Mineralocorticoid Receptor Inhibition Ameliorates the Transition to Myocardial Failure and Decreases Oxidative Stress and Inflammation in Mice With Chronic Pressure Overload

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Background—Although aldosterone, acting via mineralocorticoid receptors, causes left ventricular (LV) hypertrophy in experimental models of high-aldosterone hypertension, little is known about the role of aldosterone or mineralocorticoid receptors in mediating adverse remodeling in response to chronic pressure overload.

Methods and Results—We used the mineralocorticoid receptor–selective antagonist eplerenone (EPL) to test the role of mineralocorticoid receptors in mediating the transition from hypertrophy to failure in mice with chronic pressure overload caused by ascending aortic constriction (AAC). One week after AAC, mice were randomized to regular chow or chow containing EPL (200 mg/kg per day) for an additional 7 weeks. EPL had no significant effect on systolic blood pressure after AAC. Eight weeks after AAC, EPL treatment improved survival (94% versus 65%), attenuated the increases in LV end-diastolic (3.4±0.1 versus 3.7±0.1 mm) and end-systolic (2.0±0.1 versus 2.5±0.2 mm) dimensions, and ameliorated the decrease in fractional shortening (42±2% versus 34±4%). EPL also decreased myocardial fibrosis, myocyte apoptosis, and the ratio of matrix metalloproteinase-2/tissue inhibitor of matrix metalloproteinase-2. These beneficial effects of EPL were associated with less myocardial oxidative stress, as assessed by 3-nitrotyrosine staining, reduced expression of the adhesion molecule intercellular adhesion molecule-1, and reduced infiltration by macrophages.

Conclusions—Mineralocorticoid receptors play an important role in mediating the transition from LV hypertrophy to failure with chronic pressure overload. The effects of mineralocorticoid receptor stimulation are associated with alterations in the interstitial matrix and myocyte apoptosis and may be mediated, at least in part, by oxidative stress and inflammation. (Circulation. 2005;111:420-427.)

Key Words: aldosterone ■ stress, oxidative ■ inflammation

Chronic pressure overload due to hypertension is a major cause of heart failure. Although left ventricular (LV) hypertrophy may cause clinical heart failure due to diastolic dysfunction, progressive adverse remodeling may also lead to LV dilation and pump failure, which are associated with a poor prognosis.1,2 The mechanisms responsible for this transition from myocardial hypertrophy to dilated failure are poorly understood.

Elevated plasma aldosterone levels correlate with mortality in patients with heart failure.3 Despite recent human trials showing a mortality benefit with aldosterone inhibition,4,5 little is known about the role of aldosterone in the development of myocardial remodeling and the progression to cardiac failure. Aldosterone has been implicated in adverse cardiac remodeling associated with cardiac hypertrophy,6,7 fibrosis,8-10 and perivascular inflammation.11,12 Although the mechanisms underlying these aldosterone-induced changes are incompletely understood, some studies have suggested that these effects of aldosterone are independent of blood pressure or angiotensin levels13 and may involve increased oxidative stress and inflammation.12,14 However, because these data originate from experimental models of high-aldosterone hypertension involving unilateral nephrectomy, aldosterone infusion, and/or high-salt diet,11,12,15,16 it is unclear whether aldosterone is involved in myocardial remodeling due to pressure overload and, if so, whether the effects of aldosterone are associated with oxidative stress and inflammation, as observed in the models of high aldosterone.

Myocardial failure is associated with apoptotic death of cardiac myocytes and degradation of the interstitial matrix.15,17 Pressure overload leads to increased myocyte apoptosis17 that may contribute to myocardial failure. Recently,
we\textsuperscript{19} and others\textsuperscript{20} have shown that aldosterone, probably acting via mineralocorticoid receptors, can induce apoptosis both in vitro and in vivo. In addition, it has been shown that activation of matrix metalloproteinases (MMP), which degrade most components of the interstitial matrix, precedes LV dilation in high-salt hypertension.\textsuperscript{18} Mineralocorticoid receptors affect MMP activity,\textsuperscript{10,21} and ACE inhibition suppresses MMP activation and prevents LV remodeling and dysfunction.\textsuperscript{22} Thus, it is possible that aldosterone, acting via the mineralocorticoid receptors, is involved in mediating at least some of the cellular events observed in pressure overload--induced myocardial failure.

