Aldosterone Stimulates Matrix Metalloproteinases and Reactive Oxygen Species in Adult Rat Ventricular Cardiomyocytes

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Abstract—Matrix metalloproteinases (MMPs), aldosterone, and reactive oxygen species (ROS) are implicated in myocardial remodeling. Although ROS, cytokines, and neurohormones regulate MMP in cardiac fibroblasts, it is unknown whether aldosterone regulates MMP in cardiomyocytes. Therefore, we tested the hypothesis that aldosterone regulates MMP in cultured adult rat ventricular myocytes (ARVMs). ARVMs were treated with aldosterone for 24 hours, and MMP-2 and MMP-9 activities were measured by zymography. Aldosterone (50 nmol/L) increased MMP-2 (43±5%) and MMP-9 (55±15%; P<0.001 for both) activities. Pretreatment with spironolactone (100 nmol/L) abolished the aldosterone-induced increase in MMP activities. Aldosterone (50 nmol/L; 30 minutes) increased mitogen/extracellular signal-regulated kinase (MEK) (31±3%) and extracellular signal-regulated kinase 1/2 (ERK1/2; 41±7%; P<0.001 for both) phosphorylation. U0126 (10 μmol/L), an MEK1/2 inhibitor, abolished the aldosterone-induced increase in MMP activities. Aldosterone increased intracellular ROS as assessed by dichlorofluorescein diacetate (27±4%; P<0.05). This increase was inhibited by apocynin, an NADPH oxidase inhibitor. Apocynin likewise inhibited aldosterone-induced ERK1/2 phosphorylation and the increase in MMP activities. Furthermore, the antioxidants MnTMPyP and N-acetylcysteine inhibited the aldosterone-induced increase in ERK1/2 phosphorylation and MMP activities, respectively. Protein kinase C (PKC) is implicated in the nongenomic effects of aldosterone. To test the role of PKC, ARVMs were pretreated with chelerythrine, a PKC inhibitor. Chelerythrine prevented the aldosterone-induced increase in ERK1/2 phosphorylation and MMP activities. Thus, aldosterone induces MMP activity in ARVM via activation of the mineralocorticoid receptor, PKC, and ROS-dependent activation of the MEK/ERK pathway. NADPH oxidase is a likely source of ROS in this system. (Hypertension. 2005;46:555-561.)

Key Words: aldosterone • mineralocorticoids • oxidative stress • cardiomyocytes

The renin-angiotensin-aldosterone system is activated in heart failure (HF). Aldosterone levels are often elevated in patients with HF and associated with poor clinical outcomes.1,2 Mineralocorticoid receptor (MR) antagonists decrease morbidity and mortality in patients with severe HF resulting from left ventricular (LV) systolic dysfunction3 and LV dysfunction after acute myocardial infarction.4 However, little is known about the role of aldosterone in the heart despite the presence of aldosterone synthase and MR in the myocardium.5,6

In experimental conditions, aldosterone may increase collagen synthesis7 by stimulating cardiac myocytes and fibroblast proliferation as well as a reparative response to inflammation and cell death.8,9 We and others have shown that aldosterone infusion causes myocardial fibrosis.10,11 Aldosterone mediates “genomic” (classical) effects by binding to a cytoplasmic MR and interacting with target genes. Nongenomic (rapid) action results from its interaction with a receptor that is thought to be distinct from the classical steroid receptor.12 Rapid aldosterone action may involve protein kinase C (PKC), Ca²⁺, eAMP, and inositol 1,4,5-triphosphate (IP3).13–15 Downstream of these second messengers, aldosterone activation of the mitogen-activated protein kinase 1/2 (MAPK1/2) signaling pathway promotes cardiac fibroblast proliferation.16

Aldosterone increases cardiac remodeling, fibrosis, and collagen secretion from cardiac fibroblasts.17,18 The resultant myocardial fibrosis is independent of organ hypertrophy and arterial pressure.19,20 Under nonpathological conditions, the balance of collagen synthesis and degradation is crucial to the homeostasis of the extracellular matrix. Increased matrix metalloproteinase (MMP) activity and decreased tissue inhibitors of MMP are observed in the failing remodeled myocardium.21 Myocardial cells, including ventricular myocytes, express a variety of MMPs22,23 and may participate in myocardial remodeling.24 Neurohormones stimulate MMP...
synthesis and release from ventricular myocytes.\textsuperscript{25} MMPs may trigger and promote LV remodeling, and inhibition of these MMPs prevents LV remodeling and dysfunction.\textsuperscript{26} Aldosterone and MMPs are implicated in the regulation of myocardial remodeling via their effects on the interstitium.\textsuperscript{10,17,27} It remains unknown whether aldosterone regulates MMP activity in cardiomyocytes.

