Original Article

Cardioprotective Mechanisms of Spironolactone Associated with the Angiotensin-Converting Enzyme/ Epidermal Growth Factor Receptor/Extracellular Signal-Regulated Kinases, NAD(P)H Oxidase/Lectin-Like Oxidized Low-Density Lipoprotein Receptor-1, and Rho-Kinase Pathways in Aldosterone/Salt-Induced Hypertensive Rats

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Studies were performed to test the hypothesis that the angiotensin-converting enzyme (ACE)/epidermal growth factor receptor (EGFR)/extracellular signal-regulated kinases (ERK) pathway, nicotinamide adenine dinucleotide phosphate (NAD(P)H) oxidase/lectin-like oxidized low-density lipoprotein receptor-1 (LOX-1) pathway, and Rho-kinase pathway contribute to the pathogenesis of aldosterone/salt-induced hypertensive rats. Wistar rats were given 1% NaCl to drink and treated with one of the following combinations for 6 weeks: vehicle; aldosterone (0.75 μg/h); aldosterone plus a mineralocorticoid receptor antagonist, spironolactone (20 mg/kg/day); aldosterone plus an ACE inhibitor, imidapril (1 mg/kg/day); aldosterone plus an NAD(P)H oxidase inhibitor, apocynin (0.5 mmol/l); and aldosterone plus an Rho-kinase inhibitor, Y-27632 (3 mg/kg/day). Upregulated expression of ACE and EGFR and p44/p42ERK phosphorylation were suppressed by spironolactone or imidapril. Upregulated NAD(P)H oxidase subunits and LOX-1 expression were inhibited by spironolactone or apocynin. Increased expression of RhoA and Rho-kinase and myosin light chain phosphorylation were decreased by spironolactone or Y-27632. Moreover, these drugs effectively inhibited the vascular lesion formation, as measured by the medial thickness and level of perivascular fibrosis, and suppressed the expression of transforming growth factor-β1, type I and III collagen, and monocyte chemoattractant protein-1 mRNA. Spironolactone may be useful as a cardioprotective agent to prevent cardiovascular remodeling *via* the ACE/EGFR/ERK, NAD(P)H oxidase/LOX-1, and Rho-kinase pathways. (*Hypertens Res* 2005; 28: 925–936)

***Key Words*: angiotensin, aldosterone, oxidative stress, Rho, signal transduction**

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Introduction

The survival benefit conferred by angiotensin-converting enzyme (ACE) inhibitors in patients with chronic heart failure and in patients with left ventricular (LV) dysfunction post-myocardial infarction attests to the importance of the renin-angiotensin-aldosterone system (RAAS) in the pathophysiology of cardiac hypertrophy and heart failure (*1*). Elevated aldosterone levels have several important consequences, including worsening sodium retention, potassium and magnesium loss, myocardial collagen production and interstitial fibrosis, ventricular hypertrophy, myocardial norepinephrine release, and endothelial dysfunction (*2*). Structural remodeling of the interstitial collagen matrix is regulated in part by aldosterone (*3*). The pathophysiological roles of aldosterone have been well characterized in clinical studies such as the Randomized Aldactone Evaluation Study (RALES) and the Eplerenone Post-Acute Myocardial Infarction Heart Failure Efficacy and Survival Study (EPHESUS) (*4*, *5*). These studies demonstrate that low doses of mineralocorticoid receptor (MR) antagonists led to a dramatic improvement of mortality in patients with severe congestive heart failure or after myocardial infarction. However, the underlying mechanisms of these important actions of aldosterone remain unknown.

Several mechanisms have been proposed for the pathological actions of aldosterone, including stimulation of reactive oxygen species (ROS), and decreased nitric oxide availability (*6*). In experimental myocardial infarction in rats obtained by occlusion of the left coronary artery, the remaining cardiac tissue undergoes marked hypertrophy and remodeling, in association with increases in myocardial ACE (*7*). Thus, ACE could serve a number of important cardiovascular functions, including regulation of peripheral regional blood flow and modulation of local sympathetic nerve activity, as well as stimulation of growth and hypertrophy. Interestingly, Harada *et al*. (*8*) showed that aldosterone increased the expression of ACE mRNA and that this effect was blocked by the MR antagonist spironolactone in neonatal rat cardiocytes *in vitro*. In addition, pharmacological *in vivo* studies have reported that mineralocorticoids enhance epidermal growth factor (EGF)-induced contraction of arteries (*9*), and that spironolactone reduces the expression of EGF receptor (EGFR) mRNA after cerebral ischemia (*10*). In addition, Krug *et al*. (*11*) demonstrated that EGFR is an aldosterone-induced protein and is involved in the manifold pathobiological actions of aldosterone, and also that an interaction between aldosterone and EGFR plays a role in activating the extracellular signal-regulated kinases p44ERK and p42ERK. Moreover, other studies have proposed that, in addition to p44/p42ERK, the transcriptional nuclear factor-κB (NF-κB) and activator protein (AP)-1 are also involved in the pathological actions of aldosterone (*12*, *13*). On the other hand, the production of ROS by the nicotinamide adenine dinucleotide phosphate (NAD(P)H) oxidase may be involved in smooth muscle cell (SMC) growth and the pathogenesis of hypertension. The phagocyte NAD(P)H oxidase is composed of a membrane-associated 22-kD α-subunit (p22phox) and 91-kD β-subunit (gp91phox), with cytosolic components composed of p47phox, p67phox, and p40phox (*14*). Moreover, oxidized low-density lipoprotein (Ox-LDL) plays a role in early atherosclerosis, eliciting endothelial dysfunction by reducing the expression of endothelial nitric oxide synthase (eNOS) (*15*). The lectin-like Ox-LDL receptor-1 (LOX-1) is a type II membrane protein that is a member of the C-type lectin family and acts as a cell-surface receptor for Ox-LDL (*16*). Recent studies indicate that aldosterone increases ROS production, and vascular NAD(P)H oxidase activity and ROS production have been shown to be increased in aldosterone/salt-treated hypertensive rats (*17*). On the other hand, some studies have reported that Rho-kinase, a target protein of small GTP-binding protein Rho, plays crucial roles in various cellular functions, and in mediating cellular events such as changes in cell morphology, cell motility, focal adhesions, and cytokinesis (*18*). The possibility that Rho is involved in vascular proliferation and migration is suggested by the involvement of Rho in the growth of nonvascular cells in response to heterotrimeric G protein receptor stimulation and in the migration of endothelial cells in response to mechanical strain or tyrosine kinase growth factors (*19*). Indeed, we recently demonstrated that Rho-kinase is involved in vascular remodeling in angiotensin II (Ang II)-induced and Dahl salt-sensitive hypertensive rats *in vivo* (*20*, *21*). However, it remains to be determined whether Rho-kinase is involved in vascular proliferation in aldosterone/salt-treated hypertensive rats *in vivo*. Therefore, to elucidate the underlying mechanisms of these important actions of aldosterone, studies were performed to test the hypothesis that the ACE/EGFR/ERK pathway, NAD(P)H oxidase/LOX-1 pathway, and Rho-kinase pathway contribute to the LV remodeling of aldosterone/salt-induced hypertensive rats. Moreover, we evaluated the cardioprotective effect of the MR antagonist spironolactone and its relation to the ACE/EGFR/ERK, NAD(P)H oxidase/LOX-1, and Rho-kinase pathways in this model.