Accordingly, the goal of this study was (1) to test the hypothesis that a selective mineralocorticoid receptors antagonist, eplerenone (EPL), inhibits the progression from LV hypertrophy to failure with pressure overload and (2) to examine the role of mineralocorticoid receptors in mediating pressure overload--induced events in the myocardium, including myocyte apoptosis, interstitial matrix degradation, oxidative stress, and inflammation.

**Methods**

**Experimental Animals**

Male FVB mice were purchased from Charles River (Cambridge, Mass). The Institutional Animal Care and Use Committee at Boston University School of Medicine approved all study procedures related to handling and surgery of the mice.

**Ascending Aortic Constriction**

Ascending aortic constriction (AAC) was performed as previously described.\textsuperscript{23} Briefly, 10-week-old FVB mice (Charles River, Cambridge, Mass) were anesthetized with pentobarbital 15 mg/kg IP and ventilated on a Harvard rodent respirator (model 683). Aortic constriction was performed by ligation of the ascending aorta around a 27-gauge needle with the use of a 7-0 silk suture via an anterolateral thoracotomy. This technique has been shown to provide minimal variability of transconstriction pressure gradients in mice.\textsuperscript{23} Sham-operated mice underwent a similar procedure without ligation of the ascending aorta.

**Treatment**

After hypertrophy was established at 1 week, by echocardiography, in AAC mice, a total of 60 mice (16 sham-operated and 44 banded) were randomly assigned to regular chow or chow containing EPL (200 mg/kg body wt per day).

**Echocardiography and Blood Pressure Measurement**

Murine transthoracic echocardiography was performed in conscious mice before surgery (baseline) and 2, 4, and 8 weeks after surgery. Additional echocardiography to verify hypertrophy after AAC was conducted 1 week postoperatively. In the AAC group, only mice with established hypertrophy after 1 week postoperatively were included in the study. Echocardiography was performed as previously described.\textsuperscript{24} Hearts were imaged in the 2D parasternal short-axis view, and 3 different frames of an M-mode echocardiogram of the midcavity, and base as described.\textsuperscript{28} TUNEL-positive number (green fluorescent) and total number (blue fluorescent) of nuclei per unit area were quantified.

**Fibrosis and Myocyte Size**

Sections (4 μm) were stained with picrosirius and visualized by light microscopy. The entire section was quantified with the use of Bioquant image analysis software. Sections from 46 hearts were used for determination of myocyte cross-sectional diameter as previously described.\textsuperscript{29}

**Assessment of MMP and Tissue Inhibitor of MMP Protein Levels**

As previously described,\textsuperscript{30} tissue inhibitor of MMP (TIMP) and MMP protein levels (per 25 μg of protein) were measured by SDS-PAGE and Western blotting with polyclonal rabbit antibodies (Chemicon) recognizing TIMP-1, TIMP-2, and MMP-9 and monoclonal mouse antibody (Chemicon) recognizing MMP-2.

**Immunohistochemistry**

Immunohistochemistry for 3-nitrotyrosine was performed as described previously.\textsuperscript{31} Briefly, sections (4 μm thick) were deparaffinized, rehydrated, and treated with 10 mmol/L citric acid (pH 6.0). Sections were washed with a microwave (2 minutes, 3 times at 700 W) to recover antigenicity. Nonspecific binding was blocked with 10% normal goat serum in PBS (pH 7.4) for 30 minutes before incubation with polyclonal anti–3-nitrotyrosine antibody (1 μg/mL; Upstate Biotechnology) in PBS with 1% BSA overnight at 4°C. Tissue sections were then incubated for 30 minutes at room temperature with a biotinylated anti-rabbit IgG (1:800) secondary antibody by using the Vectastain ABC kit (Vector). Vector Red alkaline phosphatase substrate (Vector) was used to visualize 3-nitrotyrosine. Specificity of anti–3-nitrotyrosine antibodies was confirmed by preincubation of the antibody with free 3-nitrotyrosine (10 mmol/L) or by using a nonimmune rabbit IgG (Vector) isotypic control.

Intercellular adhesion molecule-1 (ICAM-1) staining was performed with the use of a 1:50 dilution of mouse monoclonal ICAM-1 (G-5) antibody (Santa Cruz Biotechnology) in PBS with 1% BSA overnight at 4°C. Nonspecific binding was blocked by incubation with 10% horse serum in PBS (pH 7.4) for 30 minutes before incubation with the antibody. Tissue sections were then incubated for 30 minutes at room temperature with a biotinylated secondary anti-mouse antibody (Vector) diluted in 1% BSA in PBS (pH 7.4). Vector Red alkaline phosphatase substrate (Vector) was used to visualize ICAM-1.

