Aldosterone Directly Induces Myocyte Apoptosis Through Calcineurin-Dependent Pathways

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Background—Aldosterone has recently attracted considerable attention for its involvement in the pathophysiology of heart failure, in which apoptotic cell loss plays a critical role. This study examined whether aldosterone directly induces myocyte apoptosis via its specific receptors.

Methods and Results—Neonatal rat cardiac myocytes were exposed to aldosterone (10^{-8} to 10^{-5} mol/L). Nuclear staining with Hoechst 33258 showed that aldosterone induced myocyte apoptosis in a dose- and time-dependent fashion. Treatment of myocytes with 10^{-5} mol/L aldosterone significantly increased the percentage of apoptosis (15.5±1.4%) compared with serum-deprived control (7.3±0.6%). Radio ligand binding assay revealed the existence of plasma membrane receptor with high affinity (K_{d}, 0.2 nmol/L) for aldosterone in cardiac myocytes but not in fibroblasts. Aldosterone rapidly (∼30 seconds) mobilized [Ca^{2+}], that was blocked by neomycin. Aldosterone induced dephosphorylation of the proapoptotic protein Bad, enhancement of mitochondrial permeability transition, decrease in mitochondrial membrane potential, and release of cytochrome c from the mitochondria into the cytosol with concomitant activation of caspase-3. These effects of aldosterone were inhibited by concurrent treatment with either an L-type Ca^{2+} channel antagonist, nifedipine, or inhibitors for the Ca^{2+}-dependent phosphatase calcineurin, cyclosporin A and FK506.

Conclusions—The present study demonstrates for the first time that the specific plasma membrane receptor (coupled with phospholipase C) for aldosterone is present on cardiac myocytes and that aldosterone accelerates the mitochondrial apoptotic pathway through activation of calcineurin and dephosphorylation of Bad, suggesting that the proapoptotic action of aldosterone may directly contribute to the progression of heart failure. (Circulation. 2004;110:317-323.)

Key Words: angiotensin ■ apoptosis ■ heart failure

Heart failure is a common, lethal condition associated with various cardiovascular diseases and remains a major cause of morbidity and mortality worldwide.1 Cardiac myocytes are known to undergo apoptosis in some pathological conditions, such as hypoxia and ischemia-reperfusion.2,3 Moreover, recent studies provide direct evidence that the progressive loss of cardiac myocytes by apoptosis is one of the most important components in the pathogenesis of heart failure.4 Several lines of evidence suggest that neurohormonal mechanisms play a central role in the progression of heart failure.5 In addition to activation of the sympathetic nervous system, the renin-angiotensin-aldosterone system is known to have a deleterious effect on the heart. Recent studies report that aldosterone is produced in failing human hearts6 and that aldosterone receptor antagonists, such as spironolactone and eplerenone, dramatically reduce morbidity and mortality from heart failure.7,8 Aldosterone-mediated nongenomic action, such as rapid activation of protein kinase C and rise in [Ca^{2+}], associated with phosphoinositide hydrolysis, is demonstrated in vascular smooth muscle cells (VSMCs) and endothelial cells.9,10 However, it remains to be determined whether a specific plasma membrane receptor is present on cardiac myocytes.

Aldosterone-mediated action may cause various intracellular responses associated with elevation of [Ca^{2+}]. Recent studies have suggested that elevation of [Ca^{2+}] induces apoptosis in some types of cells by activation of calcineurin, a Ca^{2+}-dependent phosphatase.11 Because calcineurin affects the function of the proapoptotic protein Bad, which accelerates the mitochondrial death signaling pathway,11 we hypothesized that aldosterone can directly induce myocyte apoptosis by activation of calcineurin.

This study was designed to determine whether cardiac myocytes have a plasma membrane receptor specific for aldosterone and whether aldosterone-mediated nongenomic
signaling affects myocyte apoptosis. We found that aldosterone directly induces myocyte apoptosis by activating its membrane receptor-mediated mitochondrial death signaling associated with the calcineurin-Bad pathway.

