Aldosterone-Induced Activation of Signaling Pathways Requires Activity of Angiotensin Type 1a Receptors

Catherine A. Lemarié, Stefania M.C. Simeone, Anna Nikonova, Talin Ebrahimian, Marie-Eve Deschênes, Thomas M. Coffman, Pierre Paradis, Ernesto L. Schiffrin

Rationale: Aldosterone has been shown to induce vascular damage, endothelial dysfunction, and myocardial fibrosis, which depend in part on activation of angiotensin II (Ang II)–mediated pathways. However, mechanisms underlying crosstalk between Ang II type 1 receptor (AT1R) and mineralocorticoid receptor (MR) are mostly unknown.

Objectives: We tested whether the lack of Ang II type 1a receptor (AT1aR) or Ang II type 1b receptor (AT1bR) would decrease cellular effects induced by aldosterone.

Methods and Results: We examined the effect of Ang II or aldosterone after transfection of mesenteric vascular smooth muscle cells (VSMCs) from C57Bl/6 mice with small interference RNA for AT1aR, AT1bR, or MR for 48 hours. Ang II and aldosterone separately induced ERK1/2, c-Jun NH2-terminal protein kinase (JNK), and nuclear factor (NF)-κB phosphorylation after a 20-minute stimulation. Small interference RNA for AT1aR downregulated phosphorylation of ERK1/2, JNK, and NF-κB after aldosterone stimulation compared to controls. Downregulation of AT1aR or MR only abolished the activation of NF-κB. In VSMCs from C57Bl/6 mice, aldosterone and Ang II induced the activation of the c-fos promoter from 30 minutes to 1 hour. This effect was blocked when using VSMCs from AT1aR knockout mice.

Conclusion: We show for the first time that nongenomic and genomic effects of aldosterone are differentially dependent on activity of both AT1aR and AT1bR. Our data suggest that aldosterone augments AT1R-dependent activation of ERK1/2, JNK, and NF-κB in VSMCs. We provide mechanistic understanding and experimental in vitro support for the benefit of combination therapy with dual blockade of AT1R and MR to treat hypertension and progression of heart failure as reported in clinical studies and animal models. (Circ Res. 2009;105:852-859.)

Key Words: aldosterone □ AT1R □ AT1aR □ AT1bR □ intracellular signaling

Pathophysiologically synergistic effects between angiotensin II (Ang II) and aldosterone have been described on vascular cells and support the concept that combination of aldosterone and Ang II receptor blockade may be therapeutically beneficial.1–4 In the past few years, investigators have demonstrated that blockade of mineralocorticoid receptor (MR) reduced mortality caused by progressive heart failure and sudden death from cardiac causes, as well as rate of hospitalization for heart failure. These results were observed in patients with severe heart failure who were also being treated with an angiotensin-converting enzyme inhibitor and included subjects developing heart failure after myocardial infarction.5,6

Growing evidence has shown that aldosterone could influence the signaling or trafficking of the Ang II type 1 receptor (AT1R). Indeed, mineralocorticoids such as deoxycorticosterone and aldosterone caused upregulation of Ang II binding to blood vessels and cultured VSMCs.4,7,8 Spironolactone, a specific antagonist of MR, has been shown to inhibit Ang II–stimulated proliferation of VSMCs.9 Recent studies have unraveled further evidence of a crosstalk between AT1R and MR. Ang II could directly stimulated nuclear localization of MR in human coronary and aortic VSMCs, supporting a role of MR in gene expression after Ang II stimulation.10 Spironolactone also inhibits Ang II–induced senescence of VSMCs. These studies suggest that vascular responses to Ang II could be mediated via direct signaling crosstalk between MR and AT1R.11

Among rodents, mice have 2 subtypes of AT1R, AT1aR and Ang II type 1b receptor (AT1bR). In the present study, we hypothesized that AT1aR and AT1bR interact differentially with MR to signal intracellularly. We sought to understand more precisely the molecular mechanisms underlying crosstalk between the 2 subtypes of AT1R present in the mouse, AT1aR and AT1bR, and MR. We focused on the effects that potential crosstalk had on activation of extracel-
ular signal-regulated kinase (ERK)\textsubscript{1/2}, c-Jun NH\textsubscript{2}-terminal protein kinase (JNK), and nuclear factor (NF)-\kappa B. We also questioned the possible interaction between AT\textsubscript{1}R and MR signaling at a genomic level.