For ED-1 staining to visualize macrophages, sections were deparaffinized, rehydrated, and treated with 20 μg proteinase K per milliliter of Tris-HCl (pH 8.5) for 25 minutes at room temperature to recover antigenicity. Sections were then stained with a 1:25 dilution of rat anti-mouse CD68 primary antibody (Serotec) in PBS with 1% BSA overnight at 4°C. Nonspecific binding was blocked by incubation with 10% horse serum in PBS (pH 7.4) for 30 minutes before incubation with the antibody. A biotinylated anti-rat antibody (Vector) was used as secondary antibody, and Vector Red alkaline...
Systemic blood pressure was decreased by AAC (Figure 1). During the 7 subsequent weeks of drug treatment, mice alive at the time of treatment randomization, 1 week after AAC, had no effect on wall thickness at any time point. In contrast, at 8 weeks after AAC, EPL significantly reduced the increases in LVEDD and LVESD and ameliorated the decrease in fractional shortening (Figure 2A to 2D).

**LV Geometry and Systolic Function**

Posterior wall thickness was increased 2 weeks after AAC and increased only slightly more by 8 weeks (Figure 2A). In contrast, AAC caused progressive increases in LVEDD and LVESD that were first significant at 4 weeks and increased further at 8 weeks (Figure 2B and 2C). After AAC, LV dilation was associated with a progressive decrease in fractional shortening, which was significant at 4 weeks and decreased further at 8 weeks (Figure 2D). EPL, which was begun 1 week after AAC, had no effect on wall thickness at any time point. In contrast, at 8 weeks after AAC, EPL significantly reduced the increases in LVEDD and LVESD and ameliorated the decrease in fractional shortening (Figure 2A to 2D).

**Myocardial Fibrosis**

Picrosirius staining showed increased interstitial and perivascular fibrosis 8 weeks after AAC in both EPL-treated and untreated mice (Figure 3A and 3B). EPL decreased AAC-induced fibrosis by 43%, primarily because of a decrease in interstitial fibrosis (Figure 3A and 3B).

**Myocardial MMP and TIMP**

At 8 weeks after AAC, MMP-2 protein expression was increased 76±43% versus sham mice (P<0.05). EPL attenuated the increase in MMP-2 expression after AAC by 33±25% (P<0.05 versus AAC). MMP-9 was decreased by 29±8% after AAC (P<0.05 versus sham) and was not affected by EPL (−26±12%; P=NS versus AAC). TIMP-2 expression was decreased after AAC (−83±4%; P<0.05 versus sham) and was not affected by EPL (−79±8%; P=NS versus AAC). The resulting MMP-2/TIMP-2 ratio, an index of net MMP activation, was increased >14-fold after AAC, and the increase was markedly attenuated to ∼4-fold by EPL (Figure 4).

**Myocyte Apoptosis**

Eight weeks after AAC, there was minimal myocyte apoptosis in sham-operated mice (∼2.7 TUNEL-positive myocytes sectional diameter (Table). Likewise, AAC resulted in an increase in lung wet/dry weight, indicative of pulmonary congestion. In mice subjected to AAC, HW/BW, LV weight, myocyte diameter, and lung wet/dry weight all tended to decrease with EPL treatment, although only the decrease in lung wet/dry weight was significant. The liver wet/dry weight was not affected by AAC or EPL treatment.

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**Results**

**Survival**

Mice alive at the time of treatment randomization, 1 week after surgery, were included in a Kaplan-Meier analysis (Figure 1). During the 7 subsequent weeks of drug treatment, EPL reduced the mortality of mice subjected to AAC from 35% to 6%.

**Blood Pressure and Cardiac Morphology 8 Weeks After AAC**

Systemic blood pressure was decreased by ∼10 mm Hg in mice subjected to AAC (Table). EPL treatment did not affect blood pressure or heart rate in either sham or AAC mice. BW was not affected by AAC or EPL treatment. AAC caused increases in HW, HW/BW, LV weight, and myocyte cross-sectional diameter (Table). Likewise, AAC resulted in an increase in lung wet/dry weight, indicative of pulmonary congestion. In mice subjected to AAC, HW/BW, LV weight, myocyte diameter, and lung wet/dry weight all tended to decrease with EPL treatment, although only the decrease in lung wet/dry weight was significant. The liver wet/dry weight was not affected by AAC or EPL treatment.
per 100,000 nuclei), and this was not affected by EPL. AAC increased the number of apoptotic myocytes to ≈16.2 TUNEL-positive myocytes per 100,000 nuclei, and the increase was reduced almost to the sham level by EPL treatment (Figure 5).