Methods

Cultured Neonatal Rat Cardiac Myocytes

Primary cultures of neonatal rat cardiac myocytes were prepared from neonatal Wistar rat hearts by digestion with 0.2% collagenase as described previously. All experiments were performed 36 to 48 hours after incubation with DMEM containing 0.5% FBS.

Experimental Protocols

Myocytes were incubated with aldosterone (10⁻⁸ to 10⁻⁵ mol/L) for 24 hours or incubated with 10⁻³ mol/L aldosterone for the indicated periods (12 to 48 hours). To evaluate the effects of Ca²⁺ and calcineurin, myocytes were stimulated by 10⁻⁵ mol/L aldosterone for 24 hours after pretreatment with nifedipine (10⁻⁹ mol/L), calcineurin inhibitors, cyclosporin A (10⁻⁶ mol/L), and FK506 (10 µg/mL) for 1 hour. To examine Bad dephosphorylation, myocytes were incubated with 10⁻⁵ mol/L aldosterone for 4 hours. Control myocytes were incubated in serum-free DMEM.

Histochemical Determination of Apoptosis

Histochemical staining of myocytes was performed. The cells were visualized by fluorescein microscopy, and the images were generated by dual-exposure photography. Apoptotic myocytes were identified on the basis of distinctive condensed or fragmented nuclear morphology, and apoptotic cell counts were expressed as a percentage of the total number of nuclei counted.

Radioligand Binding Assay

Membrane fraction of myocytes and fibroblasts were prepared as previously described. The fraction (50 µg of protein) was incubated for 1 hour at 37°C in a assay buffer. [3H]-aldosterone (Amer sham, UK), specific activity of 39.8 Ci/mol, was added at concentrations from 0.01 to 10 nmol/L. Incubates were transferred to Whatman GF/C filters (Whatman), and radioactivity was measured in a liquid scintillation counter. Specific binding was determined experimentally from the difference between counts in the absence and presence of 10 nmol/L cold unlabelled aldosterone. The Kd and Bmax values were estimated by Scatchard analysis of the saturation data.

Intracellular Ca²⁺ Levels

Determination of [Ca²⁺]i was performed as previously described. Myocytes grown on glass base dishes were loaded with 2×10⁻⁶ mol/L fura-2 AM (Molecular Probes) for 1 hour at 37°C. Then myocytes were incubated with PBS and [Ca²⁺], images were visualized using I on Optics dual-wavelength imaging system. Integration time was 0.17 seconds at each wavelength (340 and 380 nm), with a time increment of 0.1 seconds. The autofluorescence level was subtracted from each reading before calculation of [Ca²⁺]i. The system was calibrated by the method of Grynkiewicz et al.

Mitochondrial Permeability Transition and Transmembrane Potential

Myocytes were loaded with 5×10⁻⁶ mol/L calcein-acetoxyxymethylester (calcein-AM, Molecular Probes) in the presence of 2 to 5×10⁻³ mol/L cobalt chloride to quench the cytoplasmic signals. Then fluorescent myocytes were determined. Loss of ∆Ψm was assessed by Dye JC-1. Cells grown on coverslips were incubated in PBS containing 10⁻³ mol/L JC-1 at 37°C for 5 minutes. Fluorescence emission at 527 and 590 nm was determined after excitation at 480 nm.

Immunoblotting

Antibodies for cytochrome c (7H8.2C12, PharMingen), Bad (Transduction Laboratories), phospho-Bad (Cell Signaling), and horseradish peroxidase–conjugated anti-IgG (Amer sham) were used, and immunoblotting was performed as described. Chemoluminescence was detected with ECL Western blot detection kits (Amer sham).

Caspase-3 Activity

Caspase-3 enzymatic activity was determined with a CPP32 assay kit (MBL), which detects the production of the chromophore p-nitroanilide after its cleavage from the peptide substrate DEVD-p-nitroanilide, as described previously.

Statistical Analysis

Data are expressed as mean±SE of at least 6 samples derived from more than 6 separate experiments. Skewed data (see Figure 3) were expressed as median (interquartile range). Differences were analyzed by 1-way ANOVA combined with the Bonferroni test. P<0.05 was considered to indicate statistical significance.