**Methods**

**Cell Culture and Transfection**

VSMCs derived from mesenteric arteries of 8 weeks male C57Bl/6 and AT\textsubscript{1}R knockout mice were isolated and characterized as described previously.\textsuperscript{12,13} AT\textsubscript{1}R knockout mice were produced at Duke University\textsuperscript{14} and later bred in the animal facility at the Lady Davis Institute. Briefly, mesenteric arteries were cleaned of adipose and connective tissue and VSMCs were dissociated by the enzymatic digestion of vascular arcades. The tissue was filtered and the cell suspension centrifuged and resuspended in DMEM containing heat-inactivated FBS, HEPES, L-glutamine, penicillin, and streptomycin. VSMCs were cultured in DMEM containing 10% FBS and maintained at 37°C in a humidified incubator (5% CO\textsubscript{2}/95% air). Early passage cells (passes 3 to 6) were used. Before experimentation, cells were rendered quiescent by serum deprivation for 24 hours. VSMCs were then transfected with a negative control small interfering (si)RNA (luciferase) or siRNA (5 mmol/L) targeting AT\textsubscript{1}R, AT\textsubscript{1b}R, or MR (Qiagen) and then stimulated with Ang II or aldosterone (10\textsuperscript{−7} mol/L) after 48 hours in presence of siRNA. Where indicated, cells were also treated with losartan (10\textsuperscript{−5} mol/L) or eplerenone (10\textsuperscript{−5} mol/L) 30 minutes before stimulation with Ang II or aldosterone.

**Quantitative RT-PCR**

Efficiency of siRNA targeting AT\textsubscript{1}R, AT\textsubscript{1b}R, and MR was verified by quantitative real-time polymerase chain reaction (QRT-PCR). VSMC RNA was isolated by homogenization in TRIzol reagent (Invitrogen) followed by chloroform extraction and isopropanol precipitation. One microgram of total RNA was reversed-transcribed with random hexamers and Superscript II (Invitrogen). QRT-PCR was performed using 2 X QuantiTect SYBR Green PCR Kit (Qiagen) with the Mx3005P (Stratagene) detection system. Each QRT-PCR sample was normalized to the expression of ribosomal protein S16 and is expressed as fold change in siRNA targeting AT\textsubscript{1}R, AT\textsubscript{1b}R, or MR-treated samples compared to negative control siRNA samples. Primers were designed using Primer3 (Massachusetts Institute of Technology Center for Genome Research, Cambridge). Annealing temperature were 58°C to 61°C. Primers are described in Online Table I.

**Western Blot**

For Western blot analysis, VSMCs were homogenized in lysis buffer (final concentrations in PBS: 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mmol/L PMSF, 1 μg/mL leupeptin, 1 μg/mL aprotinin, 1 μg/mL pepstatin, and 1 mmol/L NaV\textsubscript{O}\textsubscript{4}). Lysates containing 30 μg of protein were subjected to electrophoresis on polyacrylamide gels and transferred to nitrocellulose membranes. Membranes were incubated overnight with anti–phospho-ERK\textsubscript{1/2}, anti–phospho-JNK, and anti–phospho-AT1aR antibodies (Cell Signaling Technology). Monoclonal antibodies for β-actin were used to reprobe blots to confirm equal loading in all lanes (Cell Signaling Technology). For preparation of nuclear extracts, VSMCs were first lysed in 1 mL of hypotonic buffer (20 mmol/L HEPES [pH 7.9], 20 mmol/L Na\textsubscript{2}F, 1 mmol/L Na pyrophosphate, 1 mmol/L Na orthovanadate, 1 mmol/L EDTA, 1 mmol/L EGTA, 0.25 mmol/L Na molybdate, and antiproteases cocktail) and the nuclear fraction was then extracted using a high-salt buffer (hypotonic buffer supplemented with 20% glycerol and 0.4 mol/L NaCl). Nuclear extracts were subjected to electrophoresis and incubated with anti–NF-κB p65 antibody (Cell Signaling Technology). Monoclonal antibody for β-actin was used to reprobe blots (Sigma). An enhanced chemiluminescence system was used as the detection method (Pierce).

