Original Article

Aldosterone Nongenomically Produces NADPH Oxidase–Dependent Reactive Oxygen Species and Induces Myocyte Apoptosis

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The roles of aldosterone in the progression of heart failure have not been fully elucidated. This study examined whether aldosterone nongenomically activates reactive oxygen species (ROS) production, causing myocyte apoptosis. Addition of aldosterone to neonatal rat cardiac myocytes caused the activation of NADPH oxidase and intracellular ROS production in a dose-dependent manner (10^{-9}–10^{-7} mol/L). NADPH oxidase activation was evident as soon as 5 min after aldosterone treatment. Neither an inhibitor for nuclear transcription (actinomycin D) nor an inhibitor of new protein synthesis (cycloheximide) blocked this rapid activation, and specific binding of aldosterone to plasma membrane fraction was inhibited by eplerenone, suggesting a nongenomic mechanism. Aldosterone did not affect the mRNA or protein levels of NOX2, which is a major subunit of NADPH oxidase in myocytes, after 48 h. Nuclear staining with DAPI showed that aldosterone (10^{-7} mol/L) increased the myocyte apoptosis (2.3 fold, $p<0.001$), coincident with the activation of caspase-3 (1.4 fold, $p<0.05$), compared with the serum-deprived control after 48 h. Aldosterone also induced phosphorylation of apoptosis signal–regulating kinase 1 (ASK1). These effects of aldosterone on myocyte ROS accumulation, ASK1 activation, and apoptosis were abolished by eplerenone, a mineralocorticoid receptor (MR) antagonist, apocynin, an inhibitor of NADPH oxidase activation, and tempol, a free radical scavenger, but by neither RU486, a glucocorticoid receptor antagonist, nor butylated hydroxyanisol (BHA), a mitochondrial ROS scavenger. In conclusion, aldosterone-mediated ROS production is blocked by eplerenone and induced by the nongenomic activation of NADPH oxidase, leading to myocyte apoptosis associated with ASK1 activation. These proapoptotic actions of aldosterone may play a role in the progression of heart failure. (*Hypertens Res* 2008; 31: 363–375)

***Key Words*: aldosterone, myocyte, apoptosis, heart failure, reactive oxygen species**

Introduction

Aldosterone is a major mineralocorticoid hormone secreted by the adrenal cortex, and a neurohormonal mediator of the renin-angiotensin-aldosterone system. Classically, it regulates electrolyte homeostasis and fluid balance by promoting sodium retention and potassium excretion in the kidney. Recent studies, however, have suggested that aldosterone is synthesized locally in the heart (*1*, *2*) and have revealed that aldosterone receptor (mineralocorticoid receptor [MR]) is present in rodent hearts (*3*, *4*) and human hearts (*5*).

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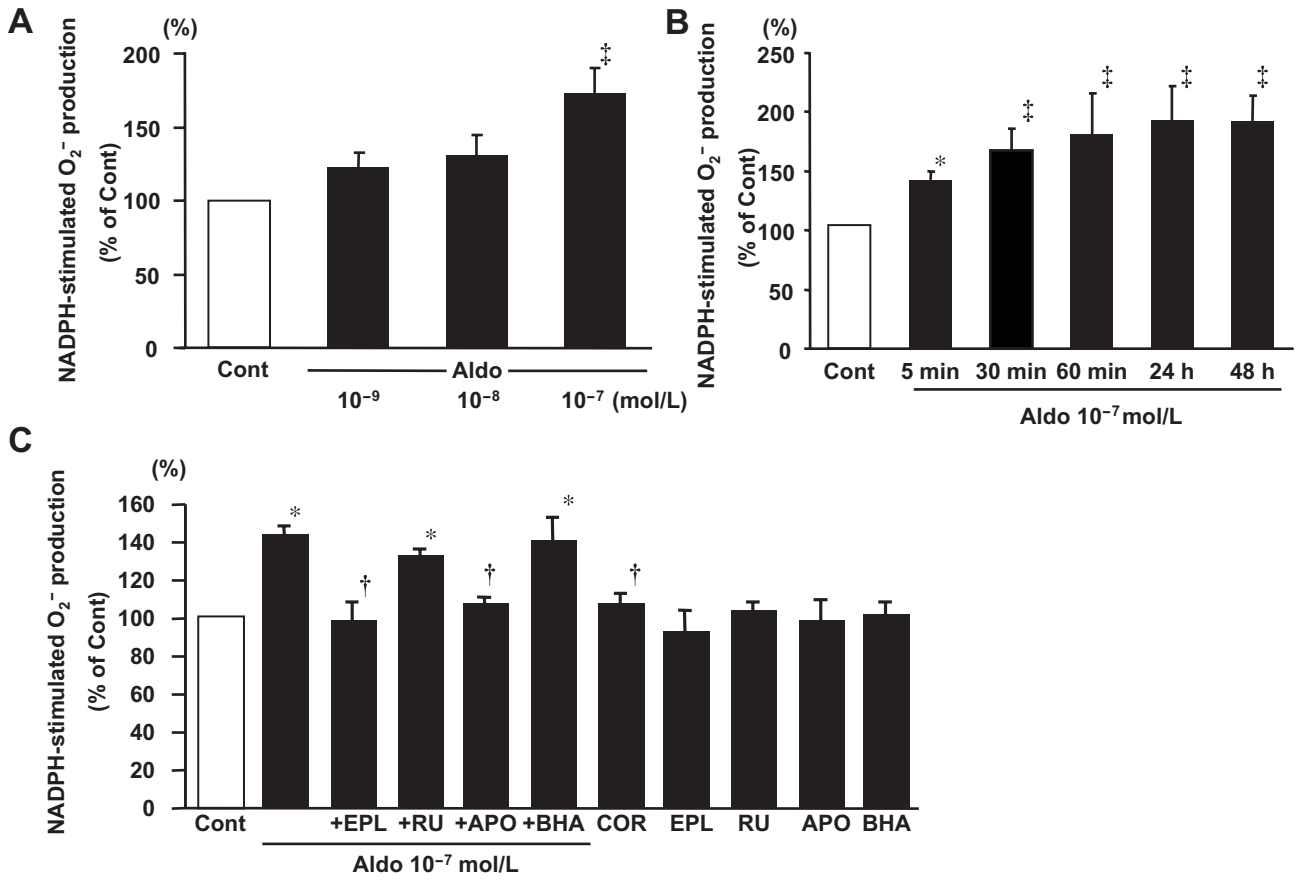

Fig. 1. *Effect of aldosterone (Aldo) on NADPH oxidase activity in myocytes. NADPH oxidase–dependent O_2^- production was measured by lucigenin-enhanced chemiluminescence. A: Myocytes were treated with the indicated concentrations of Aldo (10^{-9}–10^{-7} mol/L) for 30 min (n = 7–8). ‡p < 0.001 vs. control (Cont). B: Myocytes were treated with 10^{-7} mol/L Aldo for the indicated time periods (n = 5–7). *p < 0.05, ‡p < 0.001 vs. Cont. C: Myocytes were stimulated by Aldo (10^{-7} mol/L) or corticosterone (COR, 10^{-7} mol/L) for 30 min in the presence or absence of eplerenone (EPL, 10 μmol/L), RU486 (RU, 10 μmol/L), apocynin (APO, 300 μmol/L), or butylated hydroxyanisol (BHA, 10 μmol/L) (n = 5–7). *p < 0.01 vs. Cont; †p < 0.01 vs. Aldo. Data are expressed as the relative difference compared with Cont.*

Recently, two large clinical trials demonstrated that MR antagonists improve morbidity and mortality in patients with heart failure (*6*, *7*). Local aldosterone production in the heart tissue (*1*, *8*) as well as the plasma aldosterone level (*9*, *10*) have been reported to be increased in patients with heart failure. The plasma aldosterone level also positively correlates with the mortality in patients with heart failure (*9*). Furthermore, recent reports provide evidence that excess aldosterone has direct adverse effects on the heart (*11–14*). However, the underlying mechanisms of these deleterious effects have not been fully defined.

Reactive oxygen species (ROS) are exacerbating factors in the progression of heart failure (*15*). Experimental and clinical studies have demonstrated that excessive levels of ROS are produced in failing hearts, and are associated with the severity of heart failure (*16–18*). In addition, ROS are important apoptosis inducers (*19*), and progressive loss of cardiac myocytes by apoptosis has a critical role in the progression of heart failure (*20*).