Methods

All procedures were performed in accordance with our institutional guidelines for animal research and with the National Institutes of Health "Guide for the Care and Use of Laboratory Animals."

Animal Models and Experimental Designs

Experiments were performed on 6-week-old male normotensive Wistar rats (Oriental Bioservice, Kanto Inc., Tsukuba, Japan). The rats were randomly divided into six groups and treated with one of the following combinations for 6 weeks: group 1, 1% NaCl plus vehicle (CON, $n=6$); group 2, 1% NaCl plus aldosterone (ALD-V, 0.75 μg/h, s.c., $n=6$); group

Table 1. General Characteristics and Remodeling in Rats Administered 1% NaCl, Aldosterone, Spironolactone, Imidapril, Apocynin, and Y-27632

Parameter	CON	ALD-V	ALD-S	ALD-I	ALD-A	ALD-Y
Number	6	6	7	7	6	6
BW (g)	468±7	466±8	478±11	474±10	470±9	463±10
SBP (mmHg)	121±3	169±6*	166±5*	164±6*	163±6*	166±7*
LVW (mg)	853±23	987±18*	907±18†	898±20†	902±19†	905±18†
LVW/BW (mg/g)	1.82±0.04	2.12±0.06*	1.90±0.05†	1.89±0.06†	1.92±0.05†	1.95±0.06†
HR (bpm)	454±13	478±15	446±11	471±11	463±14	467±15
Wall-to-lumen ratio	0.14±0.01	0.25±0.02*	0.17±0.01†	0.16±0.01†	0.17±0.01†	0.18±0.01†
Perivascular fibrosis	0.26±0.03	0.52±0.06*	0.33±0.05†	0.31±0.04†	0.32 ±0.05†	0.34 ±0.05†

Data are expressed as the mean±SEM. BW, body weight; SBP, systolic blood pressure; LVW, left ventricular weight; HR, heart rate; CON, 1% NaCl plus vehicle; ALD-V, 1% NaCl plus aldosterone; ALD-S, 1% NaCl plus aldosterone plus spironolactone; ALD-I, 1% NaCl plus aldosterone plus imidapril; ALD-A, 1% NaCl plus aldosterone plus apocynin; ALD-Y, 1% NaCl plus aldosterone plus Y-27632. *$p<0.01$ *vs*. CON; †$p<0.01$ *vs*. ALD-V.

3, 1% NaCl plus aldosterone plus spironolactone added to a drinking solution (ALD-S, 20 mg/kg/day, $n=7$); group 4, 1% NaCl plus aldosterone plus ACE inhibitor imidapril added to a drinking solution (ALD-I, 1 mg/kg/day, $n=7$); group 5, 1% NaCl plus aldosterone plus NAD(P)H oxidase inhibitor apocynin added to a drinking solution (ALD-A, 0.5 mmol/l, $n=6$); and group 6, 1% NaCl plus aldosterone plus Rho-kinase inhibitor Y-27632 (ALD-Y, 3 mg/kg/day, $n=6$). In all six groups the rats were anesthetized lightly with ether, and an osmotic minipump (Alzet model 2002, Alza Corp., California, USA) containing aldosterone dissolved in 0.154 mol/l NaCl+5 ml/l ethanol or ethanol alone (CON) was implanted subcutaneously. The other osmotic minipump containing Y-27632 dissolved in saline was implanted in group 6. Systolic blood pressure (SBP) and heart rate (HR) were measured in conscious rats by the tail-cuff method before treatment and at 1-week intervals thereafter.

Quantification of mRNA Using Reverse Transcription (RT)-Polymerase Chain Reaction (PCR)

All procedures used for the mRNA extraction, cDNA synthesis, PCR, and quantification of PCR product were described in detail in our previous report (*22*). PCR was done using synthetic oligonucleotide primers as previously reported (*22*, *23*). The numbers of PCR cycles for the 12 genes examined were as follows: ACE, 30; EGFR, 30; NAD(P)H oxidase p22phox, 36; p47phox, 32; gp91phox, 33; LOX-1, 38; Rho-kinase, 29; transforming growth factor (TGF)-β1, 32; type I collagen, 27; type III collagen, 30; monocyte chemoattractant protein-1 (MCP-1), 32; and GAPDH, 22.

Western Blot Analysis

The ACE, EGFR, NAD(P)H oxidase p22phox, p47phox, gp91phox, LOX-1, and RhoA proteins were measured as described previously (*20–22*, *24*). LV was homogenized (25% w/v) in 10 mmol/l HEPES buffer, pH 7.4, containing 320 mmol/l sucrose, 1 mmol/l EDTA, 1 mmol/l DTT, 10 μg/ml leupeptin, and 2 μg/ml aprotinin at 0°C to 4°C with a polytron homogenizer. Protein concentrations were determined with bovine serum albumin as a standard protein. Equal amounts of protein were loaded in each lane for sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS-PAGE) using 13% and 10% gels. The proteins in the gels were transferred electrophoretically to polyvinylidene difluoride (PVDF) sheets for 1 h at 2 mA/cm^2. The proteins transferred to the sheets were detected using an enhanced chemiluminescence (ECL) immunoblotting detection system (Amersham Life Science Inc., Arlington Heights, USA).

p44/p42ERK, p70S6K, MLC Phosphorylation

p44/p42 extracellular signal-regulated kinase (p44/p42ERK), p70 S6 kinase (p70S6K), and myosin light chain (MLC) phosphorylation were measured as described in detail previously (*20–22*). Briefly, by using rabbit polyclonal phospho-p44/p42ERK and p70S6K, and goat polyclonal phospho-MLC antibody recognizing threonine-phosphorylated forms (active forms) of p44/p42ERK, p70S6K, and MLC, we measured LV phosphorylated p44/p42ERK, p70S6K, and MLC proteins with Western blot analysis.

Determination of NADPH Oxidase Activity

The NADPH oxidase activity in the LV was assessed by the measurement of superoxide-enhanced lucigenin chemiluminescence as described previously (*22*).

