Mineralocorticoid Receptor Blocker Increases Angiotensin-Converting Enzyme 2 Activity in Congestive Heart Failure Patients

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Abstract—Aldosterone plays an important role in the pathophysiology of congestive heart failure (CHF), and spironolactone improves cardiovascular function and survival rates in patients with CHF. We hypothesized that the mineralocorticoid receptor blockade (MRB) exerted its beneficial effects by reducing oxidative stress and changing the balance between the counter-acting enzymes angiotensin-converting enzyme (ACE) and ACE2. Monocyte-derived macrophages were obtained from 10 patients with CHF before and after 1 month of treatment with spironolactone (25 mg/d). Spironolactone therapy significantly \( (P < 0.005) \) reduced oxidative stress, as expressed by reduced lipid peroxide content, superoxide ion release, and low-density lipoprotein oxidation by 28%, 53%, and 70%, respectively. Although spironolactone significantly \( (P < 0.01) \) reduced macrophage ACE activity by 47% and mRNA expression by 53%, ACE2 activity and mRNA expression increased by 300% and 654%, respectively. In mice treated for 2 weeks with eplerenone (200 mg \( \times \) kg\(^{-1} \times \) day\(^{-1} \)), cardiac ACE2 activity significantly \( (P < 0.05) \) increased by 2-fold and was paralleled by increased ACE2 activity in macrophages. The mechanism of aldosterone antagonist action was studied in mouse peritoneal macrophages (MPMs) in vitro. Although ACE activity and mRNA were significantly increased by 250 nmol/L aldosterone, ACE2 was significantly reduced. Cotreatment with eplerenone \( (2 \mu\text{mol}/L) \) attenuated these effects. In MPM obtained from p47 knockout mice, where NADPH oxidase is inactive, as well as in control MPMs treated with NADPH oxidase inhibitor, aldosterone did not increase ACE or decrease ACE2. MRB reduced oxidative stress, decreased ACE activity, and increased ACE2 activity, suggesting a protective role for MRB by possibly increasing generation of angiotensin (1–7) and decreasing formation of angiotensin II. These effects are mediated, at least in part, by NADPH oxidase. \((\text{Circ. Res. 2005;97:946-953.})\)

Key Words: heart failure ■ mineralocorticoid receptor blockade ■ ACE2

Aldosterone has an important role in the pathophysiology of heart failure.\(^{1,2} \) It has been shown that mineralocorticoid receptor blockade (MRB) in addition to standard therapy results in improved cardiovascular function and higher survival rates.\(^{3,4} \)

The mechanisms underlying the adverse effects of aldosterone include induction of fibrosis,\(^{1} \) inflammation,\(^{5} \) and oxidative stress,\(^{5-7} \) and we have recently shown that eplerenone administration to atherosclerotic mice reduced oxidative stress and attenuated atherogenesis.\(^{8} \) One of the major stimulants of aldosterone in patients with congestive heart failure (CHF) is angiotensin II (Ang II). Although aldosterone is located downstream from angiotensin, it was shown to upregulate the expression and activity of angiotensin-converting enzymes (ACE) in cardiomyocytes via the mineralocorticoid receptor.\(^{9,10} \) Adverse effects of aldosterone in the myocardium might be mediated, at least in part, by increasing Ang II, a peptide involved in the cardiovascular pathophysiology.

ACE2, an ACE homologue insensitive to ACE inhibitors, was recently introduced as a new member of the renin-angiotensin aldosterone system (RAAS).\(^{11,12} \) ACE2 enzymatic activities include the degradation of both AngI and Ang II, with the subsequent formation of Ang-(1–7), which is known to have biological effects opposite to those of Ang II.\(^{13,14} \) In ACE2-knockout mice, which lack ACE2 protein, abnormal heart function with severely impaired cardiac contractility occurs, along with elevated Ang II levels in the plasma, heart, and kidney.\(^{15} \) This might indicate a possible protective role for ACE2 in cardiovascular pathology. We hypothesized that
the beneficial effects of MRB in the treatment of patients with heart failure could be mediated by reduction of oxidative stress and changes in the balance between the opposing activities of ACE and ACE2.