Because MMP activation plays a critical role in LV remodeling in response to reactive oxygen species (ROS),\textsuperscript{28,29} inflammatory cytokines,\textsuperscript{30} and angiotensin II,\textsuperscript{17} we sought to test whether aldosterone regulates MMPs in cardiomyocytes via ROS and its effector the MAPK1/2 cascade. The current study demonstrates that aldosterone via the MR induces MMP activity, stimulates ROS production, and activates the MAPK1/2 cascade in cultured adult rat ventricular myocytes (ARVMs).

Materials and Methods

Isolation of Adult Rat Cardiac Myocytes

As described previously,\textsuperscript{31} ARVMs (90\% to 95\% purity) were isolated from the hearts of adult (200 to 220 g) male Sprague-Dawley rats, plated at a nonconfluent density of 30 to 50 cells/mm\textsuperscript{2} on plastic culture dishes (Fisher) precoated with laminin (1 \mu g/cm\textsuperscript{2}; Invitrogen), and maintained in ACCT medium (DMEM, 2 mg/mL BSA, 2 mmol/L L-carnitine, 5 mmol/L creatinine, 5 mmol/L taurine, 100 IU/mL penicillin, and 10 g/mL streptomycin) for 16 hours before drug treatment.

Drug Treatments

Myocytes were treated with aldosterone (10 to 100 nmol/L; Sigma) for 30 minutes for signaling and 24 hours for zymography. The mitogen/extracellular signal regulated kinase 1/2 (MEK1/2) inhibitor (U0126; 10 \mu mol/L; Calbiochem), was added 30 minutes before aldosterone, as were spironolactone (100 nmol/L; Sigma), nifedipine (10 \mu mol/L; Sigma), chelerythrine chloride (10 \mu mol/L; Sigma), 1,2-Bis(2-aminophenoxo)ethane-N',N',N',N'-tetraacetic tetrakis (acetoxymethyl) ester (50 \mu mol/L; Sigma), actinomycin D (5 \mu g/mL; Sigma), cycloheximide (10 \mu g/mL; Sigma), apocynin (100 \mu mol/L; Sigma), Mn(II/III)tetrakis(1-methyl-4-peridyl)porphyrin (MnTMPyP; 50 \mu mol/L; Calbiochem), and N-acetylcysteine (NAC; 5 mmol/L; Calbiochem).

Phosphorylation of MEK/Extracellular Signal-Regulated Kinase

Cells were collected in lysis buffer (1\% Triton X-100, 0.5\% Nonidet P-40, 10 mmol/L Tris, 1 mmol/L EDTA, 1 mmol/L EGTA, 150 mmol/L NaCl, 0.4 mmol/L PMSF, 0.2 mmol/L sodium orthovanadate, and 1 g/L leupeptin). Protein concentration was determined using Bradford assay (Bio-Rad). Equal amounts of total protein were separated by SDS-PAGE on 10\% gels and transferred to Immobilon-P transfer membrane (Amersham), which was probed with anti-phospho–extracellular signal-regulated kinase 1/2 (ERK1/2; 1:1000; Cell Signaling), anti-phospho–MEK (1:1000; Santa Cruz), anti-total ERK1 (1:1000; Cell Signaling) or anti-total MEK (1:1000; Cell Signaling) antibodies. Chemiluminescence was quantified by densitometry (Molecular Analyst; Bio-Rad).

Assessment of MMPs

MMP activity was determined by in-gel zymography as described previously.\textsuperscript{32} Briefly, ARVMs were treated for 24 hours in 100-mm dishes in media without albumin. Conditioned medium was concentrated with Centricon YM30 concentrator (Millipore), and protein was determined by the Bradford assay (Bio-Rad). MMP identity was determined by estimated molecular weights against prestained molecular weight markers. MMP protein levels were assessed by Western blotting of conditioned media using a monoclonal mouse MMP-2 antibody (Chemicon).