One of the major sources of ROS in the cardiovascular system is NADPH oxidase (*21–23*), and its activity is upregulated in failing hearts (*22*, *23*). Aldosterone has been reported to activate NADPH oxidase in several kinds of cells *in vitro* (*24*, *25*). Chronic aldosterone infusion has been shown to increase NADPH oxidase activity in the heart tissue *in vivo*, leading to enhancement of cardiac oxidative stress and cardiac fibrosis (*11*, *26*). Aldosterone action is known to be induced *via* a classical genomic mechanism, whereas we have observed the nongenomic (rapid) action of aldosterone in cardiac myocytes (*27*). Aldosterone-induced NADPH oxidase activation has been reported to be a nongenomic action in renal mesangial cells and vascular smooth muscle cells (*24*, *25*). It is not fully understood whether aldosterone nongenomically stimulates NADPH oxidase, followed by the promotion

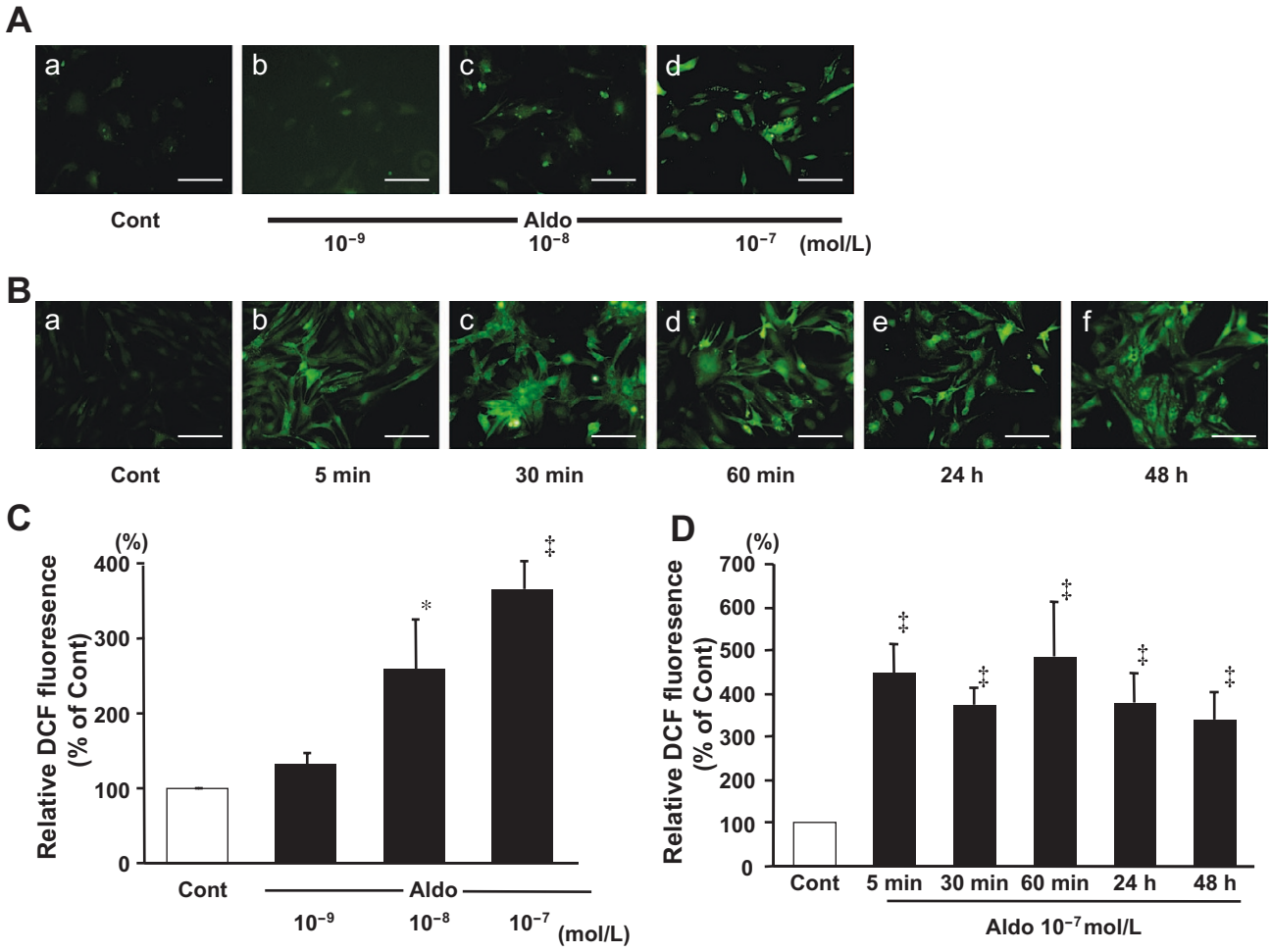

Fig. 2. *Effect of aldosterone (Aldo) on ROS production in myocytes. ROS production was evaluated by intracellular intensities of DCF fluorescence. A: Myocytes were treated with the indicated concentrations of Aldo (10^{-9}–10^{-7} mol/L) for 30 min. B: Myocytes were treated with 10^{-7} mol/L Aldo for the indicated time periods. Bars indicate 100 μm. Photomicrographs shown are representative of 5–7 independent experiments. C, D: Densitometric analysis of intensities of DCF fluorescence (n = 5–7). *$p < 0.05$, ‡$p < 0.001$ vs. controls (Cont). Data are expressed as the relative difference compared with Cont.*

of ROS production in cardiac myocytes.

The purpose of the present study was to determine whether aldosterone nongenomically increases NADPH oxidase–dependent ROS production and whether these aldosterone-mediated ROS actually induce myocyte apoptosis.

Methods

Reagents

Aldosterone, the nicotinamide adenine dinucleotide phosphate reduced form (NADPH), and butylated hydroxyanisol (BHA) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Actinomycin D and cycloheximide were purchased from Nacalai Tesque, Inc. (Kyoto, Japan). Apocynin was obtained from CALBIOCHEM (Darmstadt, Germany). RU486, 4-hydroxy-TEMPO (tempol), and *N*,*N*′-dimethyl-9,9′-biacridinium dinitrate (lucigenin) were acquired from Sigma-Aldrich Japan K.K. (Tokyo, Japan). 2′,7′-Dichlorofluorescein diacetate (H_2DCFDA) was purchased from Molecular Probes (Eugene, USA). Eplerenone was provided by Pfizer Inc. (New York, USA).

Cultured Neonatal Rat Cardiac Myocytes

All experimental procedures were performed according to the guidelines for the care and use of animals established by Kyoto Pharmaceutical University. Primary cultures of neonatal rat cardiac myocytes were prepared from Wistar rat hearts by digestion with 0.2% collagenase as described previously (*28*). We routinely obtained contractile myocyte-enriched cultures with >95% myocytes (*29*). Myocytes were cultured in Dulbecco's modified Eagle medium (DMEM) containing 10% fetal bovine serum (FBS). Bromodeoxyuridine (BrdU