Human monocyte-derived macrophages (HMDMs), which can easily be harvested, exhibit ACE activity and are known to be involved in inflammation and atherosclerosis. These cells served as a model system to test this hypothesis. We measured mRNA expression and activities of ACE and ACE2, as well as cellular oxidative status in HMDMs obtained from blood samples of CHF patients before and after treatment with spironolactone. In mice, with eplerenone, we have studied the effect of specific aldosterone antagonism on the RAAS upstream balance of ACE/ACE2 in the macrophages, as well as the modulation of their enzymatic activities in the heart. The mechanism by which aldosterone antagonist exerts its effects was studied in cultured macrophages.

Materials and Methods

Patients and Study Protocol

This trial was designed as a before–after study in which each patient served as his own control. Baseline characteristics of patients and spironolactone therapy were similar to those in the Randomized Aldactone Evaluation Study (RALES) trial. Ten male patients, aged 66.1 ± 16.5 years, with CHF attributable to ischemic heart disease (New York Heart Association functional class III-IV, ejection fraction 20.9 ± 3.3%) were enrolled into the study. Patients with other HF etiologies, history of recent myocardial infarction, renal failure, diabetes, or smoking were excluded from the trial. All patients received aspirin, ACE inhibitors (at the maximal tolerated dose), calcium channel blockers, β-adrenergic blockers, loop diuretics, and statins. Background medication remained constant throughout the duration of the study. Blood samples were collected from each patient before and at the end of 1 month of spironolactone (25 mg/d) therapy. All patients signed an informed consent and the study was supervised by the Committee of the Technion Israel Institute of Technology and was conducted in accordance with the Israeli law for animal care.

Preparation of Mouse Organs Homogenates

Hearts and kidneys were kept at −70°C immediately after harvesting. Heart left ventricle walls or kidneys were later immersed in cold phosphate-buffered saline (PBS) and cut into small pieces. Organ pieces were subjected to 2 cycles of 40-second homogenization using Polytron PT2000. Homogenates were then centrifuged (13 000g) and pellets washed twice with cold PBS. Pellets were resuspended and aliquots were subjected to activity assay and protein determination.

Serum Analyses

Serum samples were incubated with 100 mmol/L of 2,2'-Azobis(2-amidinopropane hydrochloride (AAPH, Wako Chemical Industries) for 2 hours at 37°C. AAPH is a water soluble azo compound that thermally decomposes to produce peroxyl radicals at a constant rate. Serum susceptibility to lipid peroxidation was analyzed by the thiobarbituric reactive substance (TBARS) assay.

Macrophage Lipid Peroxidation Determination

HMDMs were scraped into PBS for sonication at 4°C. Lipid peroxides were assayed in the cell sonicate as modified for the determination of lipid peroxides in cell sonicate.

Macrophages Release of Superoxides Ions

Cells (10⁶ per well) were incubated in Hanks balanced salts solution containing acetyl cytochrome-C (80 μmol/L). Superoxide cellular production was stimulated by addition of phorbol myristate acetate (0.5 μg/mL) for 30 minutes. Superoxide dismutase (30 mg/L) was added to control samples. Superoxide release was determined in the medium and expressed as nmol superoxides/mg cell protein (E₆₅₀=21 mmol/mL⁻¹ cm⁻¹).

Low-Density Lipoprotein Oxidation by Macrophages

HMDMs were incubated with low-density lipoprotein (LDL: 100 μg/mL) in RPMI-1640 medium (phenol-red-free) with CuSO₄ (2 μmol/L) for 6 hours at 37°C, and LDL oxidation was measured in the medium by the TBARS assay. Macrophage-mediated oxidation of LDL was calculated by subtraction of LDL oxidation rate without cells from that obtained with macrophages.