Results

Induction of Myocyte Apoptosis

Histochemical nuclear staining with Hoechst 33258 and immunohistochemical staining of cellular desmin showed that aldosterone increased apoptotic myocytes in a dose-dependent fashion (Figure 1A). Treatment of myocytes with 10⁻⁸ mol/L and 10⁻⁵ mol/L aldosterone significantly increased the percentage of myocyte apoptosis to 12.3% and 15.5%, respectively, compared with serum-deprived control (7.3%) (Figure 1B). Aldosterone 10⁻⁵ mol/L also increased apoptotic myocytes in a time-dependent fashion (Figures 1A and 1C). We also assessed the percentage of myocyte apoptosis by fluorescence-activated cell sorter analysis and ascertained that the percentage of apoptosis was almost compatible with that estimated by Hoechst 33258 staining (data not shown). Treatment of myocytes with 10⁻⁵ mol/L aldosterone for 48 hours did not increase necrotic cell death as estimated by calcein acetoxyxymethyl ester and ethidium homodimer-1 staining or creatine kinase activity in the medium (data not shown).

Aldosterone Receptor on Plasma Membrane of Cardiac Myocytes

Specific saturable binding of aldosterone to plasma membranes of myocytes is illustrated in Figure 2. Scatchard analysis of specific binding in plasma membrane fraction shows maximum binding of 10.3±0.4 fmol/mg protein, with a calculated Kd of 0.23±0.03 nmol/L. In contrast, no specific binding sites for aldosterone were detected in plasma membrane fraction from cardiac fibroblasts.

Intracellular Ca²⁺ Mobilization

Figure 3 illustrates the rapid induction of [Ca²⁺]i by aldosterone in myocytes. Myocytes treated with aldosterone under Ca²⁺-free conditions showed a rapid increase in fluorescence intensity reflecting the mobilization of [Ca²⁺]i (Figure 3A). This phenomenon began within 30 seconds after addition and then slowly reached a plateau after 60 seconds. The median increase of [Ca²⁺]i, induced by aldosterone was 147 nmol/L (67 to 282 nmol/L) (Figure 3B). Neomycin, an inhibitor of
phospholipase C, completely blocked this rapid effect of aldosterone (Figure 3C).

**Effects of Ca²⁺ Antagonist and Calcineurin Inhibitors**

Treatment of myocytes with aldosterone markedly increased apoptosis (2.2-fold), as estimated on the basis of nuclear morphology (Figure 4). When myocytes were pretreated with nifedipine, cyclosporin A, or FK506, the percentage of apoptotic myocytes was significantly \( P<0.0001 \) decreased. Treatment of myocytes with nifedipine, cyclosporin A, or FK506 alone did not affect the percentage of apoptosis (data not shown). Cell length determined directly from the images using an edge-detection system\(^{19}\) was decreased (43% to 48%, \( P<0.001, n=6 \) ) by addition of nifedipine (10 \( \times 10^{-6} \) mol/L) but not cyclosporin A (10 \( \times 10^{-6} \) mol/L) or FK506 (10 \( \mu \)g/mL), suggesting that contractile inhibition by Ca²⁺ channel antagonists or addition of calcineurin inhibitors is not involved in the cell survival.

**Dephosphorylation of Bad**

In the serum-deprived control, Bad consistently existed in highly phosphorylated form in the myocytes, whereas aldosterone markedly inhibited the Bad phosphorylation levels to 31% of the control and increased the dephosphorylation levels of Bad to 198% (Figure 5). This effect was maximum at 4 hours and then gradually decreased (data not shown). When myocytes were pretreated with nifedipine, cyclosporin A, or FK 506, dephosphorylation levels of Bad were substantially inhibited toward the control level.

**Mitochondrial Permeability Transition, Membrane Potential, and Cytochrome C Release**

Myocytes displayed punctate green-staining mitochondria, indicative of an intact mitochondrial membrane under control conditions. When myocytes were treated with aldosterone for 24 hours, green fluorescence in mitochondria was markedly reduced, consistent with permeability transition (PT) pore opening (Figure 6A, top). Pretreatment of myocytes with nifedipine, cyclosporin A, or FK506 significantly inhibited the loss of green fluorescence in mitochondria and thus prevented aldosterone-induced PT pore opening.