**Ang II Measurement in Media Supernatant**

Ang II levels in supernatant of VSMCs was evaluated with an ELISA kit according to the instructions of the manufacturer (AssayPro). Briefly, the kit uses a quantitative competitive sandwich enzyme immunoassay technique. A polyclonal antibody–specific for Ang II has been precoated onto a 96-well microplate. Ang II in standards and samples is competed by a biotinylated Ang II sandwiched by the immobilized antibody and streptavidin-peroxidase conjugate. All unbound Ang II is then washed away, and a peroxidase enzyme substrate is added. The color development is stopped and the color intensity measured (450 nm, Bio-Rad microplate spectrophotometer) (data not shown).

**Fluorescent–Ang II Binding Assay**

Cells were transfected with negative control or AT\textsubscript{1}R, AT\textsubscript{1b}R, or MR siRNA 48 hours before treatment with 10\textsuperscript{−7} mol/L aldosterone or Ang II for 20 minutes. Cells were then harvested and incubated 30 minutes at 37°C with Ang II–fluorescein isothiocyanate (FITC) (10\textsuperscript{−8} mol/L) (Sigma) in a binding buffer containing in mmol/L (Tris 79 mol/L, NaCl 100, KCl 5, MgCl\textsubscript{2} 5, 0.25% BSA). VSMCs form AT\textsubscript{1}R-deficient mice were used as negative control. Preparations were then fixed with 2% PAF for 20 minutes and incubated with DAPI (360c-fos luciferase construct (kindly provided by T. Hoang\textsuperscript{15}) for 48 hours before incubation with either Ang II or aldosterone for 30, 60, or 120 minutes. Cells were lysed in lysis buffer containing 100 mmol/L Tris-HCl (pH7.9), 10% Igepal-P40, and 1 mmol/L dithiothreitol and incubated 15 minutes before luciferase activity measurements in triplicate (Orion II microplate luminometer, Berthold).

**Transfection of VSMCs and Luciferase Assay**

VSMCs from C57Bl/6 and AT\textsubscript{1}R knockout mice were transfected with the mouse c-fos luciferase construct (kindly provided by T. Huang\textsuperscript{16}) for 48 hours before incubation with either Ang II or aldosterone for 30, 60, or 120 minutes. Cells were lysed in lysis buffer containing 100 mmol/L Tris-HCl (pH7.9), 10% Igepal-P40, and 1 mmol/L dithiothreitol and incubated 15 minutes before luciferase activity measurements in triplicate (Orion II microplate luminometer, Berthold).

**Statistical Analysis**

Data are presented as means±SEM. Within-group differences were assessed with 1-way ANOVA. Post hoc comparisons were tested with the Newman–Keuls test. A value of P<0.05 was considered statistically significant.

**Results**

ERK\textsubscript{1/2}, NF-κB, and JNK Activation by Aldosterone Requires a Functional AT\textsubscript{1}R

To evaluate the effect of aldosterone and Ang II on signaling pathways, we exposed VSMCs to aldosterone (10\textsuperscript{−9} to 10\textsuperscript{−10} mol/L) and Ang II (10\textsuperscript{−7} mol/L) after 48 hours in presence of siRNA. Where indicated, cells were also treated with losartan (10\textsuperscript{−5} mol/L) or eplerenone (10\textsuperscript{−5} mol/L) 30 minutes before stimulation with Ang II or aldosterone.
mol/L) or Ang II (10^{-9} to 10^{-10} mol/L) for 20 minutes and examined the activation of ERK1/2, JNK, NF-κB, and p38MAPK. Aldosterone- or Ang II–treated cells induced a concentration-dependent increase in ERK1/2, JNK (Figure 1, A, B), NF-κB, and p38MAPK phosphorylation (Figure 2A, 2B). However, 10^{-7} mol/L was the only dose of aldosterone leading to activation of JNK (Figure 1B) and was used in subsequent experiments. siRNA was used to knockdown AT1aR and AT1bR in VSMCs to examine the effect on ERK1/2, JNK, NF-κB, and p38MAPK activation. QRT-PCR with receptor-specific primers confirmed that transfection of AT1aR- and AT1bR-targeted siRNA resulted in a 70% decrease of AT1aR and 85% for AT1bR mRNA by 48 hours after transfection. Expression of AT1aR and AT1bR mRNA was unchanged in presence of siRNA targeting MR. QRT-PCR for Ang II type 2 receptor (AT2R) showed that mRNA expression of AT2R was unchanged in all conditions (Figure 3A). The binding of Ang II–FITC was decreased in presence of the acute AT1aR siRNA, and to a lesser extent after treatment with AT1bR siRNA, but was unaffected by MR siRNA compared to the negative control (Figure 3B). Western blot experiments showed that AT1aR knockdown blocked the activation of ERK1/2, JNK, NF-κB, and p38MAPK (latter data not shown) induced by Ang II. Interestingly, AT1bR knockdown abolished aldosterone-induced phosphorylation of ERK1/2, JNK, and NF-κB. However, AT1bR knockdown blunted aldosterone-induced NF-κB activation but not ERK1/2 and JNK phosphorylation (Figure 4). Nuclear translocation of NF-κB was also inhibited by the lack of AT1aR and AT1bR (Online Figure I). The activation of p38MAPK in response to aldosterone was independent of the presence of a fully active AT1aR. However, the response to Ang II required the presence of a fully active AT1aR (data not shown). The role of AT1R in aldosterone-induced signaling pathways was confirmed by AT1R blockade with losartan. The phosphorylation of ERK1/2, JNK, and NF-κB was indeed inhibited when cells were treated with losartan before stimulation with aldosterone or Ang II (Online Figure II). These results implicated AT1aR in some of the signaling pathways induced by aldosterone.