Macrophage ACE Activity Determination

HMDMs plated into 96-well plates were analyzed for their ACE activity using a commercial kit (Buhlmann). The kinetics of ACE-mediated cleavage of the synthetic substrate fufuryl-acryloyl-phenylalaninyl-glycyl-glycine to fufuryl-acryloyl-phenylalanine and glycine are measured by reduced absorbance at 340 nm. The absorbance kinetic was measured in an ultra-violet microplate reader (PowerWave, Biotech) and standardized to a known calibrator activity.

ACE2 Activity Assay

ACE2 activity determination was a modification of the method described by Huang et al. Briefly, ACE2 cleaves the leucine at the C-terminal of the decapetide angiotensin I (Asp¹ Arg² Val³ Tyr⁴ Ile⁵ His⁶ Pro⁷ Phe⁸ His⁹ Leu¹⁰) and the assay is based on measurement of free leucine released. With the addition of β-nicotinamide adenine dinucleotide and leucine dehydrogenase (LeuDH) (Sigma), NADH is formed and the latter is coupled to diaphorase-mediated conversion of resazurine to resorufin, which is fluorescent (excitation 565 nm, emission 585 nm). Fluorescence kinetic is measured for 1 hour at room temperature in the Fluostar Galaxy plate reader (BMG Labtechnologies).

The assay was adapted to measure ACE2 in intact cells. LeuDH and diaphorase concentrations were increased 5-fold (0.5 μmol/L) to minimize rate-limiting kinetics. With the addition of AngII (7.7 nmol)
to MPMs in culture, an increase in fluorescence was measured (Figure 1A). This increase is dependent on presence of leucine dehydrogenase and an external supply of nicotinamide adenine dinucleotide, indicating the specific measurement of free leucine released. Ang II (7.7 nmol) alone was unable to induce an increase in fluorescence. Preincubation of human macrophages with a specific antibody directed against the ectopic domain of human ACE2 (R&D Systems) completely abolished the AngII-induced increase in fluorescence (Figure 1B), indicating the association of the Angl degradation activity with ACE2. Activity results are expressed as fmol leucine formation per min and normalized to mg tissue protein.

ACE and ACE2 mRNA Expression by Reverse-Transcription Polymerase Chain Reaction Analysis

Total RNA was extracted from macrophages and tissues using TRI-reagent (Molecular Research Center, Inc). One μg RNA was subjected to reverse transcriptase (Boehringer Mannheim, Germany) to generate cDNA. Polymerase chain reaction (PCR) was applied using 2 μL of resulting cDNA. The forward primer for ACE was 5'-CCGATCTGGCAGAActcTc-3' and the reverse primer 5'-GGTTTCCCCAGTCGTCCTCt-3'. Amplification conditions for ACE were denaturation (95°C, 1 minute), annealing (cycles at 55°C, 45 seconds, and 35 cycles at 50°C, 45 seconds), and extension (72°C, 45 seconds). The forward primer for ACE2 was 5'-CATGGAAGAAGGTTGAGGACTTT-3' and the reverse primer 5'-GAGCTTGGCATCCTTCA-3'. Amplification conditions for ACE2 were denaturation (94°C, 2 minutes), annealing (cycles at 56°C, 45 seconds), elongation (72°C, 1.5 minutes), and extension (94°C, 45 seconds). Mouse PCR primers were p47 forward 5'-ACATCACAGGCCCCATCATCCTC-3', reverse 5'-ATGGAATTGCCCTTTGTGCC-3', ACE forward 5'-TAACGTGGTGCCGAGG-3', reverse 5'-CCACGAGTTGGCAGTCTT-3', and ACE2 forward 5'-GAAAAGCACGGAGGTATCCA-3', reverse 5'-TGAACTTGGCAATCTTGTTG-3' (Tm 58°C). Reverse transcription PCR product of β-actin gene (forward primer: 5'-GTGGGCGCCCCAGGCACCA-3' and reverse primer: 5'-CTCGCT-TAAATGCAACGGAGATT-3') served as a quantity control, and it required 25 cycles to obtain a visible product. PCR products (8 μL) were electrophoresed on a 2.0% agarose-tris-acetate-EDTA gel, which was exposed to UV to produce clear visible bands.