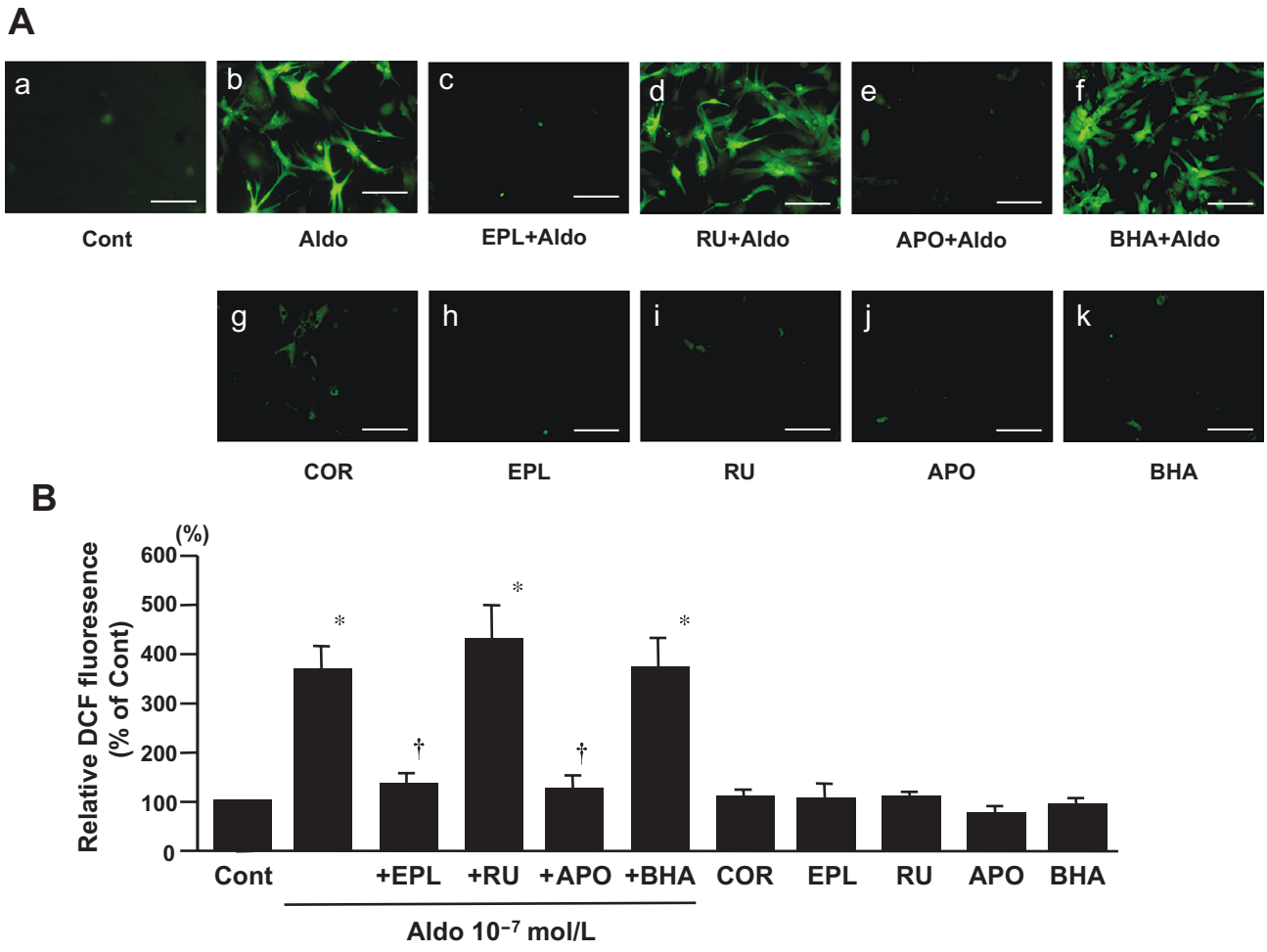

Fig. 3. *Effects of eplerenone (EPL), RU486 (RU), apocynin (APO), and butylated hydroxyanisol (BHA) on aldosterone (Aldo)-induced ROS production, and effect of corticosterone (COR) on myocyte ROS production. A: Myocytes were stimulated by Aldo (10^{-7} mol/L) or COR (10^{-7} mol/L) for 30 min in the presence or absence of EPL, RU, APO, or BHA. Bars indicate 100 μm. Photomicrographs shown are representative of 5–7 independent experiments. B: Densitometric analysis of intensities of DCF fluorescence (n = 5–7). *$p < 0.01$ vs. controls (Cont); †$p < 0.01$ vs. Aldo. Data are expressed as the relative difference compared with Cont.*

10^{-4} mol/L) was added during the first 48 h to inhibit proliferation of nonmyocytes. The myocytes were then incubated in DMEM containing 0.5% FBS without BrdU. All experiments were performed 36–48 h after this incubation.

Experimental Protocols

Cultured myocytes were washed twice with phosphate-buffered saline (PBS), followed by a final incubation in serum-deprived DMEM. During the final incubation, myocytes were treated with aldosterone (10^{-9} to 10^{-7} mol/L) for 30 min or treated with 10^{-7} mol/L aldosterone for the indicated time periods (5 min to 48 h). To evaluate the roles of MR, glucocorticoid receptor (GR), NADPH oxidase, mitochondria-derived ROS, and intracellular ROS, myocytes were stimulated by 10^{-7} mol/L aldosterone after treatment with eplerenone (10 μmol/L), a selective MR antagonist, RU486 (10 μmol/L), a selective GR antagonist, apocynin (300 μmol/L), an inhibitor of NADPH oxidase activation, butylated hydroxyanisol (BHA) (10 μmol/L), a mitochondrial ROS scavenger, and tempol (1 mmol/L), a free radical scavenger, for 15 min, respectively. To examine whether corticosterone mimics aldosterone-mediated effects, myocytes were treated with 10^{-7} mol/L corticosterone for the indicated time period (30 min or 48 h). To examine whether aldosterone nongenomically activates NADPH oxidase, we next examined the effects of actinomycin D and cycloheximide, inhibitors of transcription and protein synthesis, on aldosterone-induced NADPH oxidase activation. Myocytes were incubated with 10^{-7} mol/L aldosterone for 5 min after pretreatment with actinomycin D (5 μg/mL) or cycloheximide (20 μg/mL) for 15 min. Control myocytes were incubated in

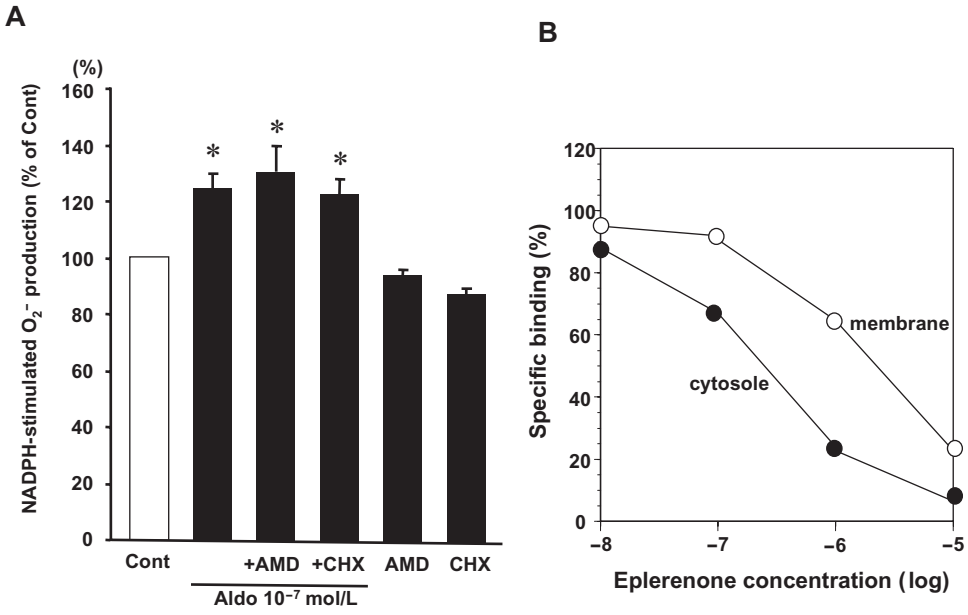

Fig. 4. *A: Effects of actinomycin D (AMD) and cycloheximide (CHX) on aldosterone (Aldo)-induced NADPH oxidase activation. Myocytes were stimulated by 10^{-7} mol/L aldosterone (Aldo) for 5 min after pretreatment with AMD (5 μg/mL) or CHX (20 μg/mL). *$p < 0.05$ vs. controls (Cont). Data are expressed as the relative difference compared with Cont ($n = 7–8$). B: Inhibition of [^{3}H]aldosterone binding to cardiac myocytes by eplerenone. The effect of eplerenone on specific binding of [^{3}H]aldosterone to the membrane fractions and cytosolic fraction of the myocytes was examined. Relative tracings are shown from 4 independent experiments.*

serum-deprived DMEM.