**NF-κB Activation Requires Crosstalk Between AT1R and MR**

Potential crosstalk between AT1aR and MR in the regulation of ERK1/2, JNK, and NF-κB activity was examined by knocking down MR using siRNA in C57Bl/6 VSMCs. Transfection efficiency was evaluated by QRT-PCR, which showed 75% knockdown of MR protein by 48 hours after transfection (Figure 3A). MR knockdown did not affect Ang II– or aldosterone-induced activation of ERK1/2 and JNK (Figure 5) but abolished the phosphorylation (Figure 5) and the nuclear translocation (Online Figure I) of NF-κB induced by either Ang II or aldosterone. The absence of MR did not affect the activation of p38MAPK (data not shown). VSMCs were also treated with eplerenone, an MR-selective antagonist. Western blot showed that eplerenone had no effect on aldosterone- or Ang II–induced ERK1/2 activation. In contrast, we found that aldosterone-induced activation of JNK was blocked by eplerenone. Furthermore, the aldosterone- or
Ang II–dependent phosphorylation of NF-κB was reduced by eplerenone (Online Figure II). These results suggest, on the one hand, that the activation of NF-κB is tightly regulated by crosstalk between AT1R and MR in response to aldosterone and Ang II. On the other hand, they demonstrate that aldosterone stimulation requires a functional AT1aR to induce activation of ERK1/2 independently of MR or partially dependent on MR for JNK activation. Ang II levels in the culture media were measured in the supernatant of VSMCs after knockdown of AT1aR, AT1bR, or MR after stimulation with vehicle, Ang II, or aldosterone for 20 minutes. Ang II was only detectable (ng/mL) in samples to which Ang II (10^{-7} mol/L) has been added exogenously during 20 minutes (data not shown).

**Aldosterone-Induced Genomic Effects Require a Functional AT1aR**

VSMCs from AT1aR-deficient mice and wild-type littermates were transfected with luciferase reporter gene −360c-fos. The time course of activation of −360c-fos in cells from wild-type mice showed that both Ang II and aldosterone increased luciferase activity from 30 minutes to 1 hour. The luciferase activity returned to basal levels after 2 hours of aldosterone stimulation but continued to increase after 2 hours when stimulated with Ang II. Interestingly, the luciferase activity induced by aldosterone or Ang II was equivalent to basal levels at any time point in cells lacking AT1aR (Figure 6).

**Discussion**

In the present study, we demonstrate that VSMC activation of signaling pathways such as ERK1/2, JNK, and NF-κB in response to aldosterone requires a functional AT1aR. These results are supported at the genomic level by the finding that AT1aR is also required for aldosterone-induced c-fos reporter gene activation. Growing evidence has demonstrated that Ang II and aldosterone may have pathophysiological synergistic effects on vascular cells. Ang II stimulates both systemic and local aldosterone production, whereas aldosterone can amplify Ang II effects by increasing the expression of angiotensin receptors and angiotensin-converting enzyme. Furthermore, the MR blocker spironolactone decreased cardiac hypertrophy, inflammation, and fibrosis induced by Ang II and Ang II–mediated oxidative stress, endothelial dysfunction, and resistance artery structural remodeling.