**Oxidative Stress and Inflammation**

To further investigate the underlying mechanisms leading to adverse remodeling after AAC, myocardial oxidative stress and inflammation were assessed. Eight weeks after AAC, staining for 3-nitrotyrosine was increased diffusely in myocytes, and the increase was markedly ameliorated by EPL (Figure 6A).

At 8 weeks after AAC, staining for ED-1 (Figure 6B), a macrophage marker, was increased in the interstitium and perivascular areas. Likewise, staining for ICAM-1 (Figure 7) was increased diffusely across the myocardium. The increases in both ED-1 and ICAM-1 were markedly ameliorated by EPL treatment.

**Discussion**

**Mineralocorticoid Receptor Activation Is Involved in the Transition to Failure**

Chronic pressure overload due to AAC results in an initial hypertrophic response during weeks 1 to 2, followed by progressive LV dilation and systolic failure during weeks 2 to 8.1-2 Treatment with the selective mineralocorticoid receptor antagonist EPL, begun 1 week after AAC, ameliorated the progressive LV dilation and systolic failure, thus providing direct evidence that mineralocorticoid receptors are involved in mediating pressure overload–induced myocardial failure and indirect evidence for the involvement of aldosterone. Because EPL treatment was not begun until 1 week after AAC, at a time when LV hypertrophy had already occurred, we cannot comment on the role of mineralocorticoid receptors in mediating LV hypertrophy. EPL also suppressed the pressure overload–induced increases in MMP activity and myocyte apoptosis, which may have contributed to the improvement in remodeling. These beneficial effects of EPL occurred without a decrease in systemic blood pressure but were associated with decreases in oxidative stress and inflammation in the myocardium.

Because aldosterone is a major agonist for mineralocorticoid receptors and plasma aldosterone levels are reportedly elevated in pressure overload,33,34 these data are consistent with the thesis that aldosterone plays a key role in mediating adverse myocardial remodeling with pressure overload. This thesis is supported by the observations that cardiac myocytes express mineralocorticoid receptors35 and that aldosterone is synthesized by the myocardium,36 as well as by studies in which elevated aldosterone levels were associated with adverse myocardial remodeling (hypertrophy, fibrosis, and LV dilation) that was inhibited by a mineralocorticoid receptor antagonist.6-10,13 It should also be noted that, under appropriate conditions, glucocorticoids may lead to mineralocorticoid receptor activation in the cardiac myocyte.37 Thus, although our data clearly implicate mineralocorticoid receptors in mediating adverse LV remodeling with pressure overload, we cannot exclude a potential contribution of glucocorticoids to mineralocorticoid receptor activation.

**Mineralocorticoid Receptors Mediate Interstitial Matrix Remodeling**

Pressure overload–induced LV dilation was associated with an increase in the MMP/TIMP ratio, which reflects a net increase in MMP activity that may contribute to ventricular dilation by impairing the integrity of the interstitial matrix.22,38 Our finding that EPL attenuated the increase in the MMP/TIMP ratio suggests that activation of mineralocorticoid receptors contributes to LV dilation in response to pressure overload. Despite LV dilation and an increase in the MMP/TIMP ratio, pressure overload was associated with increased interstitial and perivascular fibrosis, similar to that described by Rocha et al11 and Sun et al12 in rats subjected to
chronic aldosterone infusion. EPL significantly decreased the amount of pressure overload–induced myocardial fibrosis, primarily as a result of a reduction in interstitial fibrosis. Although an increase in MMP activity might be expected to cause a decrease in myocardial fibrosis, it is also possible that increased collagen turnover mediated by increased MMP activity, if opposed by an increase in collagen synthesis, may result in the accumulation of collagen that is qualitatively altered and perhaps less suitable for maintenance of normal LV structure.