NADPH Oxidase Activity

NADPH oxidase–dependent O_2^- production in cardiac myocytes was measured by lucigenin-enhanced chemiluminescence as previously described with some modifications (*24*, *30*). Briefly, myocytes were detached from the culture dishes using 0.5% trypsin/EDTA, washed with PBS, and resuspended at 1×10^6 cells/mL in Krebs-HEPES buffer (pH 7.4) containing 145 mmol/L NaCl, 5 mmol/L KCl, 1.8 mmol/L $CaCl_2$, 1.2 mmol/L $MgCl_2$, 10 mmol/L glucose, 10 mmol/L HEPES, and 0.2% BSA. The cell suspension (5×10^4 cells/50 μL) was transferred into vials containing lucigenin (50 μmol/L) in Krebs-HEPES buffer (950 μL). After preincubation for 5 min, background lucigenin chemiluminescence was measured (Lumat LB9507; Berthold Tech. Co., Ltd., Tokyo, Japan). NADPH (100 nmol/L) was then added to the incubation medium as a substrate for O_2^- production and lucigenin chemiluminescence was measured every 10 s for 5 min. The chemiluminescence value was evaluated as the area under the curve (AUC).

Intracellular ROS

A fluorescent probe, H_2DCFDA, was used to evaluate intracellular ROS production in cultured myocytes. H_2DCFDA is cleaved by cellular esterases to nonfluorescent 2′,7′-dichlorofluorescein (DCFH) and oxidized by ROS to a fluorescent product, dichlorofluorescein (DCF). H_2DCFDA (5 μmol/L) was added to the cultured myocytes 30 min before the end of the aldosterone treatment. The cardiac myocytes were then washed with PBS and fluorescence images were detected with a microscope (Olympus IX71; Olympus Corp., Tokyo, Japan). The averages of the fluorescence intensity values from myocytes were quantified using image software from the National Institutes of Health (NIH).

Radioligand-Binding Assay

Myocytes were scraped and pelleted by centrifugation at $800 \times g$ for 5 min. The membrane fraction and cytosolic fraction of myocytes were prepared as previously described (*27*). The fractions (plasma membrane, 50 μg of protein; cytosol, 20 μg of protein) were incubated for 1 h at 37°C in an assay buffer (pH 7.4) containing 140 mmol/L NaCl, 20 mmol/L HEPES, 5 mmol/L KCl, 0.5 mmol/L $MgCl_2$, and 1 mmol/L phenylmethyl sulfonyl fluoride. [1,2-^{3}H]Aldosterone (Amersham Biosciences, Buckinghamshire, UK), specific activity of 39.8 Ci/mmol, was added at 10 nmol/L in the presence or absence of eplerenone (10^{-8}–10^{-5} mol/L). Incubates were transferred to Whatman GF/C filters (Whatman, Middlesex, UK), and radioactivity was measured in a liquid scintillation counter. Specific binding was determined experimentally

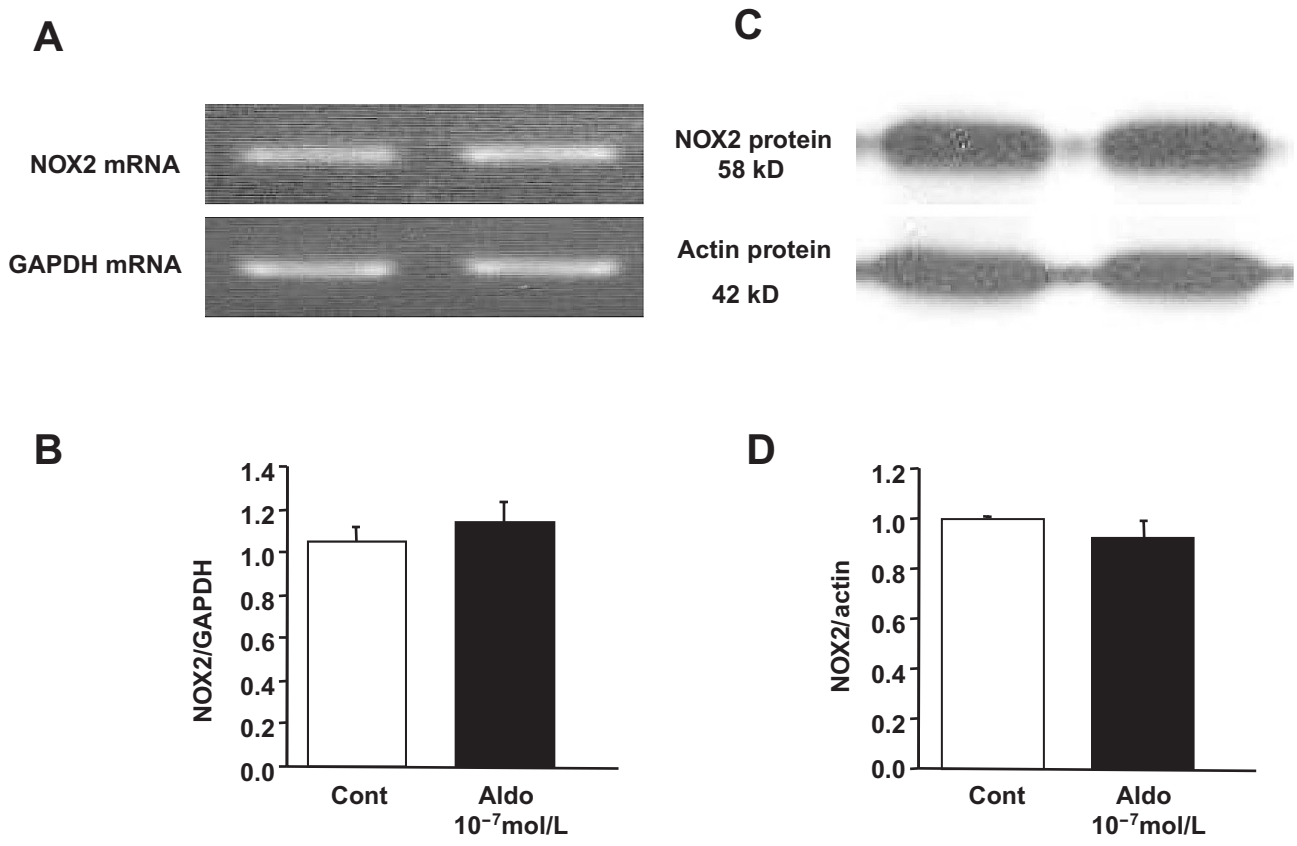

Fig. 5. *Effect of aldosterone (Aldo) on NOX2. A: The expression of NOX2 mRNA in cardiac myocytes. Myocytes were treated with 10^{-7} mol/L Aldo for 48 h. B: Densitometric analysis of NOX2 mRNA expression. C: The level of NOX2 protein in cardiac myocytes. Myocytes were treated with 10^{-7} mol/L Aldo for 48 h. D: Densitometric analysis of NOX2 protein level. Representative results are shown from 6 independent experiments.*

from the difference between counts in the absence and presence of 10 μmol/L unlabeled aldosterone.

Reverse Transcription–Polymerase Chain Reaction

The determination of NOX2 mRNA was performed by reverse transcription (RT)–polymerase chain reaction (PCR). Total RNA was isolated from cultured myocytes using Isogen regent (NIPPON GENE, Tokyo, Japan) and 1 μg of total RNA was reverse-transcribed with a Super Script II First-Strand-Synthesis System (Invitrogen Co., Carlsbad, USA). First-Strand cDNA was amplified by PCR using Taq DNA polymerase (TaKaRa EXTaq; TAKARA, Kyoto, Japan). The following protocol was used for each PCR: 94°C for 1 min (1 cycle), followed by 94°C for 1 min, 60°C (NOX2) or 57°C (GAPDH) for 1 min, and 72°C for 2 min (26 cycles), and a final extension phase at 72°C for 5 min. The primers used for the analysis of mRNA were as follows: NOX2 forward, 5′-TGACTCGGTTGGCTGCTGGCATC-3′; NOX2 reverse, 5′-CGCAAAGGTACAGGAACATGGG-3′; GAPDH forward, 5′-TCCCTCAAGATTGTCAGCAA-3′: GAPDH reverse, 5′-AGATCCACAACGGATACATT-3′. PCR products were run on a 2% agarose gel containing 0.5 μg/mL ethidium bromide. The expression of NOX2 was normalized to the expression levels of GAPDH.