Assessment of Intracellular ROS

Intracellular ROS were assessed with the ROS-sensitive fluorophore dichlorofluorescein diacetate (DCF; Molecular Probes) as described previously.\textsuperscript{15} Briefly, cells were incubated with 20 \mu mol/L DCF for
Results

Aldosterone Induces MMP Activity and Expression in ARVMs Via MR

In-gel zymography of conditioned media from ARVMs treated with aldosterone for 24 hours significantly increased MMP-2 and MMP-9 activities. Specific bands corresponded to the molecular weights of MMP-2 (72/66 kDa; gelatinase A) and MMP-9 (95/88 kDa; gelatinase B; Figure 1A). Aldosterone increased the proenzyme and active enzyme bands for MMP-2 and MMP-9. The response to aldosterone is maximal at 50 nmol/L. At this concentration, aldosterone increased MMP-2 activity (Figure 1B) by 43 ± 5% (P < 0.001 versus control) and MMP-9 activity (Figure 1C) by 55 ± 15% (P < 0.001 versus control). The increase in MMP activity was accompanied by an increase in MMP-2 protein expression (83 ± 13%; P < 0.05 versus control).

Spironolactone (100 nmol/L), an aldosterone receptor antagonist, abolished the aldosterone-induced increase in MMP-2 and MMP-9 activities (P < 0.01 versus aldosterone for both; Figure 1A through 1C).

Increased MMP Activity Is Mediated Via PKC and MEK and ERK Phosphorylation

U0126 (10 μmol/L), an MEK1/2 inhibitor, abolished the aldosterone-induced increase in MMP-2 and MMP-9 activities (P < 0.001 versus aldosterone for both), suggesting that the MEK/ERK pathway is involved in the aldosterone-induced activation of these gelatinases (Figure 1A through 1C).

By Western blotting, aldosterone (50 nmol/L) induces MEK and ERK1/2 phosphorylation that was present within 10 minutes, sustained for ≥60 minutes, and maximal at 30 minutes. Aldosterone treatment (50 nmol/L) for 30 minutes increased MEK phosphorylation by 31 ± 3% and ERK1/2 phosphorylation by 41 ± 7% (P < 0.001 for both). There was minimal activation of ERK1/2 at 6 and 24 hours (data not shown). Thirty-minute pretreatment with spironolactone (100 nmol/L) inhibited the aldosterone-induced increase in MEK and ERK1/2 phosphorylation (P < 0.001 versus aldosterone for both; Figure 2).

Aldosterone increases calcium13–15,33 and induces cell death pathways in the presence of calcium.9 Thus, we sought to test whether calcium was involved in aldosterone-induced MMP regulation. Pretreatment with nifedipine (10 μmol/L), an L-type calcium channel antagonist, did not inhibit aldosterone-induced ERK1/2 phosphorylation (Figure 3A).

Chelerythrine chloride, a PKC inhibitor, was used to determine whether PKC signaling was involved in aldosterone-induced ERK1/2 phosphorylation and an increase in MMP activities. Pretreatment with chelerythrine chloride (10 μmol/L) inhibited aldosterone-induced ERK1/2 phosphorylation, suggesting involvement of PKC. Additionally, chelerythrine abolished the aldosterone-induced increase in MMP-2 (P < 0.05 versus aldosterone) and MMP-9 (P < 0.01 versus aldosterone) activities (Figure 3A and 3B). This suggests that PKC is involved in aldosterone-induced activation of gelatinases.

Nongenomic Effects of Aldosterone: Effects of Actinomycin D and Cycloheximide

Although the effects of aldosterone-induced ERK1/2 phosphorylation are rapid, we investigated whether they occur in the presence of the RNA and protein synthesis inhibitors actinomycin D (5 μg/mL) and cycloheximide (10 μg/mL), respectively (Figure 4A and 4B). The aldosterone-induced ERK1/2 phosphorylation was not inhibited by actinomycin D but was increased an additional 21 ± 3% (P < 0.05 versus aldosterone). Additionally, pretreatment with cycloheximide increased aldosterone-induced ERK1/2 phosphorylation by 38 ± 11% (P < 0.01 versus aldosterone).

