Mineralocorticoid Receptor Blockade Improves Vasomotor Dysfunction and Vascular Oxidative Stress Early After Myocardial Infarction

Carmem Luíza Sartório, Daniela Fraccarollo, Paolo Galuppo, Meike Leutke, Georg Ertl, Ivanita Stefanon, Johann Bauersachs

Abstract—Mineralocorticoid receptor blockade improves mortality early after myocardial infarction (MI). This study investigated the vascular effects of mineralocorticoid receptor blockade in the early phase postinfarction in rats. Starting immediately after coronary ligation, male Wistar rats were treated with placebo or eplerenone (100 mg/kg/d). After 7 days, hemodynamic assessment was performed and endothelial function was determined. Maximum acetylcholine-induced relaxation was significantly attenuated in aortic rings from rats with heart failure after MI, and ameliorated by eplerenone treatment. Endothelium-independent relaxation by DEA-NONOate was similar among the groups. Endothelial NO synthase phosphorylation was reduced in the aorta of MI rats and restored by eplerenone therapy. Angiotensin I–induced vasoconstriction as well as angiotensin-converting enzyme protein levels were enhanced in aortas from MI placebo rats, and reduced by mineralocorticoid receptor inhibition. Aortic reactive oxygen species formation as well as the expression of the NAD(P)H oxidase subunit p22phox were increased after MI and normalized in eplerenone treated rats. In conclusion, mineralocorticoid receptor antagonism improved endothelial dysfunction in the early phase post-MI. Underlying mechanisms involve inhibition of vascular angiotensin-converting enzyme upregulation and improvement of endothelial NO synthase–derived NO bioavailability. (Hypertension. 2007;50:919-925.)

Key Words: aldosterone ■ acute myocardial infarction ■ endothelial dysfunction ■ oxidative stress

Chronic heart failure is accompanied by endothelial dysfunction. Increased production of vascular reactive oxygen species, especially superoxide anion (O$_2^-$), as well as diminished antioxidant defense in heart failure contribute to a reduced bioavailability of NO. The renin angiotensin aldosterone system (RAAS) is markedly activated and displays a central role in heart failure progression. Aldosterone synthesis and mineralocorticoid receptors (MR) have been described in the vascular wall. Aldosterone stimulates vascular ACE expression/activity and potentiates angiotensin II effects. Angiotensin II and aldosterone stimulate vascular O$_2^-$ production, promoting NO scavenging and reducing its bioavailability. In healthy subjects, contradictory effects of aldosterone on endothelial function are reported. Farquharson et al$^{14}$ showed that intravenous infusion of aldosterone reduced the forearm blood flow response to acetylcholine but did not affect sodium nitroprusside-induced vasodilation, thus inducing acute endothelial dysfunction. Others reported that aldosterone increases forearm blood flow and induces vasodilation by stimulating NO release through rapid non-genomic effects.$^{15}$ In a recent investigation, short-term and chronic aldosterone excess did not affect forearm blood flow, but enhanced the vasodilator response to exogenous NO and improved endothelium-dependent NO-mediated vasodilation in the forearm vasculature of healthy men.$^{16,17}$ However, in subjects with damaged vasculature and endothelial dysfunction, aldosterone infusion induced vasoconstriction, and MR blockade improved NO bioavailability.$^{9,16}$ Also in experimental chronic heart failure, MR antagonism improved endothelial dysfunction.$^{18}$

MR antagonism reduces morbidity and mortality among patients with myocardial infarction (MI) complicated by ventricular dysfunction.$^{19-21}$ However, the underlying mechanisms are still under investigation. Immediate MR antagonism with spironolactone for 1 month improved LV dilation and function in patients with first anterior MI.$^{22}$ Perrier et al$^{23}$ showed that MR antagonism prevents the electrical remodeling that precedes cellular hypertrophy after MI such as upregulation of myocardial calcium current (I$_{Ca}$), downregulation of transient outward potassium current (I$_{to}$), and prolongation of action potential duration. On the other hand, cardiomyocyte-specific MR overexpression leads to ion channel remodeling, resulting in prolonged ventricular repolarization and in severe ventricular arrhythmias.$^{24}$
However, no information is available on the effects of MR antagonism in the vascular system early post-MI. Therefore, this study was designed to evaluate the effects of eplerenone on the vasomotor function, RAAS activation, and oxidative stress in rats early after MI induced by coronary artery ligation.