Mineralocorticoid Receptors Mediate Myocyte Apoptosis
Pressure overload was associated with a modest increase in myocyte apoptosis, as previously observed in rats with aortic constriction.17 Although the contribution of apoptosis to LV failure in this model is not known, there is increasing evidence that myocyte apoptosis can be an important contributor to LV failure.39 Our finding that EPL markedly attenuated the increase in apoptosis thus raises the possibility that mineralocorticoid receptor–mediated myocyte apoptosis contributes to LV failure in pressure overload. The mechanism by which EPL prevented apoptosis is not known. However, in vitro studies have shown that aldosterone can cause apoptosis in cardiomyocytes,20 thymocytes, and neuronal cells.40,41 Likewise, we found that chronic aldosterone infusion in mice is associated with myocyte apoptosis.19

Oxidative Stress and Inflammation
Pressure overload was associated with increased 3-nitrotyrosine staining diffusely in myocytes, indicative of oxidative nitration of tyrosines. A similar observation of increased nitrotyrosine staining has been made in animals subjected to chronic aldosterone infusion12 or pressure overload due to aortic constriction.42 Although our data do not allow us to determine the precise oxidant species, they are consistent with increases in reactive oxygen species (eg, superoxide), reactive nitrogen species (eg, nitric oxide), or both. Superoxide and nitric oxide may react...
chemically to yield peroxynitrite, a potent oxidant that is capable of causing tyrosine nitration. Our finding that pressure overload–induced nitrotyrosine staining is markedly inhibited by EPL implicates mineralocorticoid receptors in mediating at least some of the increased oxidative stress that has been appreciated in myocardium subjected to pressure overload.43

The mechanism by which mineralocorticoid receptors mediate oxidative stress remains to be determined. However, Sun et al12 observed that inhibition of NADPH oxidase ameliorates the adverse myocardial effects of aldosterone, suggesting that NADPH oxidase may be a source of reactive oxygen species in response to mineralocorticoid receptors activation. Furthermore, the proinflammatory effect mediated by mineralocorticoid receptors may lead to induction of nitric oxide synthase 2, resulting in increased levels of nitric oxide.

Pressure overload was also associated with the increased expression of ICAM-1 and increased numbers of macrophages in the myocardium, indicative of an inflammatory response. The increases in ICAM-1 and macrophage infiltration were both prevented by EPL, thus strongly implicating mineralocorticoid receptors. Rocha et al11 and Sun et al12 observed a qualitatively similar inflammatory response in rats subjected to chronic aldosterone infusion, and in both cases the response was inhibited by mineralocorticoid receptor blockade. The relationship between oxidative stress and inflammation is complex and bidirectional. Inflammatory pathways may lead to the generation of reactive oxygen species by several mechanisms. Conversely, oxidative stress is a stimulus for inflammation. For example, reactive oxygen species can activate reactive oxygen species–sensitive transcription factors such as nuclear factor-κB and activator protein-1, which mediate the expression of ICAM-1,44 leading to the recruitment of inflammatory cells and the elaboration of inflammatory cytokines such as tumor necrosis factor-α.45 Because both reactive oxygen species and inflammatory cytokines have been shown to mediate alterations in the interstitial matrix46 and myocyte apoptosis,47 it is possible that the beneficial effects of EPL are due, at least in part, to reductions in both oxidative stress and inflammation.

**Location of Mineralocorticoid Receptors**

From our data we cannot determine the site of the mineralocorticoid receptors involved in adverse remodeling. However, it was shown that transgenic mice with myocyte-specific overexpression of 11β-hydroxysteroid dehydrogenase type 2, resulting in loss of the normal opposition to mineralocorticoid receptor activation that is provided by occupancy by glucocorticoids, develop a dilated cardiomyopathy associated with interstitial fibrosis.13 Although this observation suggests that stimulation of mineralocorticoid receptors on cardiac myocytes is sufficient to cause many of the features we observed, it is also likely that mineralocorticoid receptors on other cell types such as fibroblasts are involved in mediating effects on the interstitial matrix.

**Summary**

It has been shown that mineralocorticoid receptors mediate adverse myocardial remodeling in response to chronic aldosterone infusion.11 Our findings provide the first evidence that mineralocorticoid receptors are involved in mediating the
adverse remodeling that occurs in response to a common form of hemodynamic overload. The beneficial effects of EPL occurred in the absence of a decrease in systemic blood pressure but were associated with decreases in myocardial oxidative stress and inflammation, which may be involved in mediating the adverse effects of mineralocorticoid receptor activation in pressure overload. These findings may help to explain the beneficial effects of mineralocorticoid receptor inhibition in clinical trials and, in addition, suggest that the early institution of mineralocorticoid receptor inhibition may have utility in preventing the development of adverse remodeling and failure in response to hemodynamic overload.

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