Aldosterone causes both rapid, non-genomic, and genomic effects on cardiovascular function. Rapid signaling by aldosterone can be mediated through classical MR (ie, that can be inhibited by spironolactone or eplerenone), which potentiate Ang II/AT1R-mediated actions through noncanonical MR, which have also been implicated in aldosterone/Ang II signaling. Aldosterone synergistically augments ERK1/2 activation, JNK phosphorylation, Ki-ras2A induction, and oxidative stress, processes involved in VSMC growth, senescence, fibrosis, and inflammation. In agreement with those studies, our results demonstrated that aldosterone induced activation of ERK1/2, JNK, and NF-κB through noncanonical and classical MR. We not only found that aldosterone and Ang II signaling crosstalk, but most importantly, that aldosterone signaling requires a functional AT1aR. More importantly, these results were confirmed by pharmacological blockade of AT1R and MR, demonstrating the clinical relevance of the resulted from the present study.
Several studies have suggested that Ang II and aldosterone in combination, at low or physiological doses, via AT1R and MR, induce the activation of ERK1/2 and c-Src, leading, respectively, to growth and migration of VSMCs.11,21 Mazak et al showed that in VSMCs from Sprague–Dawley rats, Ang II or aldosterone alone or in combination at higher concentration (10⁻⁷ mol/L) induced the activation of ERK1/2 and JNK. Spironolactone treatment of VSMCs from Sprague–Dawley rats was able to reduce aldosterone-induced activation of ERK1/2 and JNK.2 In the present study, we treated cells with relatively high concentrations of aldosterone, based on the fact that aldosterone levels as high as 10⁻⁷ mol/L have been reported in individuals with congestive heart failure.22,23,24 We found that high doses of Ang II or aldosterone increased the phosphorylation of ERK1/2, JNK, and NF-κB. However, activation of ERK1/2 was independent of MR, and JNK activation was only partially dependent on MR, suggesting that aldosterone may bind to a plasma membrane MR different from that blocked by spironolactone as proposed by Wehling et al.25 Differences between these results and those of Mazak et al may be explained in part by the use of different animal species (rat versus mouse). The use of siRNA in the present study may be more specific than spironolactone, which has been shown for example to also block glucocorticoid receptor.2 Nonetheless, we demonstrate here for the first time that some aldosterone-induced signaling pathways require a functional AT1aR. This effect is specific to ERK1/2, JNK, and NF-κB pathways, as shown by the fact that aldosterone-induced activation of p38MAPK was not dependent on activity of AT1aR.

Activation of ERK1/2 and JNK was independent of AT1bR. In contrast, the activation of NF-κB required the presence of a functional AT1bR. The role of AT1bR in the vasculature is poorly understood. This receptor subtype appears to have a more important role in the kidney and adrenal glands, where it has been shown to contribute to 20% of Ang II binding compared to 80% for AT1aR.26 However, Chen et al demonstrated that AT1bR is an important component of AT1R mRNA in major mouse vessels, such as abdominal aorta and femoral artery. They found that the Ang II concentration–response curves in abdominal aorta and femoral artery were similar between AT1aR-deficient and wild-type mice, suggesting that AT1bR is able to maintain a normal Ang II response in the absence of AT1aR. However, AT1bR-deficient mice do not exhibit abnormalities in basal blood pressure.27 The present is the first report to our knowledge demonstrating that the Ang II–induced activation of NF-κB is mediated by both AT1R subtypes.
Ang II production by VSMCs after 20 minutes of aldosterone stimulation was undetectable, suggesting that aldosterone could transactivate AT1aR, leading to increased phosphorylation of ERK1/2, JNK, and NF-κB. This hypothesis was first proposed by Wehling et al, who showed unequivocal rapid effects of aldosterone that were neither mimicked by cortisol nor blocked by spironolactone, and postulated that these nongenomic effects were mediated by a plasma membrane receptor distinct from the classical MR.25 Wildling et al recently identified specific aldosterone interaction sites on the endothelial cell surface structurally and functionally distinct from cytosolic receptors. These authors hypothesized that aldosterone interacts with a plasma membrane receptor site that mediates calcium influx into the cell cytosol.28 Supporting this hypothesis, Yamada et al demonstrated that the vasoconstrictor effect of aldosterone is nongenomic and independent of classical MR and is not inhibited by spironolactone but by AT1R blockers. These investigators reported that aldosterone induced calcium-dependent activation of intracellular transglutaminase, leading to dimerization of AT1R and to its activation in a manner distinct from the activation induced by binding of Ang II.29 Although we were unable to detect Ang II release by VSMCs in our experiments, this does not imply that blocking AT1R would not provide effective blockade of these aldosterone actions. Zou et al have shown that AT1R antagonists such as candesartan could still be effective in blocking the activation of AT1R even in the absence of Ang II. These investigators proposed that candesartan could act as an inverse agonist, that is, as an agent that stabilizes AT1R in an inactive conformation, thus suppressing its activation.30