Western Blot Analysis

Cardiac myocytes were lysed in a lysis buffer containing 20 mmol/L HEPES (pH 7.7), 2.5 mmol/L $MgCl_2$, 0.1 mmol/L EDTA, 500 μmol/L DL-dithiothreitol (DTT), 10 μmol/L Na_3VO_4, 1% Nonidet P-40, 0.1% Triton X-100, 1 mmol/L phenylmethylsulfonyl fluoride (PMSF), and 1% Protease Inhibitor Cocktail (Nacalai Tesque, Inc.) on ice for 10 min. Cell extracts were clarified by centrifugation and resolved on SDS-polyacrylamide gel electrophoresis followed by electroblotting onto PVDF membranes (Millipore, Bedford, USA). The membranes were blocked with 5% BSA in Tris-buffered saline containing 0.1% Tween 20 and then incubated with rabbit polyclonal antibody against phospho (P)-apoptosis signal–regulating kinase 1 (ASK1) (1:500; Cell Signaling Technology, Beverly, USA), or mouse monoclonal antibody against NOX2 (1:1,000; BD Transduction Laboratories, Lex-

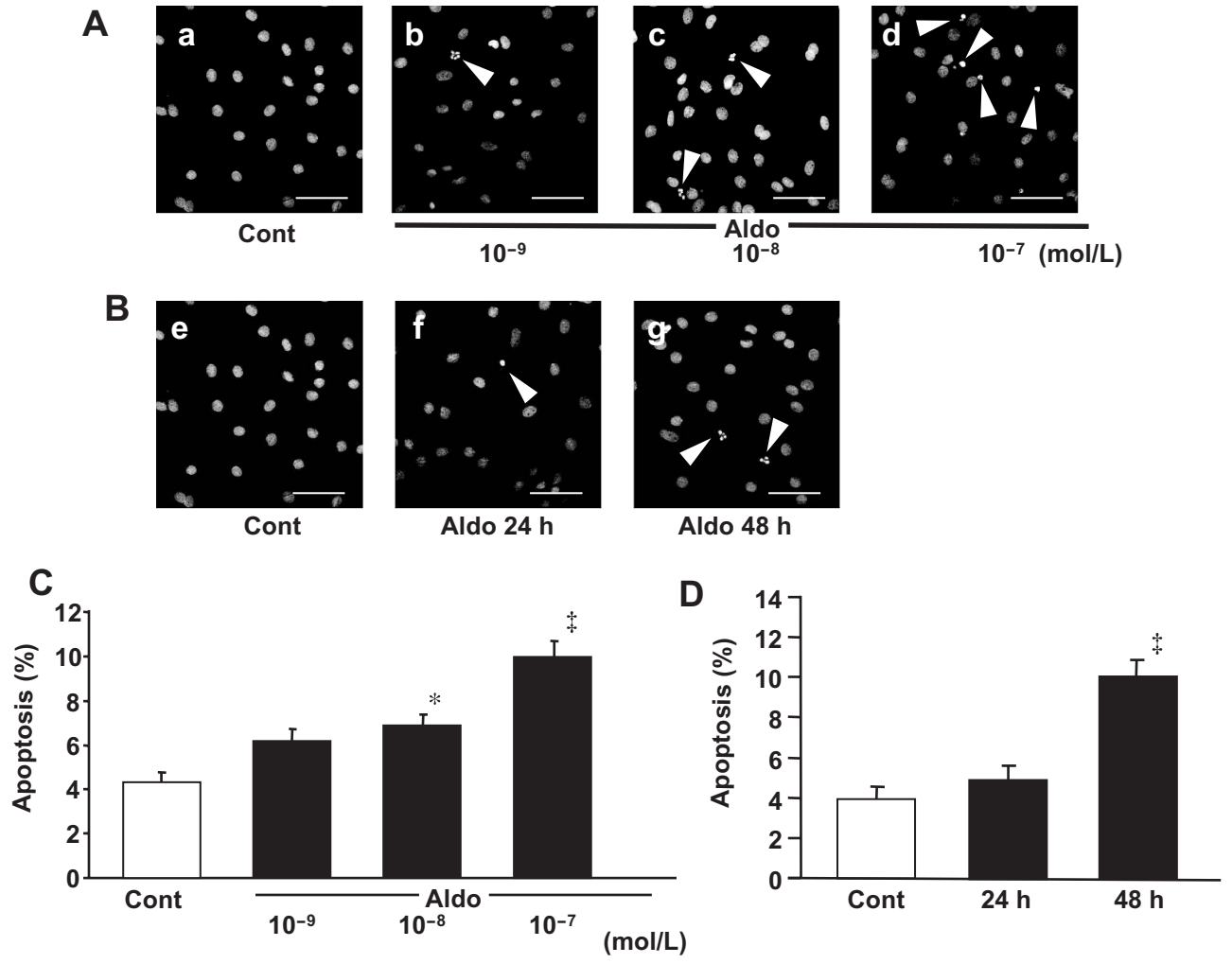

Fig. 6. *Dose- and time-dependent effects of aldosterone (Aldo) on myocyte apoptosis. A: Myocytes were treated with Aldo (10^{-9}–10^{-7} mol/L) for 48 h and then stained with DAPI. B: Myocytes were treated with 10^{-7} mol/L Aldo for the indicated time periods and then stained with DAPI. Representative photomicrographs are shown. Arrowheads indicate a typical feature of apoptotic myocytes. Bars indicate 50 μm. C, D: Percentage of apoptotic myocytes. Apoptotic myocytes were calculated as described in Methods (n = 5–7). *$p < 0.05$, ‡$p < 0.0001$ vs. controls (Cont).*

ington, USA). The membranes were incubated with goat anti-rabbit IgG or anti-mouse IgG conjugated with horseradish peroxidase (HRP), respectively. The detection of chemiluminescence was carried out with enhanced chemiluminescence (ECL; Amersham Biosciences). The bands were quantified by scanning densitometry, and the optical density of samples was normalized with respect to the amount of actin (1:5,000, mouse anti-actin monoclonal antibody, Chemicon International, Temecula, USA; 1:2,500, anti-mouse IgG HRP conjugated, Amersham Biosciences).

Caspase-3 Activity

Caspase-3 activity was determined using an APOPCYTO Caspase-3 Colorimetric Assay Kit (MBL, Nagoya, Japan). According to the manufacturer's protocol, cell lysates were incubated with 10 mmol/L DTT and the labeled caspase-3 substrate DEVD-*p*-nitroanilide (DEVD-pNA) for 48 h at 37°C. Cleavage of a substrate was quantified by measuring the absorbance at 405 nm using a microplate reader.

Determination of Myocyte Apoptosis

Cultured myocytes were fixed with 2% paraformaldehyde, and permeabilized with 0.5% Triton X-100. Nuclei were then stained with 4′,6′-diamidino-2-phenylindole (DAPI). Apoptotic cells were identified by the distinctive condensed or fragmented nuclei. The numbers of nuclei were counted in ten randomized, nonoverlapping fields at a magnification of ×400, and apoptotic cell counts were expressed as a percent-