Histologic Examination and Evaluation of Cardiovascular Remodeling

Histologic examination was performed as described in detail previously (*20–24*). The wall-to-lumen ratio (the area of the

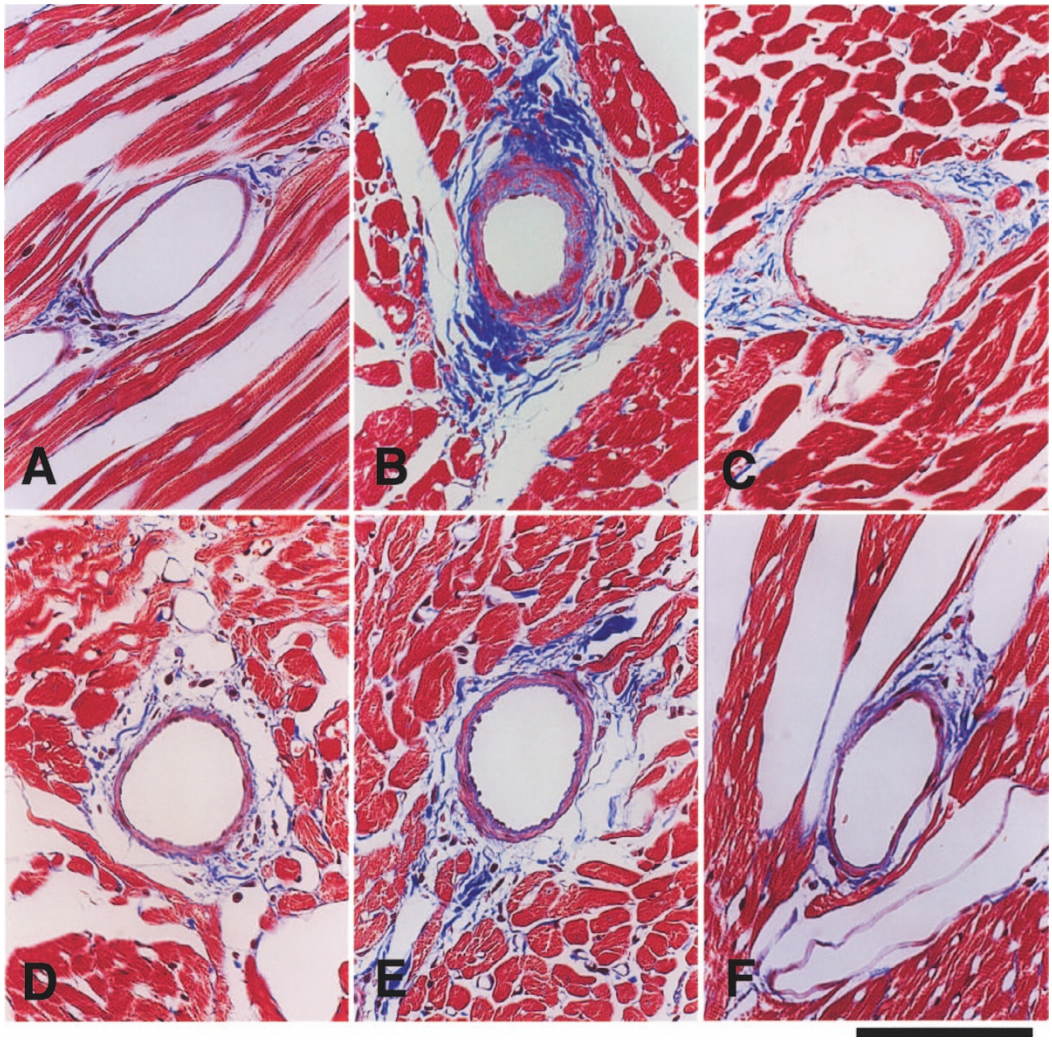

Fig. 1. *Typical histological findings in the rats administered 1% NaCl plus vehicle (A), 1% NaCl plus aldosterone (B), 1% NaCl plus aldosterone plus spironolactone (C), 1% NaCl plus aldosterone plus imidapril (D), 1% NaCl plus aldosterone plus apocynin (E), and 1% NaCl plus aldosterone plus Y-27632 (F). The bar indicates 100 μm.*

vessel wall divided by the area of the total blood vessel lumen) was determined. The area of fibrosis immediately surrounding the blood vessels was calculated, and perivascular fibrosis was determined as the ratio of the area of fibrosis surrounding the vessel wall to the total area of the vessel in the microscopic field of each Masson's trichrome-stained section.

Comparison of Aldosterone Infusion in the Absence and Presence of Salt Loading

The present study was designed to assess the effects of aldosterone in the presence of salt loading on the development of cardiovascular remodeling. Some investigators have shown that aldosterone excess in the presence of salt loading is associated with cardiovascular remodeling (*25–27*). However, it is unclear whether chronic aldosterone infusion in the absence of salt loading induces cardiovascular remodeling *in vivo*. To evaluate whether chronic subcutaneous aldosterone infusion in the absence of salt loading has sufficient potency to increase SBP and deteriorate fibrosis factor in 6-week-old male normotensive Wistar rats *in vivo*, we performed a supplemental experiment with infusion of aldosterone alone (0.75 μg/h, s.c., $n=6$) for 6 weeks.

Statistical Analysis

All values are expressed as the mean±SEM. The mean values were compared among groups by ANOVA and the Bonferroni post hoc test for multiple comparisons. A value of