Statistical Analysis

Results are expressed as mean±SEM. Two-tailed Student t test was used to determine statistical significance when comparing 2 arrays of data, treatment versus control. For comparison of data obtained in patients after spironolactone treatment to data obtained before treatment, Student paired t test was used.

Results

Effect of Spironolactone Therapy on Patients’ Serum and HMDM Oxidative Stress

Patients were hemodynamically stable throughout the study and no medications were added. No changes in blood pressure and serum potassium were observed in the patients after spironolactone therapy (data not shown).

Patients treated with spironolactone were first analyzed for their serum oxidative status. The patients’ serum susceptibility to lipid peroxidation, measured by the TBARS assay, was significantly reduced by 31% (P<0.001) after spironolactone treatment compared with values obtained before treatment (Figure 2A).

HMDMs isolated from the patients’ blood after spironolactone therapy exhibited significantly decreased (28%) lipid peroxide content (P<0.001) and superoxide ion release (53%; P<0.005) compared with values obtained before spironolactone therapy (Figure 2B and 2C). Reduced HMDM oxidative status after spironolactone therapy was translated into a significantly lower ability (70%) to oxidize LDL (P<0.005) compared with values obtained before treatment (Figure 2D).

Effect of Spironolactone Therapy on ACE and ACE2 Activity and mRNA Expression in HMDM of CHF Patients

HMDMs obtained from patients treated with spironolactone (25 mg/d) for 1 month exhibited a significant (P<0.01) increase in ACE activity, with an average increase of 400% compared with the activity measured before therapy (Figure 3A). Although ACE activity was upregulated by spironolactone therapy, ACE activity was reduced to 47% of the activity measured before treatment (Figure 3B).

The changes in mRNA expression of both ACE and ACE2 after spironolactone therapy were analyzed. ACE2 mRNA expression in HMDMs (Figure 3C) was significantly (P<0.01) upregulated by 6.54-fold and ACE mRNA (Figure 3D) was downregulated by 53% (P<0.05) compared with values before spironolactone treatment. ACE2 mRNA levels were negligible in HMDMs obtained from CHF patients before spironolactone therapy, as shown in a representative agarose gels of PCR products for ACE2 and β-actin (Figure 3C, insert). Although the extent of the effects varied between patients, all the patients...
responded to spironolactone therapy with increased ACE2 activity and decreased ACE activity and mRNA expression.

**Effect of the Aldosterone Receptor Blockade by Eplerenone on Cellular and Cardiac ACE and ACE2 Activities in Mice**

To determine whether the effect observed with spironolactone on macrophage ACE2 relates to cardiac ACE2, we studied Balb/C mice treated with the specific mineralocorticoid receptor antagonist eplerenone. Two weeks administration of eplerenone (200 mg·kg⁻¹·d⁻¹) had no effect on blood pressure of mice. Systolic pressures of control and eplerenone-treated mice were 102/3.8 and 101/7.7 mm Hg, respectively.

ACE2 activity in MPMs of aldosterone receptor blocker-treated mice was 2.54-fold higher than in MPMs obtained from control untreated mice, and ACE activity was reduced to 51.6% of that measured in MPMs of control untreated mice (Figure 4). Similar effects on ACE2 and ACE activities were observed in hearts of treated mice. Although ACE2 activity showed an increase of 99%, ACE activity decreased by 48% of control (Figure 4).

In the kidney, the natural target for aldosterone inhibition, both ACE2 and ACE activities were higher in treated mice by 8% and 38%, respectively, compared with control mice (Figure 4), but these changes were not statistically significant. The relative ACE2 activity level in the various control tissues was significantly different (n=4, P<0.05 versus MPMs). When normalized to milligram protein, the highest activity was measured in the kidney (to a lesser extent in the MPMs) and the least activity in heart tissue, with 3063±640, 1584±147, and 58±7 fmol leucine formation per minute, respectively. ACE2 mRNA significantly (P<0.05) increased in hearts of eplerenone-treated mice and was 3-fold higher compared with control untreated mice (299±10%). In the kidney, however, ACE2 mRNA expression in eplerenone-treated mice was 111±19% of control untreated mice.