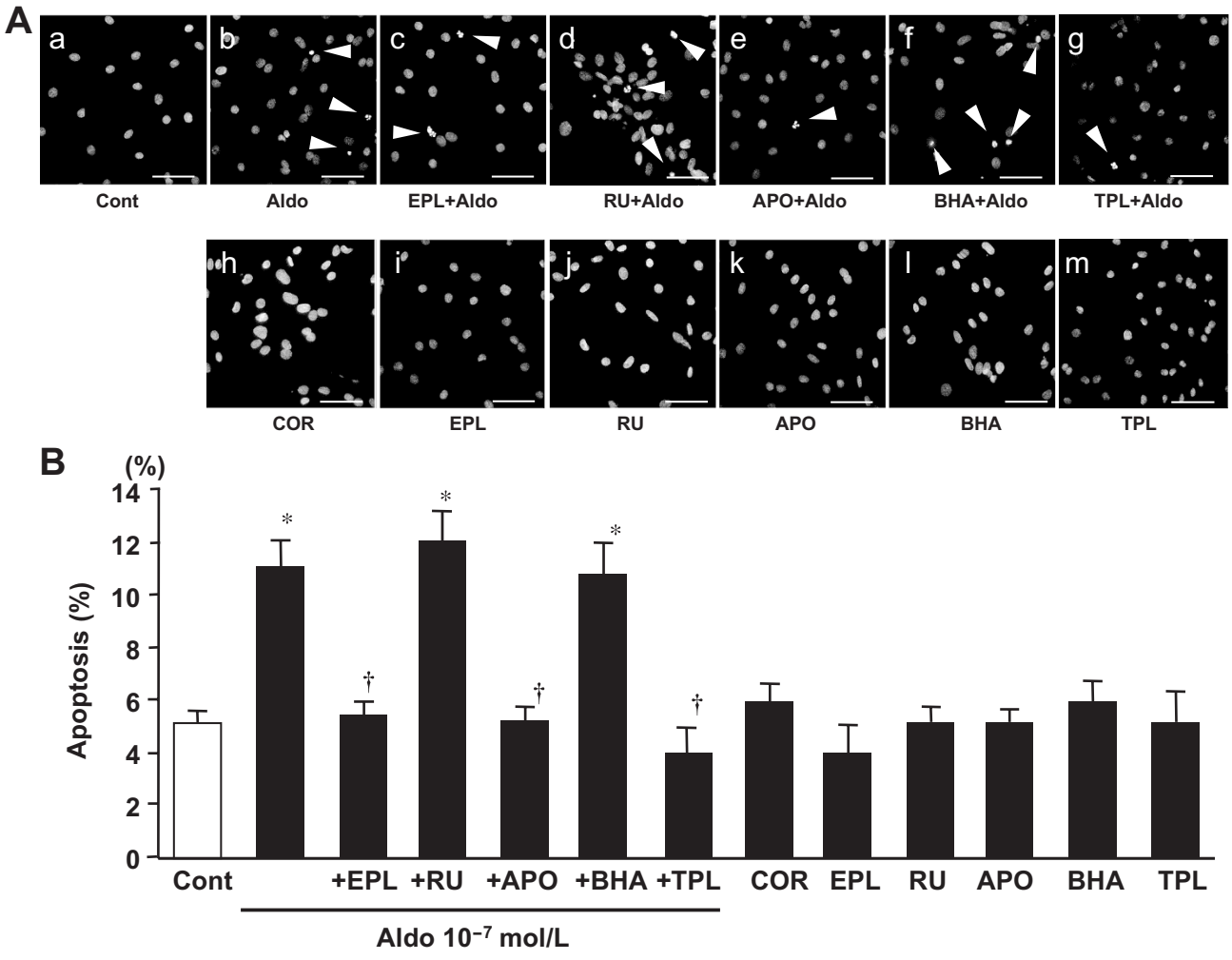

Fig. 7. *Effects of eplerenone (EPL), RU486 (RU), apocynin (APO), butylated hydroxyanisol (BHA), and tempol (TPL) on aldosterone (Aldo)-induced myocyte apoptosis, and the effect of corticosterone (COR) on myocyte apoptosis. Myocytes were stimulated by Aldo (10^{-7} mol/L) or COR (10^{-7} mol/L) for 48 h in the presence or absence of EPL, RU, APO, BHA, or TPL, and then stained with DAPI. A: Representative photomicrographs are shown. Arrowheads indicate a typical feature of apoptotic myocytes. Bars indicate 50 μm. B: Percentage of apoptotic myocytes. Apoptotic myocytes were calculated as described in Methods ($n=5–7$). *$p<0.01$ vs. controls (Cont); †$p<0.01$ vs. Aldo.*

age of the total number of nuclei counted.

Statistical Analysis

All results are expressed as the means±SEM. Statistical analysis was performed by one-way ANOVA combined with Fisher's post-hoc test. Values of $p<0.05$ were considered to be significant.

Results

Effect of Aldosterone on NADPH Oxidase Activity

A very low level of O_2^- was detected in cardiac myocytes in the absence of NADPH using lucigenin-derived chemiluminescence (data not shown). Administration of NADPH (100 nmol/L) elicited substantial production of O_2^- in myocytes. Aldosterone activated NADPH oxidase in a dose-dependent fashion, and the treatment with 10^{-7} mol/L aldosterone significantly enhanced NADPH-dependent O_2^- production (172.9%) (Fig. 1A). Aldosterone 10^{-7} mol/L activated NADPH oxidase in a time-dependent fashion. NADPH oxidase activity increased significantly as early as 5 min after aldosterone treatment, then reached a plateau level at 30 min and remained at this level for 48 h (Fig. 1B). This aldosterone-mediated enhancement of NADPH oxidase activity was significantly attenuated by preincubation with eplerenone or apocynin, by 31.6% or 25.7%, respectively. Neither RU486 nor BHA affected this aldosterone-induced NADPH oxidase activation, and corticosterone also did not change NADPH

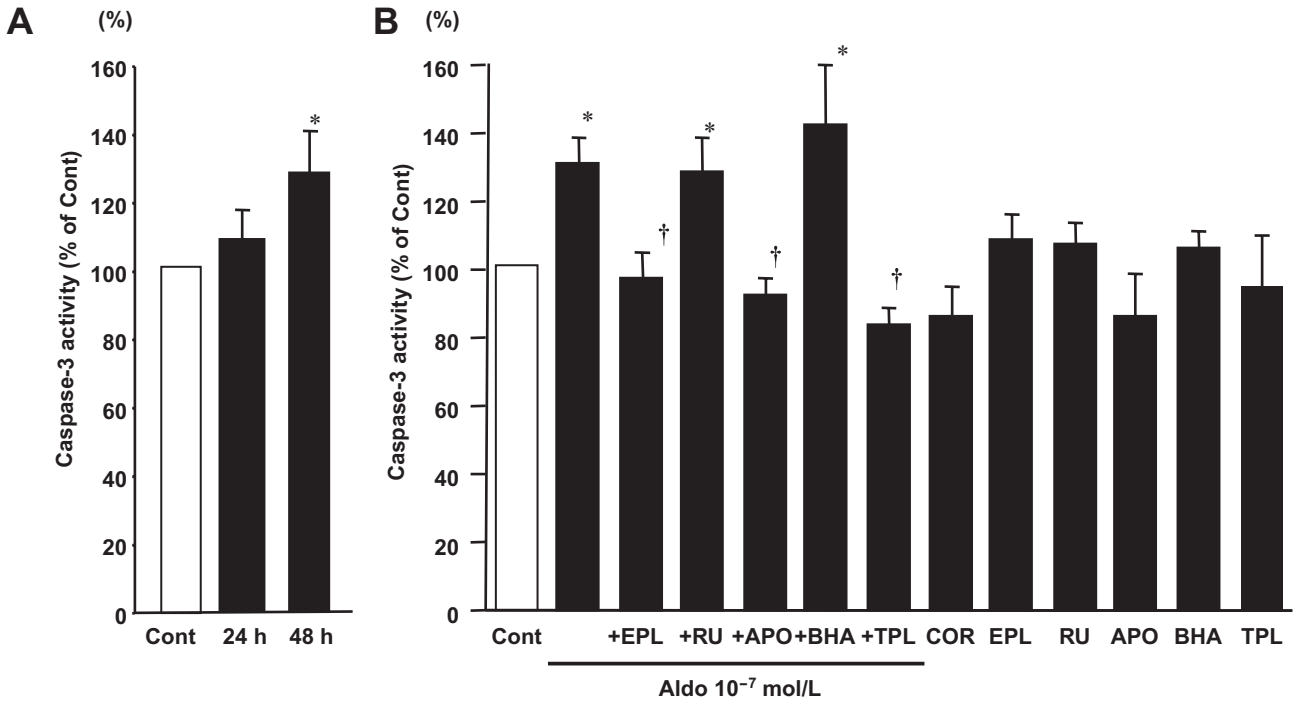

Fig. 8. *Caspase-3 activity in myocytes. A: Myocytes were treated with 10^{-7} mol/L aldosterone (Aldo) for the indicated time periods (n = 7). B: Myocytes were stimulated by Aldo (10^{-7} mol/L) or corticosterone (COR) (10^{-7} mol/L) for 48 h in the presence or absence of eplerenone (EPL), RU486 (RU), apocynin (APO), butylated hydroxyanisol (BHA), or tempol (TPL) (n = 6–9). *p < 0.05 vs. controls (Cont); †p < 0.05 vs. Aldo. Data are expressed as the relative difference compared with Cont.*

oxidase activity (Fig. 1C).