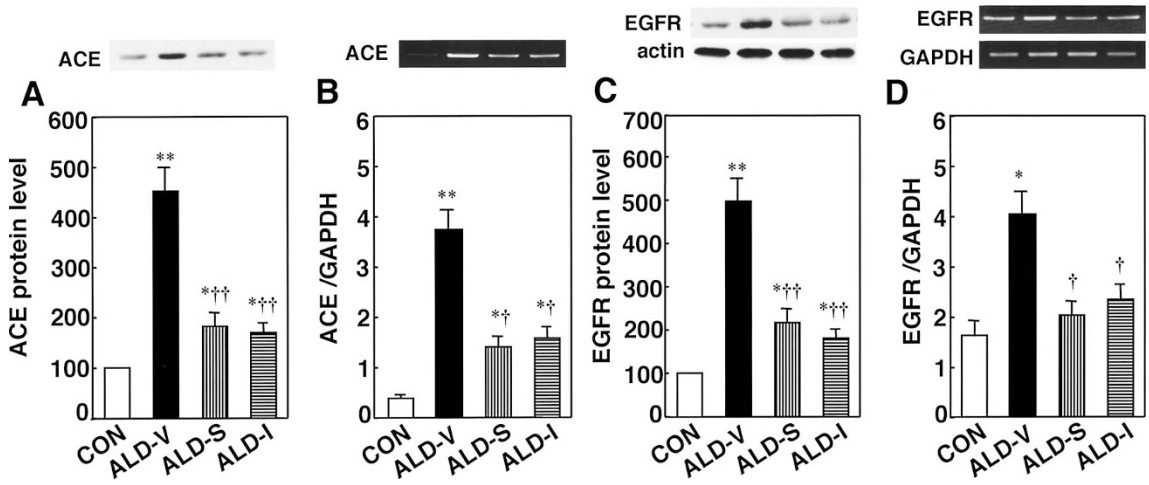

Fig. 2. *Effects of chronic spironolactone or imidapril treatment on ACE protein (A), ACE mRNA (B), EGFR protein (C), and EGFR mRNA (D). Values are the means±SEM.* n = *5 per group.* *p < *0.05,* **p < *0.01* vs. *CON;*†p < *0.05,* ††p < *0.01* vs. *ALD-V. CON, 1% NaCl plus vehicle; ALD-V, 1% NaCl plus aldosterone; ALD-S, 1% NaCl plus aldosterone plus spironolactone; ALD-I, 1% NaCl plus aldosterone plus imidapril.*

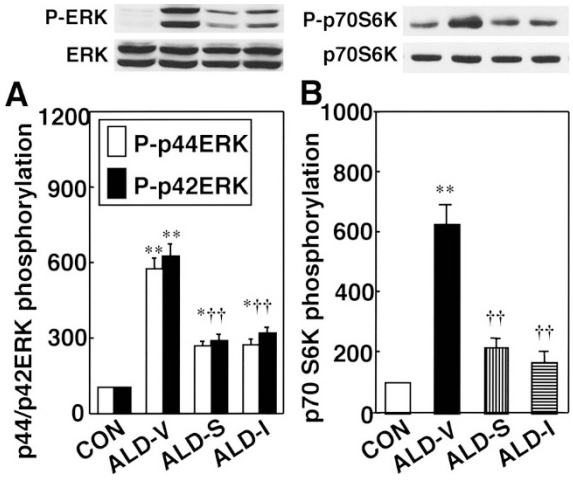

Fig. 3. *Effects of chronic spironolactone or imidapril treatment on phospho-p44/p42ERK (A) and phospho-p70S6K (B) phosphorylation. Values are the means±SEM.* n = *5 per group.* *p < *0.05,* **p < *0.01* vs. *CON;*††p < *0.01* vs. *ALD-V. CON, 1% NaCl plus vehicle; ALD-V, 1% NaCl plus aldosterone; ALD-S, 1% NaCl plus aldosterone plus spironolactone; ALD-I, 1% NaCl plus aldosterone plus imidapril.*

$p < 0.05$ was considered statistically significant.

Results

Physiologic Profiles after 6 Weeks of Treatment in Aldosterone-Salt Rats

Body weight (BW), SBP, LV weight (LVW) to BW ratio, and HR in the 6 groups are presented in Table 1. ALD-V rats had markedly higher SBP by the tail-cuff method than did CON rats. Long-term spironolactone, imidapril, apocynin, or Y-27632 therapy did not significantly affect the SBP. HR and BW were similar in CON and ALD-V, and were not changed by the administration of spironolactone, imidapril, apocynin, or Y-27632. In contrast, ALD-V rats had a higher LVW/BW compared with CON rats. Long-term spironolactone, imidapril, apocynin, or Y-27632 treatment in aldosterone-salt rats significantly decreased LVW/BW, respectively.

Cardiovascular Remodeling

The morphological appearance, wall-to-lumen ratio and perivascular fibrosis of the coronary arteries in the 6 groups are shown in Fig. 1 and Table 1. The wall-to-lumen ratio and perivascular fibrosis were significantly increased in ALD-V rats compared with CON rats. Long-term spironolactone, imidapril, apocynin, or Y-27632 treatment caused significant amelioration of these ratios.

ACE and EGFR Expression

ACE and EGFR mRNA and protein levels in the LV were significantly increased in ALD-V rats compared with CON rats (Fig. 2). Long-term spironolactone treatment significantly decreased ACE and EGFR expression, and the degree of suppression of both proteins by spironolactone was similar to that by treatment with imidapril.