**The Direct Effects of Aldosterone and Its Antagonist on ACE and ACE2 in Cultured MPM**

MPMs obtained from C57Bl mice were grown in a serum-free medium for 36 hours in the presence or absence of aldosterone. In the presence of 250 nmol/L aldosterone, ACE2 activity decreased by 65% (Figure 5A) compared with control, and ACE activity increased by 30% (Figure 5B). These effects were dose-dependent (data not shown). Coadministration with eplerenone (2 μmol/L) attenuated the effects observed with aldosterone alone. ACE2 and ACE mRNA expression levels were similarly changed (Figure 5C and 5D).

To further investigate whether aldosterone-mediated control of ACE and ACE2 is related to oxidation, we examined the involvement of the cellular reactive oxygen species (ROS) generator NADPH oxidase. In MPMs obtained from C57Bl mice, expression of the p47 subunit of NADPH oxidase increased by 47% after incubation with aldosterone. Furthermore, in MPMs with inactive NADPH oxidase obtained from p47ko mice, aldosterone did not induce the

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**Figure 2.** Effect of spironolactone treatment on CHF patients serum and HMDM oxidative stress. A, Serum samples from CHF patients treated with spironolactone (25 mg/d, 1 month) were analyzed for their susceptibility to lipid peroxidation (n=10). Patients’ HMDMs were analyzed for their lipid peroxides content (B; n=10), superoxides release (C; n=9), and ability to oxidize LDL (D; n=9). Values are means±SEM. * P<0.005 after vs before spironolactone.
changes in ACE and ACE2 that were observed in C57Bl mice.

We further investigated whether the aldosterone-induced effects are related to increased levels of ROS or require the activation of NADPH oxidase. Thus, MPMs obtained from C57Bl mice were incubated with aldosterone in the absence or presence of either superoxide dismutase (SOD) or apocynin. SOD, which inactivates superoxides, did not alter the aldosterone-induced effect (Figure 6A and 6B). Coadministration of aldosterone with apocynin (50 μmol/L), which blocks association of p47 with membrane-associated subunits,23 abolished the effects of aldosterone on ACE and ACE2 activities, however, as shown in Figure 6A and 6B, respectively. Neither SOD nor apocynin altered the basal level of ACE and ACE2 in the absence of aldosterone (data not shown). These data strongly implicate NADPH oxidase signaling in the control of both angiotensinas.

Discussion
In the present study, we have demonstrated for the first time a substantial increase in ACE2 activity and mRNA in HMDMs obtained from CHF patients after 1 month of spironolactone therapy. ACE activity and mRNA were significantly decreased. These effects co-occurred with decreased oxidative stress. In mice treated with the specific aldosterone receptor blocker eplerenone, we demonstrated that, similar to what was seen with MPMs, ACE2 activity increased and ACE activity decreased in their hearts. In cultured MPMs treated with aldosterone, reduced ACE2 and increased ACE activities were observed, and coadministration with eplerenone attenuated these effects. The absence of response to aldosterone in MPMs obtained from p47ko mice, as well as in control mice treated with NADPH oxidase inhibitor, strongly implicates NADPH oxidase activation in the mediation of these effects.

We have previously shown that aldosterone treatment increased ACE activity and mRNA expression in mouse peritoneal macrophages.24 In the present study, we show that blocking of the mineralocorticoid receptor in control mice, with no background therapy, resulted in decreased ACE in both MPMs and in the heart. ACE downregulation after spironolactone administration was also reported in cardio-
Increased ACE2 could result in increased formation of Ang-(1–7), which is known to have biological activities opposite to Ang II. These events may lead to decreased Ang II and increased Ang-(1–7) and could account for the improvement in CHF patients after mineralocorticoid receptor antagonism shown in the RALES and Eplerenone Post-Acute Myocardial Infarction Heart Failure Efficacy and Survival Study (EPHESUS) studies.