Effect of Aldosterone on ROS Production

Treatment of cardiac myocytes with aldosterone (10^{-9}–10^{-7} mol/L) increased intracellular ROS as measured by DCF fluorescence in a dose-dependent fashion (Fig. 2A, C). This aldosterone (10^{-7} mol/L)-mediated increase in DCF fluorescence was evident 5 min after treatment and detectable even after 48 h (Fig. 2B, D). Pretreatment with eplerenone or apocynin, but neither RU486 nor BHA, markedly attenuated the aldosterone-induced increase in DCF fluorescence. Corticosterone, unlike aldosterone, did not augment DCF fluorescence (Fig. 3).

Nongenomic Effect of Aldosterone

Aldosterone rapidly activated NADPH oxidase and generated ROS within 5 min (Figs. 1B and 2B, D). Hence, we examined whether this effect was dependent on the nongenomic action of aldosterone. As shown in Fig. 4, pretreatment with actinomycin D or cycloheximide, inhibitors of transcription or protein synthesis, respectively, did not affect aldosterone-mediated NADPH oxidase activation. Recently, we identified the presence of a plasma membrane receptor specific for aldosterone in cardiac myocytes, and this receptor was associated with the nongenomic action of aldosterone (*27*). Therefore, we next examined whether eplerenone could inhibit aldosterone binding to this plasma membrane receptor. Eplerenone blocked aldosterone binding to the plasma membrane receptor in a dose-dependent fashion, and the level of this inhibition was less than the blocking of aldosterone binding to cytosolic receptors by eplerenone (Fig. 4B). Moreover, aldosterone-induced NADPH oxidase activation and intracellular ROS accumulation continued as long as 48 h after treatment. However, aldosterone did not increase the mRNA expression or protein level of gp91phox (NOX2), which is a major isoform of NADPH oxidase in cardiac myocytes (Fig. 5) (*31*).

Effect of Aldosterone on Myocyte Apoptosis

Nuclear staining with DAPI showed that aldosterone increased the number of apoptotic myocytes in a dose-dependent fashion. Treatment of myocytes with 10^{-8} and 10^{-7} mol/L aldosterone for 48 h significantly increased the percentage of apoptotic myocytes to 6.9% and 9.9%, respectively, compared with the serum-deprived control (4.3%) (Fig. 6A, C). Aldosterone 10^{-7} mol/L increased apoptotic myocytes in a time-dependent fashion (Fig. 6B, D). Serum-deprivation for 24 and 48 h slightly increased the percentage of apoptotic myocytes, but the difference was not significant compared with the baseline level. Pretreatment with eplerenone, apocy-

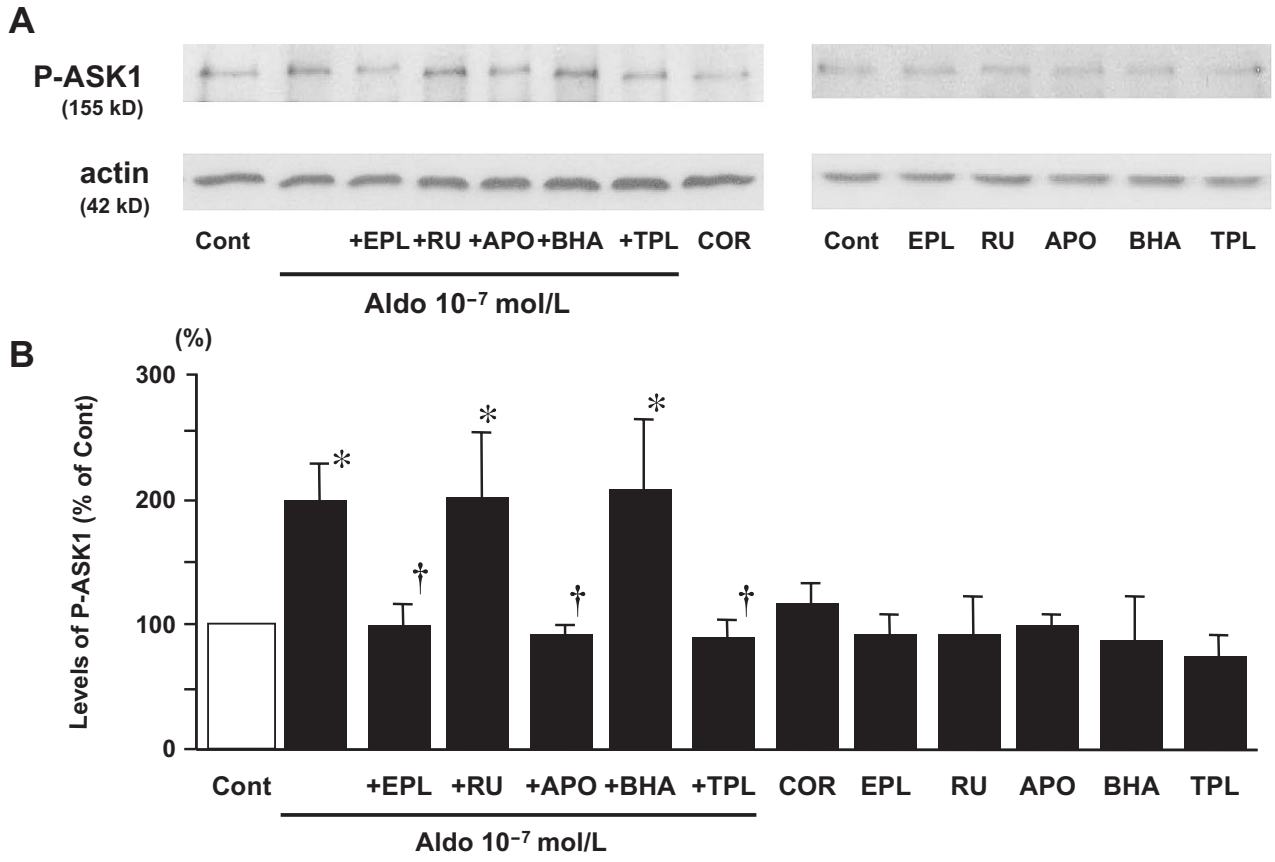

Fig. 9. *Western blot analysis of phospho (P)-ASK1. Myocytes were stimulated by aldosterone (Aldo) (10^{-7} mol/L) or corticosterone (COR) (10^{-7} mol/L) for 48 h in the presence or absence of eplerenone (EPL), RU486 (RU), apocynin (APO), butylated hydroxyanisol (BHA), or tempol (TPL). Cell extracts were probed with anti–P-ASK1 or anti-actin antibodies. A: Representative Western blotting for P-ASK1. B: Densitometric analysis of P-ASK1 (n = 5). *$p < 0.05$ vs. controls (Cont); †$p < 0.05$ vs. Aldo. Data are expressed as the relative difference compared with Cont.*

nin, or tempol reduced the aldosterone-induced increase in the percentage of apoptotic myocytes to 5.4%, 5.1%, or 4.0%, respectively. However, neither RU486 nor BHA inhibited aldosterone-induced myocyte apoptosis, and corticosterone did not increase the percentage of myocyte apoptosis. There was no significant change in the level of myocyte apoptosis after treatment with eplerenone, RU486, apocynin, BHA, or tempol alone (Fig. 7).

Effect of Aldosterone on Caspase-3 Activity

Caspase-3 activity in the myocytes treated with aldosterone for 48 h was significantly increased by 35.8% compared with that in the serum-deprived control (Fig. 8A). Pretreatment with eplerenone, apocynin, or tempol, but neither RU486 nor BHA, significantly inhibited the aldosterone-stimulated caspase-3 activation. Treatment with corticosterone, eplerenone, RU486, apocynin, BHA, or tempol alone did not change caspase-3 activity (Fig. 8B).