p44/p42ERK and p70S6K Phosphorylation

Phosphorylation of p44/p42ERK and p70S6K in the LV was

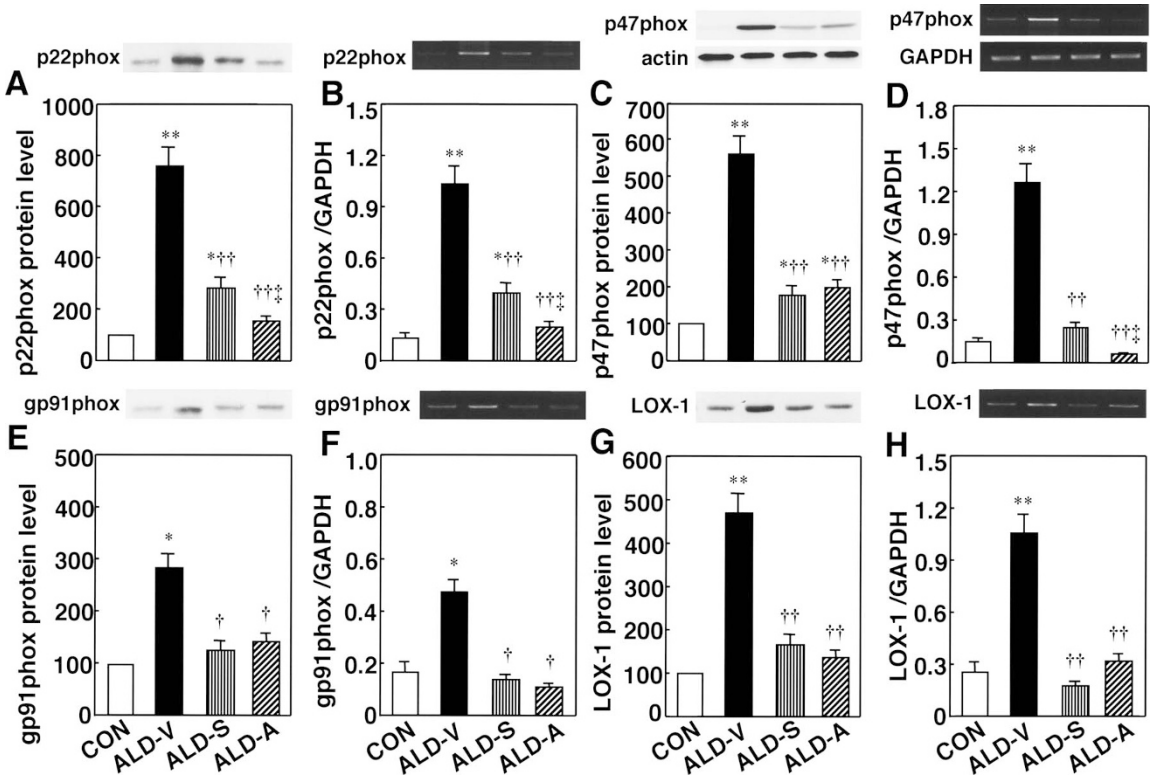

Fig. 4. *Effects of chronic spironolactone or apocynin treatment on NAD(P)H oxidase p22phox protein (A), p22phox mRNA (B), p47phox protein (C), p47phox mRNA (D), gp91phox protein (E), gp91phox mRNA (F), LOX-1 protein (G), and LOX-1 mRNA (H). Values are the means±SEM.* n=*5 per group.* *p<*0.05,* **p<*0.01* vs. *CON;* †p<*0.05,* ††p<*0.01* vs. *ALD-V;* ‡p<*0.05* vs. *ALD-S. CON, 1% NaCl plus vehicle; ALD-V, 1% NaCl plus aldosterone; ALD-S, 1% NaCl plus aldosterone plus spironolactone; ALD-A, 1% NaCl plus aldosterone plus apocynin.*

significantly higher in ALD-V than in CON rats. Long-term spironolactone or imidapril therapy significantly decreased the phosphorylation of p44/p42ERK and p70S6K (Fig. 3).

NAD(P)H Oxidase Subunits and LOX-1 Expression

NAD(P)H oxidase p22phox, p47phox, gp91phox, and LOX-1 expression levels in the 4 groups are shown in Fig. 4. NAD(P)H oxidase p22phox, p47phox, gp91phox, and LOX-1 mRNA and protein levels in the LV were significantly higher in ALD-V rats than in CON rats. Long-term spironolactone therapy significantly decreased the p22phox, p47phox, gp91phox, and LOX-1 expression, and the degree of suppression of these proteins by spironolactone was similar to that by treatment with apocynin.

NADPH Oxidase Activity

NADPH oxidase activity in the LV was significantly higher in ALD-V rats than in CON rats (184.3±9.5 *vs*. 100±4.7, $p<0.01$). Long-term spironolactone or apocynin treatment significantly reduced NADPH oxidase activity (141.6±6.3, 132.7±5.1 *vs*. ALD-V, $p<0.01$, respectively).

Involvement of RhoA and Rho-Kinase Expression and Phosphorylation

RhoA and Rho-kinase expression and MLC phosphorylation in the 4 groups are shown in Fig. 5. To quantify the activity of Rho-kinase in the hypertensive heart, we performed Western blot analysis for phosphorylated MLC in the LV. RhoA protein and Rho-kinase mRNA and phosphorylation in the LV were significantly higher in ALD-V rats than in CON rats. Long-term spironolactone therapy significantly decreased the RhoA and Rho-kinase expression and phosphorylation, and the levels of these effects by spironolactone were similar to those by treatment with Y-27632.

Gene Expression of TGF-β1, Type I and III Collagen, and MCP-1

The gene expressions of TGF-β1, type I and III collagen, and MCP-1 mRNA in the 6 groups are shown in Fig. 6. The gene expressions of TGF-β1, type I and III collagen, and MCP-1 in the LV was significantly higher in ALD-V rats than in CON

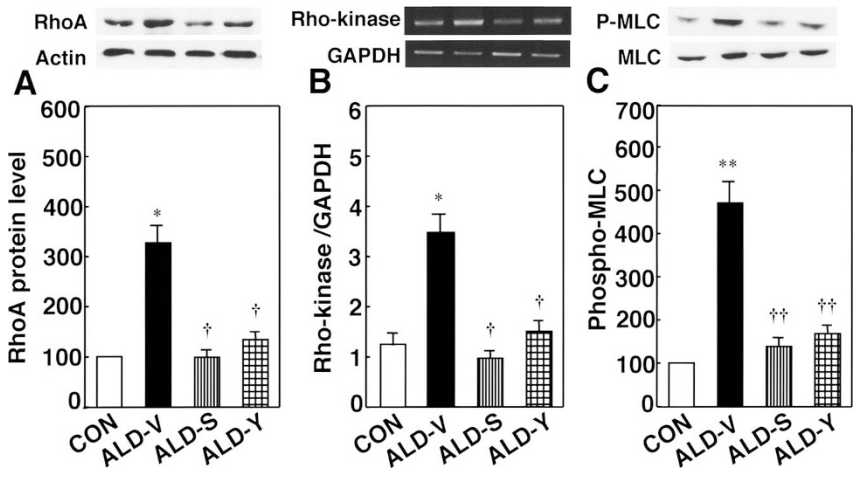

Fig. 5. *Effects of chronic spironolactone or Y-27632 treatment on RhoA protein (A), Rho-kinase mRNA (B), and phospho-MLC (C). Values are the means±SEM.* n = *5 per group.* *p < *0.05,* **p < *0.01* vs. *CON;* †p < *0.05,* ††p < *0.01* vs. *ALD-V. CON, 1% NaCl plus vehicle; ALD-V, 1% NaCl plus aldosterone; ALD-S, 1% NaCl plus aldosterone plus spironolactone; ALD-Y, 1% NaCl plus aldosterone plus Y-27632.*