Aldosterone and ROS

Treatment of ARVM with aldosterone (50 nmol/L; 30 minutes) induced a significant increase in intracellular ROS as measured by DCF fluorescence (27 ± 4%; P < 0.05 versus control; Figure 5A). Pretreatment with apocynin, an NADPH oxidase inhibitor, inhibited the aldosterone-induced increase in DCF fluorescence (P < 0.001 versus aldosterone).
To test the role of ROS in aldosterone-induced ERK1/2 phosphorylation and the increase in MMP activities, the ROS scavenger MnTmPyp and the antioxidant NAC were used. MnTmPyp abolished the increase in ERK1/2 phosphorylation in response to aldosterone (P<0.01 versus MnTmPyp; Figure 5B). Furthermore, MnTmPyp (data not shown) and NAC (5 nmol/L; Figure 6A and 6B) abolished the aldosterone-induced increase in MMP-2 activity by 28±2% after treatment with aldosterone (P<0.05 versus control). Chelerythrine also inhibited the increase in MMP-9 activity by 35±6% after treatment with aldosterone (P<0.01 versus control). Chelerythrine prevented the increase in MMP-2 activity seen with aldosterone (P<0.05 versus aldosterone). MMP-9 activity was increased by 35±6% after treatment with aldosterone (P<0.001 versus control). Chelerythrine also inhibited the increase in MMP-9 activity by 35±6% after treatment with aldosterone (P<0.01 versus aldosterone). Data are mean±SEM from 3 experiments.

Discussion
Aldosterone increases MMP activity and expression in cultured ARVMs. The aldosterone-induced increase in MMP activity is mediated via activation of the MR, PKC, and ROS-dependent activation of the MEK/ERK pathway. Our data further indicate that NADPH oxidase is a likely source of ROS in response to aldosterone stimulation. Aldosterone-induced activation of ERK1/2 occurs within minutes and is not affected by inhibitors of transcription and translation, suggesting that the ERK-dependent increase in MMP activity may be attributable to rapid, nongenomic effects of aldosterone (Figure 7). However, we do not exclude genomic effects of aldosterone.

Neurohormonal stimulation of cardiac myocytes and fibroblasts in culture regulates MMP production and activity. For example, stimulation of cardiac fibroblasts with inflammatory cytokines or increasing oxidative stress increases MMP expression and activity, whereas stimulation of cardiac fibroblasts with angiotensin II decreases MMP expression and activity. Aldosterone does not appear to have a direct effect on cardiac fibroblast MMP regulation in culture. In cardiac myocytes, endothelin and angiotensin II also increase MMP expression and activity. This study now extends the direct regulation of cardiac MMP activity to aldosterone via stimulation of cardiac myocytes. Inhibition of MMP activity has been shown to attenuate LV remodeling and dysfunction in a number of animal models. These data suggest that one of the mechanisms by which MR antagonists exert their beneficial effects is via downregulation of myocardial MMP activity.

Our current findings show that aldosterone increases apocynin-sensitive ROS in cardiac myocytes, suggesting activation of NADPH oxidase. We and others have shown that cardiac myocytes express NADPH oxidase, and that its activity is regulated by neurohormones. In cardiac myocytes, the antioxidants MnTMPyP, NAC, and the NADPH...
oxidase inhibitor apocynin inhibit aldosterone-stimulated ERK1/2 activation and MMP activity. Similarly, in vivo studies demonstrate an increase in NADPH oxidase subunits in the heart after aldosterone infusion. Furthermore, in these studies, cardiac fibrosis or hypertrophy are inhibited by antioxidants and apocynin.\textsuperscript{38,39} Our data implicate cardiac myocytes, but do not exclude infiltrating inflammatory cells, as the site of aldosterone-stimulated NADPH oxidase activity.