Effect of Aldosterone on Phosphorylation of ASK1

As a mechanism of ROS-induced myocyte apoptosis, we examined whether aldosterone activates ASK1 in myocytes. As shown in Fig. 9, treatment of myocytes with 10^{-7} mol/L aldosterone significantly increased the P-ASK1 level by 99.0%, compared with the serum-deprived control. Pretreatment with eplerenone, apocynin, or tempol inhibited the aldosterone-induced activation of ASK1 by 49.7%, 54.3%, or 54.9%, respectively. Neither RU486 nor BHA affected this aldosterone-induced ASK1 activation, and corticosterone did not activate ASK1.

Discussion

The present study demonstrates for the first time that aldosterone (10^{-7} mol/L) increases intracellular ROS synthesis in cardiac myocytes by activating NADPH oxidase *via* a non-genomic pathway, and that these changes are prevented by the

MR antagonist eplerenone and the NADPH oxidase activation inhibitor apocynin, whereas neither the GR antagonist RU486 nor the mitochondrial ROS scavenger BHA has significant effects. Furthermore, we found that aldosterone activated ASK1 and induced myocyte apoptosis, and that these effects were abolished by pretreatment with eplerenone, apocynin, or the ROS scavenger tempol. These findings suggest that aldosterone-induced ROS are mainly mediated by NADPH oxidase activation *via* MR, and lead to myocyte apoptosis.

Plasma aldosterone levels in patients with heart failure have been reported to be approximately 10^{-8} mol/L (*32*), and the aldosterone level in heart tissue has been reported to be 10^{-11}–10^{-10} mol/g-wet weight (10^{-8}–10^{-7} mol/L) (*33*). The present study showed that 10^{-9}–10^{-7} mol/L of aldosterone causes a time- and dose-dependent increase in myocyte apoptosis, suggesting that aldosterone might induce myocyte apoptosis in the failed myocardium. Regarding the signaling pathway of this aldosterone-induced myocyte apoptosis, we previously reported that aldosterone (10^{-5} mol/L, 24 h) induced myocyte apoptosis *via* a rapid increase in intracellular Ca^{2+} concentration and Ca^{2+}-induced Bad activation (*27*). Although intracellular Ca^{2+} concentration was not significantly increased by 10^{-9}–10^{-7} mol/L aldosterone (data not shown), we found that aldosterone at 10^{-7} mol/L markedly activated NADPH oxidase and generated ROS in myocytes (Figs. 1, 2). NADPH oxidase activation inhibitor, free radical scavenger, and MR blockade by eplerenone significantly attenuated aldosterone-induced myocyte apoptosis. Although undetectable levels of increase in intracellular Ca^{2+} might be partially involved in aldosterone (10^{-7} mol/L)-induced apoptosis *via* activation of NADPH oxidase and Bad, our present study demonstrates that ROS have a critical role in aldosterone (10^{-7} mol/L)-induced myocyte apoptosis. Kuster *et al*. reported that eplerenone markedly attenuated pressure overload–induced myocyte apoptosis together with a decrease in cardiac oxidative stress levels using an ascending aorta constriction model (*34*). Our present *in vitro* study is consistent with the *in vivo* observation by Kuster *et al*. and clearly shows that the MR antagonist eplerenone directly blocks myocyte apoptosis by suppressing oxidative stress production.

Aldosterone-induced NADPH oxidase activation occurred within minutes and was not affected by inhibitors of transcription or translation, suggesting that the aldosterone-induced ROS production may be attributed to rapid, nongenomic actions of aldosterone. In the present study, we found that eplerenone inhibited the specific binding of aldosterone to the plasma membrane of myocytes by using a radioligand-binding assay, indicating that an aldosterone-mediated nongenomic action such as NADPH oxidase activation may be attributed to plasma membrane receptor signaling. In addition, aldosterone did not alter the mRNA expression or protein level of the NOX2 isoform of NADPH oxidase after 48 h. These results are consistent with previous studies showing that aldosterone did not affect the mRNA expression of NADPH oxidase, but activated NADPH oxidase by translocation into the membrane fraction in mesangial cells (*24*), and that none of the mRNAs for NADPH oxidase components showed significant differences in the microarrays on aldosterone-stimulated hearts (*35*). Taken together, these results suggest that aldosterone-mediated NADPH oxidase activation is likely due to a nongenomic mechanism.

The mechanisms underlying aldosterone-induced activation of NADPH oxidase were not determined in this study. Activation of protein kinase C is a well known signaling event upstream of NADPH oxidase in some other types of cells, such as neutrophils, macrophages, vascular endothelial cells, and smooth muscle cells (*36–38*). Recently, Zhang *et al*. reported that glycated BSA stimulates cardiomyocyte ROS production through a protein kinase C–dependent NADPH oxidase activation (*31*). Protein kinase C has several kinds of isoforms, including Ca^{2+} dependent isoforms and Ca^{2+} independent isoforms, and both isoforms activate NADPH oxidase (*36*, *37*), implying that NADPH oxidase might be activated by PKC under the present conditions without appreciable Ca^{2+} change. Considering that aldosterone rapidly and nongenomically represses protein kinase C activity in cultured neonatal cardiac myocytes (*39*), our present study might suggest that aldosterone enhances NADPH oxidase *via* nongenomic protein kinase C activation.

ASK1 was originally identified as a proapoptotic signaling intermediate, and was recently shown to be a ROS-sensitive mitogen-activated protein (MAP) kinase kinase kinase (*40*). Overexpression of ASK1 induces apoptosis in various cells through mitochondria-dependent caspase activation (*40–42*). Overexpression of a constitutively active mutant of ASK1 induces myocyte apoptosis in isolated rat neonatal cardiac myocytes, and neonatal ASK1 knockout cardiac myocytes are resistant to H_2O_2-induced apoptosis (*43*, *44*). The present study showed that aldosterone activated ASK1 and this activation was abolished by an inhibitor of NADPH oxidase activation and a free radical scavenger, suggesting that aldosterone-induced ROS activated ASK1. Although we did not examine whether the blocking of ASK1 activation attenuates aldosterone-induced myocyte apoptosis, it is possible that activation of ASK1 is involved in the aldosterone-induced myocyte apoptosis.

In the present study, corticosterone, unlike aldosterone, neither augmented ROS production nor induced myocyte apoptosis, although corticosterone and aldosterone exhibit a similar affinity to the MR (*45*). In epithelial tissue, corticosterone is converted into cortisone, which is inactive at MR, by 11β-hydroxysteroid dehydrogenase type 2 (11β-HSD2) (*46*). However, in cardiac myocytes 11β-HSD2 is not appreciably expressed (*47*, *48*). In this regard, it is noteworthy that corticosterone could bind MR in our experimental protocol. The precise mechanism whereby aldosterone and corticosterone had different effects remains unclear. Qin *et al*. have reported that overexpression of 11β-HSD2 in cardiac myocytes results in cardiac hypertrophy and heart failure, and

these effects are attenuated by eplerenone, suggesting that increased access of aldosterone to MR has detrimental effects, and aldosterone has a more detrimental effect than corticosterone on MR (*49*). In addition, aldosterone has been shown to alter the conformation of MR, while corticosterone does not, leading to the distinct physiological actions of the two hormones in non-epithelial cells, where 11β-HSD2 is not present (*50*). The present results are consistent with these findings from previous studies.

In this study using cultured cardiac myocytes, we demonstrated that aldosterone increased myocyte ROS production *via* NADPH oxidase and thereby triggered apoptosis, while in the *in vivo* experiments the significance of NADPH oxidase-derived ROS in heart failure remained unclear. Targeted deletion of NADPH oxidase subunit gp91phox (NOX2) did not affect left ventricular remodeling after myocardial infarction (*51*). On the other hand, in mice lacking the p47phox subunit of NADPH oxidase, left ventricular remodeling/dysfunction were markedly attenuated after myocardial infarction (*52*). Therefore, further examinations will be required to determine the importance of aldosterone-mediated NADPH oxidase activation in the progression of heart failure.

In conclusion, the present study demonstrates that aldosterone nongenomically activates NADPH oxidase, resulting in excessive ROS production in cardiac myocytes. The ROS then activate ASK1 and induce myocyte apoptosis. Even if the ratio of myocyte apoptosis is low, continuous cell loss due to aldosterone-induced myocyte apoptosis could promote a cascade leading to heart failure. Aldosterone thus plays an important role in the progression of heart failure, and NADPH oxidase in the heart may potentially represent a useful therapeutic target for patients with heart failure.

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