Myocytes showed red-orange mitochondrial staining by JC-1, indicative of normal high membrane potentials under control conditions. When myocytes were treated with aldosterone for 24 hours, JC-1 fluorescence was significantly decreased, indicative of mitochondrial membrane depolarization (Figure 6A, bottom). Pretreatment of myocytes with nifedipine, cyclosporin A, or FK506 showed increased red-fluorescent intensity, indicating that \( \Delta \psi_m \) was markedly preserved.

As shown in Figure 6, cytochrome c was detected only in the mitochondrial fraction under control conditions. Aldosterone inhibited the immunoreactivity of cytochrome c in
mitochondria to 36% of the control, whereas the activity in the cytosolic fraction was increased up to 395% of the control, which was inhibited by pretreatment with nifedipine, cyclosporin A, or FK506.

Activation of Caspase-3

Caspase-3 activity in the myocytes treated with aldosterone for 24 hours significantly increased by 1.4-fold compared with the serum-deprived control (Figure 7). Pretreatment with nifedipine, cyclosporin A, or FK506 inhibited the aldosterone-induced activation of caspase-3 to 1.1-fold, 1.1-fold, and 1.0-fold, respectively.

Discussion

The present study demonstrates for the first time that (1) aldosterone directly induces myocyte apoptosis in a dose- and time-dependent fashion; (2) plasma membrane receptor with high-affinity binding sites for aldosterone exists on cardiac myocytes but not cardiac fibroblasts; (3) aldosterone rapidly increases \([\text{Ca}^{2+}]_i\) associated with phospholipase C hydrolysis and induces dephosphorylation of Bad with enhancement of mitochondrial PT, decrease in \([\text{Ca}^{2+}]_i\) release of cytochrome c from the mitochondria into the cytosol, and activation of caspase-3; and (4) aldosterone-mediated effects are inhibited by nifedipine, cyclosporin A, or FK506. Thus, our data clearly demonstrate that aldosterone induces a nongenomic intracellular response through phosphoinositide hydrolysis, resulting in stimulation of mitochondrial apoptotic pathway associated with calcineurin signaling and dephosphorylation of Bad.

In addition to the classic adrenal biosynthetic pathway, previous clinical and experimental studies have demonstrated the production of aldosterone and the presence of mineralocorticoid receptor in cardiovascular tissue.21–23 Recent studies provide evidence that aldosterone rapidly increases \([\text{Ca}^{2+}]_i\) in endothelial cells and VSMCs by prompting transport of \([\text{Ca}^{2+}]_i\) into the cytosol.
from storage sites via activation of phospholipase C, followed by an increase in inositol-1,4,5-triphosphate.9,10 Such aldosterone-mediated nongenomic action was not observed in isolated adult rat cardiac myocytes; Benitah and Vassort24 reported that Ca²⁺ current was increased by long-term incubation but not during short-term incubation (up to 6 hours) of aldosterone. This discrepancy between cardiac myocytes and VSMCs suggested that nongenomic aldosterone effects are restricted to specific target tissues. The present study showed for the first time the existence of the plasma membrane receptor specific for aldosterone in cardiac myocytes but not in cardiac fibroblasts, the binding affinity of which was very similar to that already reported in membrane fractions from human mononuclear leukocytes and pig kidneys (Kd, 0.23 nmol/L vs 0.1 to 0.4 nmol/L, respectively).9 Furthermore, we found that nuclear fractions from myocytes have specific binding sites for aldosterone, with maximum binding of 504 fmol/mg protein and Kd of 1.2 nmol/L, suggesting that myocardial plasma membrane receptor is apparently different from intracellular receptor. Taken together, these data clearly indicate that cardiac myocytes and VSMCs possess a similar membrane receptor for aldosterone that is coupled with phospholipase C and that its activation leads to a rapid intracellular signaling cascade associated with [Ca²⁺], and protein kinase C.

