synthesis of Ki-ras2A is involved in down-regulation of MKP-1, thereby resulting in late phase activation of ERK and enhanced VSMC proliferation.

In this study, we further demonstrated that rapid ERK activation associated with EGF receptor transactivation in
response to Aldo and Ang II has an impact on genomic induction of Ki-ras2A, and downregulation of MKP-1. We also observed that a combination of Aldo and Ang II at lower doses significantly enhanced EGF receptor phosphorylation in the early phase. Moreover, our results indicated that both an MEK inhibitor, PD98059, and an EGF receptor tyrosine kinase inhibitor, AG1478, effectively suppressed the increased Ki-ras2A expression and reduced MKP-1 expression, and markedly inhibited DNA synthesis in VSMC induced by Aldo together with Ang II. Therefore, in VSMC proliferation, we propose that the interaction between Aldo and Ang II through EGF receptor transactivation contributes to eliciting the rapid ERK pathway via the nonclassical Aldo receptor and AT1 receptor, which then influence transcriptional activation of Ki-ras2A expression via the classical MR and a decrease in the expression of MKP-1, thereby contributing to the late phase of ERK activation.

The possible signaling interaction between Aldo and Ang II stimulation leading to ERK activation in VSMC via two different pathways is shown in Figure 8. More detailed analysis of possible cross-talk between not only ERK and the classical MR, but also ERK- and MR-regulated specific gene expression involved in VSMC proliferation could contribute to elucidation of the pathogenesis of cardiovascular disease.
Taken together, our study results provide in vitro evidence of a direct effect of Aldo on VSMC growth. Our results also suggest that Aldo synergistically augments the mitogenic effects of Ang II in VSMC via interaction of the classical MR, the nonclassical Aldo receptor with the AT1 receptor. In clinical practice, Aldo antagonism by spironolactone is highly beneficial to patients experiencing from cardiovascular disease.40 Our results suggest that inhibition of both the classical MR and rapid Aldo actions has more beneficial effects to prevent the abnormal mitogenic effects of Aldo. Our findings also support experimentally the benefit of combination therapy with blockade of Aldo and AT1 receptors to prevent hypertension and progression to end-stage congestive heart-failure reported in clinical studies and animal models.41,42 In conclusion, activation of RAAS may exert growth-promoting effects and cardiovascular damage through a synergistic interaction of Aldo and Ang II. Thus, combination therapy achieving blockade of multiple targets might be a more effective approach to prevent various cardiovascular diseases.

Acknowledgments
This work was supported by grants from the Ministry of Education, Science, Sports, and Culture of Japan, Takeda Science Foundation and the Novartis Foundation of Gerontological Research.

References


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Circ Res. 2005;97:434-442; originally published online August 4, 2005;
doi: 10.1161/01.RES.0000180753.63183.95

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### Online Table 1

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**Online Table 1**: Effect of different concentrations of Aldo and/or Ang II on DNA synthesis in VSMC

DNA synthesis was assayed by measuring [³H]-thymidine incorporation as described in *Materials and Methods*. Values are expressed as mean ± SEM of four treatment wells from three different culture lines. Aldo, aldosterone. Ang II, angiotensin II. *p<0.05 vs. control.  a*p<0.05 vs Ang II (10⁻¹⁰ mol/L), Aldo (10⁻¹² mol/L).  b*p<0.05 vs Ang II (10⁻¹⁰ mol/L), Aldo (10⁻⁹ mol/L).  c*p<0.05 vs Ang II (10⁻⁷ mol/L), Aldo (10⁻¹² mol/L).  d*p<0.05 vs Ang II (10⁻⁷ mol/L), Aldo (10⁻⁹ mol/L).