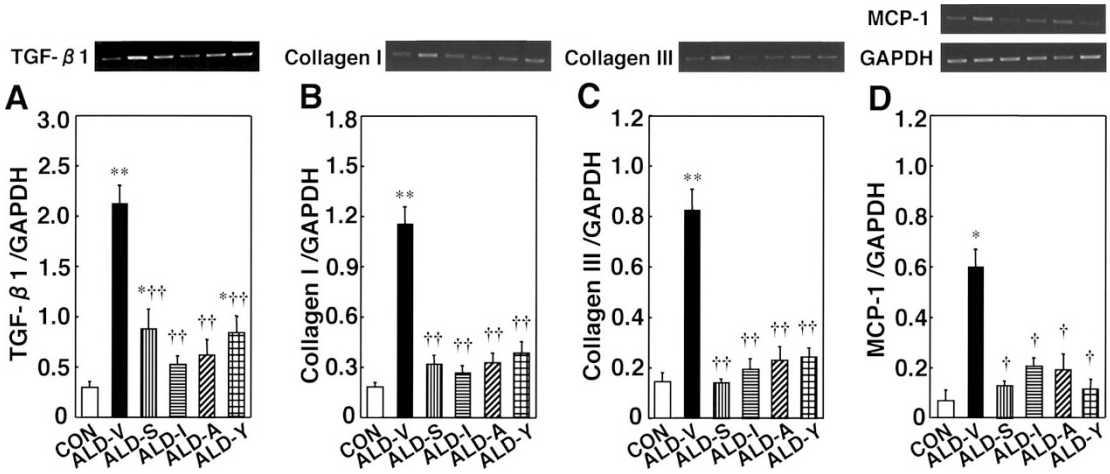

Fig. 6. *Effects of chronic spironolactone, imidapril, apocynin, or Y-27632 treatment on TGF-β1 (A), type I collagen (B), type III collagen (C), and MCP-1 (D) mRNA. Values are the means±SEM.* n = *5 per group.* *p < *0.05,* **p < *0.01* vs. *CON;* †p < *0.05,* ††p < *0.01* vs. *ALD-V. CON, 1% NaCl plus vehicle; ALD-V, 1% NaCl plus aldosterone; ALD-S, 1% NaCl plus aldosterone plus spironolactone; ALD-I, 1% NaCl plus aldosterone plus imidapril; ALD-A, 1% NaCl plus aldosterone plus apocynin; ALD-Y, 1% NaCl plus aldosterone plus Y-27632.*

rats. Long-term spironolactone therapy reduced the expression of TGF-β1, type I and III collagen, and MCP-1 mRNA, and the levels of these reductions by spironolactone were similar to those by treatment with imidapril, apocynin, and Y-27632, respectively.

Comparison of Aldosterone Infusion in the Absence and Presence of Salt Loading

There were no changes in BW (461±9 g *vs*. ALD-V, NS), SBP (167±5 mmHg *vs*. ALD-V, NS), HR (460±14 bpm *vs*. ALD-V, NS), and perivascular fibrosis (0.48±0.05 *vs*. ALD-V, NS) in aldosterone-infused rats between the absence and presence of salt loading. In contrast, LVW/BW (1.85±0.04 mg/g *vs*. ALD-V, $p<0.01$) and wall-to-lumen ratio (0.15±0.01 *vs*. ALD-V, $p<0.01$) were significantly decreased in the rats receiving aldosterone alone compared with the aldosterone/salt rats. Recently, Iglarz *et al*. (*28*) showed that chronic subcutaneous aldosterone infusion in the absence of salt loading increased SBP and induced collagen

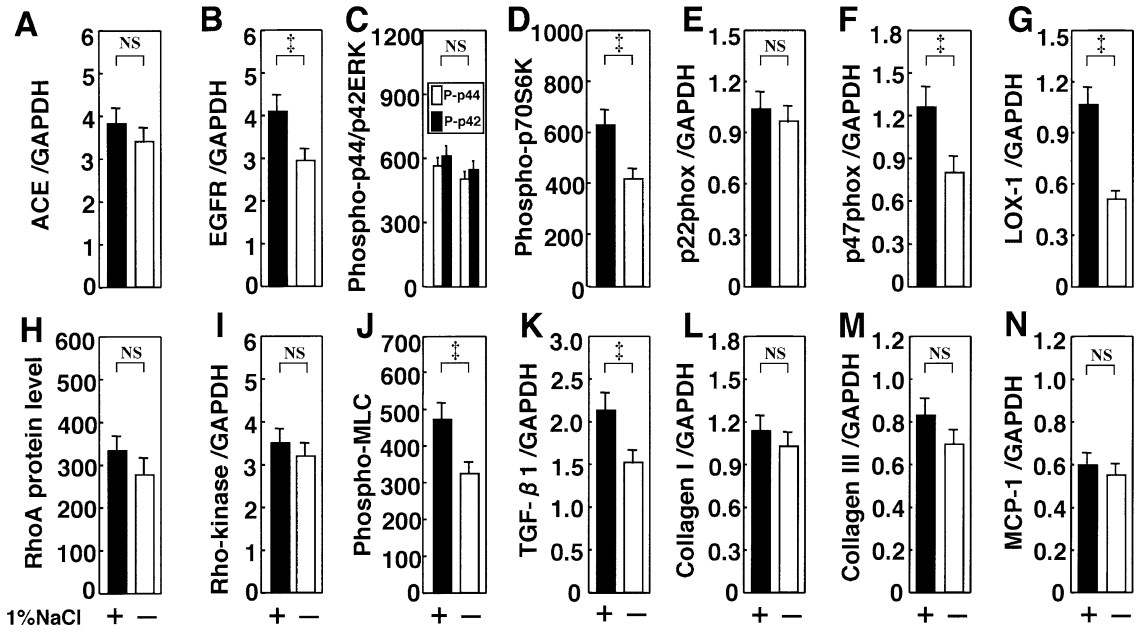

Fig. 7. *Comparison of aldosterone infusion in the absence and presence of salt loading on ACE mRNA (A), EGFR mRNA (B), phospho-p44/p42ERK (C), phospho-p70S6K (D), NAD(P)H oxidase p22phox mRNA (E), p47phox mRNA (F), LOX-1 mRNA (G), RhoA protein (H), Rho-kinase mRNA(I), phospho-MLC (J), TGF-β1 mRNA (K), type I collagen mRNA (L), type III collagen mRNA(M), and MCP-1 mRNA (N). Values are the means ± SEM.* n = *5 per group.* ‡p < *0.05* vs. *ALD-V.*

content in the heart, but not cardiac hypertrophy. These findings support our present data. The levels of ACE, NAD(P)H oxidase p22phox, Rho-kinase, type I and III collagen, and MCP-1 mRNA, RhoA protein, and p44/p42ERK phosphorylation in the group receiving aldosterone alone were similar to those in the group receiving aldosterone infusion in the presence of salt loading (*vs*. ALD-V, NS, respectively). However, the levels of EGFR, NAD(P)H oxidase p47phox, LOX-1, and TGF-β1 mRNA, p70S6K, and MLC phosphorylation were significantly decreased in the rats receiving aldosterone alone compared with the aldosterone/salt rats (*vs*. ALD-V, $p<0.05$, respectively) (Fig. 7).