Although macrophages and the heart responded similar ways to MRB, with an increased ACE2 and decreased ACE, the activity of these enzymes in the kidney was not significantly changed after eplerenone administration. Whether other substances are involved in aldosterone effects in the kidney needs to be studied. It was recently suggested that aldosterone is associated with inflammatory processes that are mediated, at least in part, via endothelin-1.

Activation of circulating RAAS, with elevated Ang II and aldosterone, induces proinflammatory response in the heart that involves resident cardiac cell proliferation and infiltration of macrophages. Possessing ACE, the attracted macrophages may play an important role in regulating the local RAAS by generating Ang II. Similarly, after eplerenone treatment, infiltrating macrophages exhibiting decreased ACE and increased ACE2 could contribute to the creation of a new balance of local RAAS that could be beneficial in a failing heart.

The role of aldosterone in the pathophysiology of heart failure involves its ability to induce inflammation and increase oxidative stress. In the present study, we have shown that spironolactone reduced the oxidative stress in HMDMs obtained from CHF patients. Thus, the reduced oxidative stress observed after spironolactone administration could result directly by antagonizing the aldosterone or via ACE-mediated regulation of Ang II levels. Elevated ACE activity was shown to increase oxidative stress, possibly via formation of Ang II, and ACE inhibition results in decreased plasma and cellular oxidation. In several studies, we have shown that ACE inhibition and ACE deficiency lead to reduced oxidative stress. In vascular smooth muscle cells, aldosterone potentiates Ang II-induced signaling with increased phosphorylation of extracellular signal-regulated kinase and c-Jun-N-terminal kinase, which are ROS generation dependent.

Animals treated with aldosterone exhibited increased NADPH oxidase activity, a cellular generator of ROS. Hypercholesterolemic rabbits treated with spironolactone exhibited reduced NADPH oxidase activity compared with placebo rabbits, suggesting a possible involvement of aldosterone in oxidative stress. In hypertensive rats treated with spironolactone, improved angiotensin-induced vascular changes and reduced oxidative stress were shown. We have recently shown that administration of eplerenone to atherosclerotic mice reduced oxidative stress and attenuated atherosclerosis development. Inhibition of NADPH oxidase ameliorates the adverse myocardial effects of aldosterone. In the present study, we observed elevated expression of the p47 subunit of the NADPH oxidase in MPMs treated with aldosterone, which was attenuated by coadministration with eplerenone. The changes in p47 expression were directly

**Figure 4.** Effect of eplerenone treatment on ACE and ACE2 in mice. Balb/C mice were randomly divided into 2 groups: Eplerenone-treated (200 mg·kg⁻¹·d⁻¹ in their drinking water for 2 weeks or control) and untreated. MPMs were harvested and cultured, and hearts and kidneys were homogenized. Aliquots of the nonsolubilized homogenate pellet were taken for ACE and ACE2 activity measurements. Results are expressed as the ratio of activity measured in eplerenone-treated mice to control untreated mice. ACE2 control activities in MPMs, hearts, and kidneys were 1584, 58, and 3063 fmol leucine formation per minute, respectively. Each bar represents mean±SEM, n=4. NS indicates non significant, eplerenone vs control. *P<0.001.
related to ACE and inversely related to ACE2. The failure of aldosterone to induce changes in ACE and ACE2 in macrophages with inactive NADPH oxidase obtained from p47ko mice strongly implicate the NADPH oxidase-related signaling pathways in the control of these 2 enzymes. Moreover, on the basis of the observations that apocynin rather than SOD had a protective role against aldosterone, it is thought that formation of active NADPH oxidase rather than ROS accumulation is involved in aldosterone-mediated regulation of the angiotensinases. Further studies are required to understand the mechanism by which NADPH oxidase and angiotensinase are coregulated in response to aldosterone signaling.

In conclusion, administration of mineralocorticoid receptor blockade can lead to differential control of angiotensinases and reduction of oxidative status, and thus could contribute to the beneficial effects of MRBs observed in the RALES and EPHESUS studies.

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