Apoptosis is governed by families of proteins with positive and negative regulatory members acting at serial steps along a programmed pathway.25 Bad is usually maintained in a phosphorylated and sequestered form in the cytosol by 14-3-3 proteins and cannot exert its death-promotive action. However, when Bad is dephosphorylated by apoptotic signals, it translocates to the mitochondria and heterodimerizes with Bcl-xL, suppressing their survival signals.26 Recently, Ca²⁺-mobilizing agents have been reported to dephosphorylate Bad by activating calcineurin to augment Bad heterodimerization with Bcl-xL, leading to apoptosis.11 In the present study, we have clearly shown that aldosterone decreases the phosphorylation levels of Bad and that this effect was significantly inhibited when myocytes were pretreated with an L-type Ca²⁺ channel antagonist or calcineurin inhibitors. Our findings therefore strongly suggest that Ca²⁺-dependent calcineurin activation and dephosphorylation of Bad have a central role in the aldosterone-induced death-signaling pathway.

Mitochondria possesses the porin channel, called voltage-dependent-anion channel, in the outer membrane.27 Binding of Bcl-xL protein to this channel usually closes (stabilizes) the PT pore. However, when Bad migrates into mitochondria and heterodimerizes with Bcl-xL, voltage-dependent-anion channel is opened. Thus, Bad increases mitochondrial PT and releases cytochrome c into the cytosol, with concomitant loss of Δψm.11,27 In our study, aldosterone induced PT pore opening, loss of Δψm, release of cytochrome c into the cytosol, and activation of caspase-3, which were inhibited by concurrent treatment with nifedipine, cyclosporin A, or FK506. These findings indicate that aldosterone accelerates the mitochondrial apoptotic pathway triggered by calcineurin-induced dephosphorylation of Bad.

The level of aldosterone in plasma is approximately 10⁻⁷ mol/L in patients with heart failure, and the level of aldosterone in myocardium is approximately 17 times higher than that in plasma.28,29 The aldosterone concentrations used in our study are therefore considered clinically relevant. Campbell et al30 reported in aldosterone-treated rats that aldosterone caused myocyte necrosis by mitochondrial injury and sarcolemmal contraction, because an increase in circulating aldosterone concentrations caused electrolyte imbalance, such as an enhanced potassium excretion, leading to activation of Na⁺/H⁺ and Na⁺/Ca²⁺ exchange in cardiac mitochondria and sarcolemma. Taken together, in the in vivo situation in which the circulating aldosterone levels and electrolyte homeostasis are maintained, aldosterone may induce myocyte apoptosis rather than necrosis.

The present data indicate that nifedipine significantly inhibits the proapoptotic effect of aldosterone, suggesting that transsarcolemmal Ca²⁺ influx is also involved in aldosterone-induced increase in [Ca²⁺]. Indeed, Benitah and Vassort24
have reported that aldosterone upregulates transsarcolemmal Ca\textsuperscript{2+} current into myocytes, probably by stimulating L-type Ca\textsuperscript{2+} channel subunit mRNA expression. Concurrent treatment of the myocytes with actinomycin D, an inhibitor of transcription, or cycloheximide, an inhibitor of protein synthesis, blunted the aldosterone-induced apoptosis (data not shown), therefore indicating that an L-type Ca\textsuperscript{2+} channel activation through a genomic effect is also partly involved in the effect of aldosterone. Thus, this evidence strongly supports the findings that calcineurin inhibitors were more effective than nifedipine in suppressing the proapoptotic effect of aldosterone.

In conclusion, our study has demonstrated that cardiac myocytes possess a plasma membrane receptor specific for aldosterone coupled with phospholipase C and that aldosterone induces myocyte apoptosis through a calcineurin-dependent mitochondrial death-signaling pathway triggered by rapid increase in intracellular Ca\textsuperscript{2+} levels. Aldosterone thus plays a crucial role in the progression of heart failure, and regulation of calcineurin and Bcl-2 family proteins may have important implications for the development of new therapeutic strategies for patients with heart failure.

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The AHA is hereby retracting these articles:

References

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