Discussion

Figure 8 shows a schematic chart of the signal transduction pathway in aldosterone/salt-induced hypertensive rats. In the present study, to elucidate the critical pathway in the effects of aldosterone on coronary vascular medial thickness and perivascular fibrosis, we evaluated the cardioprotective effects of long-term treatment with spironolactone *via* the ACE/EGFR/ERK pathway, NAD(P)H oxidase/LOX-1 pathway, and Rho-kinase pathway on cardiovascular remodeling in aldosterone/salt-induced hypertensive rats. Spironolactone, at a dose which did not reduce blood pressure, inhibited cardiovascular remodeling and gene expression of growth factors such as TGF-β1, type I and III collagen, and MCP-1 in aldosterone-salt rats. In addition, spironolactone suppressed the ACE/EGFR/ERK, NAD(P)H oxidase/LOX-1, and Rho-kinase pathway, and for each pathway the degree of suppression by spironolactone was similar to that by treatment with the ACE inhibitor imidapril, NAD(P)H oxidase inhibitor apocynin, and Rho-kinase inhibitor Y-27632, respectively. Thus, these results suggest that the cardioprotective effect of spironolactone on cardiovascular remodeling may be at least partly associated with the ACE/EGFR/ERK pathway, NAD(P)H oxidase/LOX-1 pathway, and Rho-kinase pathway in aldosterone/salt-induced hypertensive rats. However, it is difficult to determine which pathway is the most important, or whether every pathway is involved to a similar degree. Further research will be needed to answer these questions.

To elucidate whether chronic combination therapy with spironolactone and ACE inhibitor in aldosterone/salt-induced hypertensive rats has sufficient potency to suppress cardiac hypertrophy and cardiovascular remodeling to the control levels, we performed a supplemental experiment in which 6-week-old male normotensive Wistar rats ($n=6$) were treated with the combination of 1% NaCl, aldosterone (0.75 μg/h), spironolactone (20 mg/kg/day), and imidapril (1 mg/kg/day) for 6 weeks. Long-term combination therapy with spironolactone and imidapril significantly decreased the LVW/BW (1.83 ± 0.04 *vs*. CON, NS), wall-to-lumen ratio (0.13 ± 0.01 *vs*. CON, NS), and perivascular fibrosis (0.28 ± 0.03 *vs*. CON, NS), and the degree of inhibition by this combination therapy

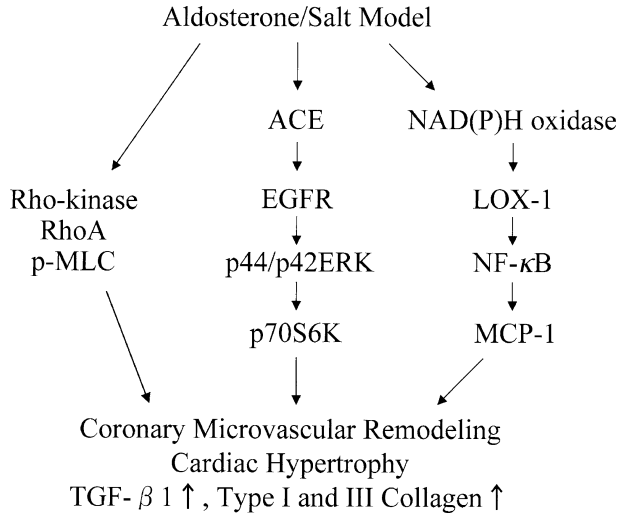

Fig. 8. *Schematic chart of signal transduction pathways in aldosterone/salt-induced hypertensive rats.*

was similar to that by the control group. In another experimental model, Asai *et al*. (*29*) reported that combination therapy with spironolactone and cilazapril in anti-Thy-1-antibody-induced nephritis in uninephrectomized rats ameliorated proteinuria and renal interstitial fibrosis. These findings suggest that chronic combination therapy may be a potentially useful treatment for hypertensive rats.

ACE formed in the heart has been implicated in the pathogenesis of various forms of cardiac hypertrophy and heart failure. Previous studies have demonstrated that in the myocardium of human explanted failing hearts, the level of ACE mRNA and the number of ACE-binding sites are increased compared with those of control hearts (*30*, *31*). In this study, we demonstrated that cardiac ACE mRNA and protein expression was upregulated, and spironolactone inhibited these ACE expressions in aldosterone/salt rats. In addition, we observed that the level of inhibition of these expressions by spironolactone was similar to that by treatment with the ACE inhibitor imidapril. Interestingly, Harada *et al*. (*8*) showed that aldosterone increased the expression of ACE mRNA and that this effect was blocked by the MR antagonist spironolactone in neonatal rat cardiocytes *in vitro*. Wang *et al*. (*32*) reported that aldosterone induced the expression of ACE mRNA in cultured rat neonatal cardiomyocytes, and that spironolactone completely blocked ACE induction, suggesting that this effect may be mediated by the endogenous MR receptor present in myocytes. In addition, Robert *et al*. (*25*) showed that aldosterone induced cardiac Ang II type 1 (AT1) receptor expression in uninephrectomized aldosterone/salt-induced hypertensive rats, and that spironolactone and the AT1 receptor antagonist losartan prevented aldosterone-induced increases in AT1 receptor density and fibrosis and collagen levels. Therefore, these findings suggest that the cardiac ACE expression stimulated by aldosterone may play a critical role in hypertension in aldosterone/salt-induced hypertensive rats, and that a positive feedback pathway from aldosterone to ACE may exist within the local cardiac RAAS (*8*).

In this study, upregulated EGFR expression was significantly prevented by spironolactone. Pharmacological *in vivo* studies have indicated that mineralocorticoids enhance EGF-induced contraction of arteries (*9*) and that spironolactone reduces the expression of EGFR mRNA after cerebral ischemia (*10*). In addition, EGFR expression supports fibrosis in cardiovascular and renal tissue (*33–35*). Moreover, endothelin-induced phosphorylation of the p44/p42ERK was completely prevented by an inhibitor of the EGFR kinase, and an interaction between aldosterone and EGF has been shown to play a role in p44/p42ERK activation (*12*, *13*). In fact, Krug *et al*. (*11*) demonstrated that EGFR is an aldosterone-induced protein and is involved in the manifold pathobiological actions of aldosterone. These results suggest that the interaction of aldosterone with EGFR *via* p44/p42ERK may play a role in the mediation of progressive cardiovascular remodeling by aldosterone.

Recently, Nishiyama *et al*. (*26*) showed that the expressions of NAD(P)H oxidase p22phox, gp91phox, and nox-4 mRNA were significantly increased in the renal cortex tissues of aldosterone/salt-treated rats. In addition, Sun *et al*. (*27*) reported that immunohistochemical staining for NAD(P)H oxidase gp91phox was increased in the hearts of aldosterone/salt-treated uninephrectomized rats. In the present study, upregulated ventricular ROS levels in aldosterone/salt-treated rats were associated with increased expression of NAD(P)H oxidase p22phox, p47phox, and gp91phox mRNA and protein and increased NAD(P)H oxidase activity. Moreover, the present study also showed that these increases in the expression and activity of NAD(P)H oxidase were prevented by spironolactone, and that the levels of these inhibitions were similar to those induced by treatment with the NAD(P)H oxidase inhibitor apocynin. These findings are consistent with those of previous studies in which increased vascular NAD(P)H oxidase activity and ROS production observed in rats with a pathological condition were reduced by treatment with spironolactone (*17*), and in which the mRNAs of NAD(P)H oxidase subunits were suppressed by eplerenone (*26*). Therefore, these findings suggest that the aldosterone/salt-induced ROS production *via* NAD(P)H oxidase plays a critical role in the progression of cardiovascular remodeling.