Mazak et al have suggested that the activation of ERK1/2 by Ang II and aldosterone modulates the expression of genes via phosphorylation of Elk-1, which controls the production of c-fos.2 Jaffe and Mendelsohn demonstrated that Ang II–stimulated nuclear localization of MR, and spironolactone inhibited Ang II–mediated MR-dependent gene expression in VSMCs. Ang II–mediated MR activation was also inhibited by losartan, demonstrating a link between AT1R activation and MR transcriptional activation. These authors highlighted a specific role for MR independently of aldosterone.10 In the present study, we questioned whether the AT1R is required for the genomic activity of aldosterone as it is for nongenomic rapid effects. In VSMCs from wild-type mice, aldosterone induced the activation of the c-fos promoter at 30 and 60 minutes, similar to what is found with Ang II. Aldosterone-induced c-fos promoter activation was blunted in VSMCs from AT1aR knockout mice. These results strongly suggest that aldosterone also requires a functional AT1aR to induce genomic effects, such as transcriptional activity of the c-fos promoter. These actions are consistent with the aldosterone-induced activation of ERK1/2 and JNK, which have been shown to increase the activation of AP-1 transcription factor.31 Thus, our results may explain how aldosterone could influence the signaling of the AT1aR and how it could mediate, in part, some of the classical cellular effects of Ang II. Finally, the present study has shown, to our knowledge for the first time, that aldosterone regulates AT1aR-dependent gene expression.

Taken together, the results of the present study provide a partial mechanistic explanation of the signaling pathways induced by the activation of AT1R and MR. They also support the combined use of AT1R and MR antagonists in the treatment of cardiovascular disease, in particular in the context of hypertension, but also in heart failure. Further understanding of the molecular mechanisms by which aldosterone induces AT1aR-dependent signaling and gene expression, and which are the genes that are most relevant for the vascular pathophysiological effects of aldosterone, may lead to the development of novel cardiovascular therapeutic strategies.
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**Disclosures**

None.

**References**


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### SUPPLEMENTAL MATERIAL

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**Online Table I.** Sequence of primers for AT_{1a}, AT_{1b}, AT_{2}, MR and Ribosomal protein S16 used in the study and product length after QRT-PCR.
Online Figure I. Nuclear translocation of NF-κB requires AT₁αR, AT₁βR and MR in response to aldosterone or Ang II. Western blot of nuclear extracts to evaluate the amount of p65 subunit of NF-κB revealed that the translocation of NF-κB to the nucleus is increased in response to aldosterone or Ang II in comparison to a negative control. However, the amount of NF-κB in the nucleus in the absence of AT₁αR or AT₁βR or MR is not increased with aldosterone or Ang II compared to the vehicle. These results correlate with the decrease of NF-κB phosphorylation when AT₁αR, AT₁βR and MR are downregulated by siRNA (n=3). *p<0.05 vs Neg CTRL+vehicle.
Online Figure II. Pharmacological blockade of AT\textsubscript{1}R inhibited the activation of ERK1/2, JNK and NF-\kappaB. VSMC were treated with the AT\textsubscript{1}R antagonist losartan (10^{-5} \text{ mol/L}) 30 minutes prior to stimulation with either Ang II or aldosterone. Aldosterone or Ang II induced ERK1/2, JNK and NF-\kappaB activation in absence of losartan (n=10). Losartan decreased the phosphorylation of ERK1/2, JNK and NF-\kappaB induced by aldosterone or Ang II (n=10). VSMC were also pre-treated with the selective MR antagonist eplerenone (10^{-5} \text{ mol/L}) for 30 minutes and then incubated with Ang II or aldosterone for 20 minutes. Pharmacological blockade of MR had no effect on aldosterone-induced activation of ERK1/2 but inhibited the activation of JNK and NF-\kappaB. Blockade of MR had no effect on Ang II-dependent ERK1/2 or JNK activation, but inhibited NF-\kappaB (n=10). *p<0.05 vs vehicle and † p<0.05 vs eplerenone.