Nongenomic effects of aldosterone have been described in a number of cell types.\textsuperscript{40–43} These effects are characterized by rapid onset of action, specificity for mineralocorticoids, and insensitivity to inhibitors of transcription (eg, actinomycin D) and translation (eg, cycloheximide). The nongenomic effects may be insensitive to MR antagonists,\textsuperscript{44} and some have suggested the involvement of a plasma membrane receptor.\textsuperscript{9} Our data show that aldosterone-stimulated MEK/ERK1/2 phosphorylation is rapid and inhibited by MR antagonists, but not by actinomycin D or cycloheximide, suggesting that the MR or a related protein mediates these nongenomic effects. This has been demonstrated previously for other steroid hormones (eg, the estrogen receptor [ER] modulates NO

\textbf{Figure 5.} A, DCF fluorescence. Aldosterone (Aldo; 50 nmol/L) increased DCF fluorescence by 27±4\% (\(P<0.05\)). Pretreatment with apocynin (APC) inhibited the aldosterone-induced increase in DCF fluorescence (\(P<0.001\) vs aldosterone). Data are from a total of 73 to 200 cells counted per condition in 3 independent experiments. B, Aldosterone signaling is mediated by ROS. Treatment of ARVM with aldosterone for 30 minutes increases ERK1/2 phosphorylation by 53±9\% (\(P<0.01\) vs control). Pretreatment with MnTmPyp (50 \(\mu\)mol/L) inhibited the aldosterone-induced increase in ERK1/2 phosphorylation (\(P<0.01\) vs MnTmPyp alone; \(P=\text{NS}\) vs aldosterone).

\textbf{Figure 6.} A, Aldosterone (Aldo)-induced gelatinase activity is mediated by ROS. MMP-2 activity was increased by 38±11\% after treatment with aldosterone (\(P<0.05\) vs control). NAC prevented the increase in MMP-2 activity seen with aldosterone (\(P<0.05\) vs aldosterone). MMP-2 activity was increased by 17±2\% after treatment with aldosterone (\(P<0.001\) vs control). NAC also inhibited the increase in MMP-9 activity seen with aldosterone (\(P<0.01\) vs aldosterone). Data are mean±SEM from 3 experiments. B, Representative zymogram of gelatinase activity. Conditioned media were taken from untreated LV myocytes and those treated with 50 nmol/L aldosterone, 5 mmol/L NAC, and NAC/Aldo. Ctl indicates control. C, Aldosterone signaling is mediated by NADPH oxidase. Aldosterone treatment of ARVMs increased ERK1/2 phosphorylation by 126±28\% (\(P<0.05\) vs control). Pretreatment with 100 \(\mu\)mol/L apocynin (APC) inhibited the aldosterone-induced increase in ERK1/2 phosphorylation. Data are mean±SEM from 3 experiments. D, Representative zymogram of gelatinase activity. Conditioned media were taken from untreated LV myocytes and LV myocytes treated with 50 nmol/L aldosterone, 100 \(\mu\)mol/L apocynin, and apocynin/Aldo.
Synthetic activity via a G-protein–coupled pathway in a rapid, nongenomic manner). ER antagonists inhibit most of the nonclassical effects of estrogen, and the cytosolic ER translocates to the cell membrane with estrogen treatment. This mechanism is distinct from the one in cardiac myofibroblast cells where aldosterone activates ERK via upregulation of Ki-ras after 6 hours of stimulation.

The nongenomic effects of aldosterone stimulation have been associated with a number of second messenger systems, including calcium, IP3, diacylglycerol, and PKC activation. In cardiomyocytes, ERK1/2 phosphorylation is inhibited by the PKC inhibitor chelerythrine, suggesting that PKC is involved in our model. However, it is unclear whether the PKC isoform involved is calcium dependent or independent.

In conclusion, aldosterone directly induces MMP-2 and MMP-9 activities in ARVMs. This response is mediated by a rapid, nongenomic effect of aldosterone that involves activation of the MR, PKC, and the MEK/ERK2 pathway. Our findings further indicate that ROS play a role in the regulation of cardiac myocyte MMP activity in response to aldosterone. ROS acts at or above the level of MEK/ERK1/2, and NADPH oxidase is the likely source of ROS in this system.

**Perspectives**

Our data suggest that the rapid, nonclassical effects of aldosterone in cardiac myocytes may modulate genomic effects of aldosterone. These results provide novel insights into aldosterone signaling in cardiac myocytes and the mechanisms by which aldosterone affects the extracellular matrix. Additionally, inhibition of ROS may be a mechanism by which MR antagonists exert their beneficial effect in myocardial hypertrophy and remodeling. These results contribute to our understanding of the mechanisms involved in myocardial hypertrophy and ventricular remodeling and may provide a basis for possible future strategies to prevent or reverse cardiac remodeling.

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