The Rho-kinase pathway plays an important role in regulating the contraction of vascular SMCs as well as in other cellular functions, such as proliferation and migration. In an *in vitro* study, Yamakawa *et al*. (*36*) examined the effects of Y-27632 on Ang II-induced leucine uptake in vascular SMCs. They showed that pretreatment of the cells with Y-27632 dose-dependently suppressed the leucine incorporation induced by Ang II. Kuwahara *et al*. (*37*) evaluated the Rho/ROCK pathway in endothelin-1 (ET-1) induced hypertrophic

signals in cardiac myocytes. They indicated that Y-27632 significantly suppressed ET-1-induced hypertrophic responses such as the augmentation of natriuretic peptide gene expression, increase in protein synthesis and cell size, and myofibrillar reorganization. With respect to the *in vivo* studies, Mukai *et al*. (*38*) examined the role of Rho-kinase in functional and structural alterations of hypertensive blood vessels in spontaneously hypertensive rats (SHR). They concluded that upregulation of Rho-kinase plays a key role in the pathogenesis of hypertensive vascular disease. Recently, we demonstrated that Rho-kinase is involved in the hypertension of Ang II-induced and Dahl salt-sensitive hypertensive rats *in vivo* (*20*, *21*). However, very few studies have specifically evaluated whether Rho-kinase is involved in the hypertension of aldosterone/salt-induced hypertensive rats. In the present study, we have shown that Rho-kinase is involved in the hypertension of aldosterone/salt-induced hypertensive rats *in vivo*, and that the Rho-kinase pathway plays a critical role in aldosterone/salt-induced cardiac hypertrophy and cardiovascular remodeling by using the specific Rho-kinase inhibitor Y-27632. Our results suggest that the cardioprotective effect of spironolactone on cardiovascular remodeling may be at least partly associated with the Rho-kinase pathway in aldosterone/salt-induced hypertensive rats.

With regard to other rat models of hypertension, some investigators, including ourselves, have shown that in SHR (*38*, *39*), stroke-prone SHR (SHRSP) (*40–43*), Ang II-induced (*20*, *44*), Dahl salt-sensitive (*23*, *45*) and deoxycorticosterone acetate-salt hypertensive rats (*22*, *46*), ACE, NAD(P)H oxidase subunits and Rho-kinase expression were upregulated in the vasculature. In these hypertensive rat models, the effect of spironolactone on these factors is unclear. Recently, however, we have demonstrated that administration of a novel selective aldosterone blocker, eplerenone, to Dahl salt-sensitive hypertensive rats reduced proteinuria and glomerulosclerosis, and suppressed the expression of the genes coding for growth factors. Moreover, eplerenone suppressed the expression of the LOX-1-mediated adhesion molecule and protein kinase C/ERK/p90RSK pathway and improved endothelial function by inhibiting the Rho-kinase pathway (*47*).

The mechanisms responsible for the cardiac fibrosis and hypertrophy seen in aldosterone/salt excess are still unclear. Robert *et al*. (*25*) showed that losartan prevented aldosterone/salt-induced fibrosis and upregulation of collagen types I and III mRNA, aldosterone/salt treatment increased AT1 receptor (AT1R) density and AT1R mRNA accumulation, and spironolactone and losartan prevented the aldosterone/salt-induced increase in AT1R density independent of blood pressure and plasma concentration of Ang II. They suggested that the mechanism by which aldosterone/salt induced cardiac fibrosis involved Ang II acting through AT1R. Moreover, Nishiyama *et al*. (*26*) demonstrated that renal injury was associated with increases in the expression of renal cortical NAD(P)H oxidase components and the activation of mitogen-activated protein kinase (MAPK) in aldosterone/salt-induced hypertensive rats, and that tempol treatment prevented the elevation of NAD(P)H oxidase expressions and MAPK activities and ameliorated renal injury. They concluded that ROS and MAPK played a role in the progression of renal injury induced by chronic elevations in aldosterone. Therefore, these results suggest that the ACE/EGFR/ERK and NAD(P)H oxidase/LOX-1 pathway may play a critical role in cardiovascular remodeling in aldosterone/salt-induced hypertensive rats.

The present study was designed to assess the effects of aldosterone in the presence of salt loading on the development of cardiovascular remodeling. However, it is unclear whether or not chronic aldosterone infusion in the absence of salt loading alters cardiovascular remodeling or any of several molecular factors. We therefore performed experiments to compare aldosterone infusion in the absence and presence of salt loading in normotensive Wistar rats. We showed that chronic aldosterone infusion in the absence of salt loading increased SBP and induced type I and III collagen expression in the LV but not cardiac hypertrophy. These results are practically identical to those of a previous report (*28*). Salt loading has been described to induce vascular remodeling in normotensive rats (*48*). Similarly, arterial wall hypertrophy in conduit arteries is only observed in the presence of salt loading (*49*). Aldosterone infusion in the absence of salt loading presented a mild profibrotic effect at the renal, vascular, and cardiac levels. This moderate effect could be related to the normal salt diet (*28*). The present findings demonstrated that the levels of the expressions of ACE, NAD(P)H oxidase p22phox, Rho-kinase, type I and III collagen, MCP-1, and RhoA, and p44/p42ERK phosphorylation in the animals administered aldosterone alone were very similar to those in the group receiving aldosterone infusion in the presence of salt loading. In contrast, the expressions of EGFR, NAD(P)H oxidase p47phox, LOX-1, and TGF-β1, and p70S6K, and MLC phosphorylation were significantly lower in the rats receiving aldosterone alone that in those of the aldosterone/salt group. The mechanism by which chronic aldosterone infusion in the absence and presence of salt loading mediates these molecular factors remains to be determined. These findings may at least suggest differential mechanisms of action of aldosterone and especially underline the role of salt loading or not.

In conclusion, chronic administration of subdepressor doses of spironolactone to aldosterone/salt-induced rats ameliorated perivascular fibrosis and coronary microvascular hyperplasia. These results suggest that the cardioprotective effect of spironolactone on cardiovascular remodeling may be at least partly associated with the ACE/EGFR/ERK pathway, NAD(P)H oxidase/LOX-1 pathway, and Rho-kinase pathway in aldosterone/salt-induced hypertensive rats. Spironolactone may have be of significant therapeutic benefit for the treatment of hypertension.

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