**Methods**

All procedures were approved by the institutional Animal Research Committee.

**Myocardial Infarction, Study Protocol, Hemodynamic Measurements**

Left coronary artery ligations were performed in adult male Wistar rats (200 to 250g, Harlan-Winkelmann, Borchern, Germany).25 Starting immediately after coronary ligation, surviving rats were randomly selected for eplerenone (100 mg/kg body wt) or placebo treatment (5% arabic gum) administered by gavage for 7 days. Sham-operated animals received placebo treatment. Eplerenone was used at a dose of 100 mg/kg per day, which is the most commonly used dose for this drug in rats with myocardial infarction25,26 and provided marked end-organ protective effects in the heart and kidney of hypertensive rats.37 Eplerenone selectively blocks the mineralocorticoid receptor. In rats, the IC50 of eplerenone for the glucocorticoid, androgen, progesterone, and estrogen receptors were >10000 nmol/L.28,29 Left ventricular systolic (LVSP) and end-diastolic pressures (LVEDP), mean arterial pressure (MAP), and dP/dt were measured 7 days after MI, under light isoflurane anesthesia and spontaneous respiration, using micromanometer (Millar Instruments). Infarct size was determined as previously described.25 Only rats with extensive infarcts (>40%) were included in the study.

**Aldosterone Radioimmunoassay**

A blood sample was collected from the right carotid artery. Aldosterone levels were measured by radioimmunoassay (Sorin Biomedica).

**Vascular Reactivity Studies**

The descending thoracic aorta was dissected after removal of the heart and cleaned of connective tissue. The upper section (~8 mm) was immediately frozen in liquid nitrogen for Western blot analysis. The lower section (~6 mm) was used for immediate measurement of O2 production. One ring was frozen in Tissue Teck and another one placed in formalin (4%) and then embedded in paraffin. The remainder was cut into 3-mm rings which were mounted in an organ bath for isometric force measurements. These rings were equilibrated for 30 minutes under a resting tension of 2g in oxygenated (95% O2; 5% CO2) Krebs-Henseleit solution (118 mmol/L NaCl, 4.7 mmol/L KCl, 1.2 mmol/L MgSO4, 1.6 mmol/L CaCl2, 1.2 mmol/L K2HPO4, 25 mmol/L NaHCO3, and 12 mmol/L glucose, pH 7.4; 37°C) containing diclofenac (1%); deoxycolate 0.5%; NaF 10 mmol/L; sodium pyrophosphate 10 mmol/L; phenylmethylsulfonyl fluoride 100 mmol/L; aprotinin 2 µg/mL; leoptein 2 µg/mL). Aortic extracts were mixed with sample loading buffer and separated under reducing conditions on 12% SDS-polyacrylamide gel. Proteins were electro-transferred on polyvinylidene fluoride (PVDF) membranes (Immun-Blot 0.2 µm, Bio-Rad). The bands were detected using a chemiluminescence assay (ECL Plus, Amershams). Primary antibodies used recognize: eNOS (1:2500, Transduction Laboratories, 610296), phosphorylated eNOS at Ser1177 (1:2000, Transduction Laboratories, 612392), Akt (1:2000, Cell Signaling Technology, 9272), phosphorylated Akt at Ser 473 (1:2000, Cell Signaling Technology, 9271), ACE (1:1000, Abcam, ab11734), p22phox (1:1000, Santa Cruz Biotechnology, sc-11712), MnSOD (1:50000, Abcam, ab13533) and GAPDH (1: 50000, Abcam, ab8245).

**Western Blot Analysis**

Aortic samples were homogenized in ice-cold RIPA buffer (NaCl 150 mmol/L; Tris-HCl 50 mmol/L; EDTA 5 mmol/L; Nonidet-P 40 1%; deoxycolate 0.5%; NaF 10 mmol/L; sodium pyrophosphate 10 mmol/L; phenylmethylsulfonyl fluoride 100 mmol/L; aprotinin 2 µg/mL; leoptein 2 µg/mL). Aortic extracts were mixed with sample loading buffer and separated under reducing conditions on 12% SDS-polyacrylamide gel. Proteins were electro-transferred on polyvinylidene fluoride (PVDF) membranes (Immun-Blot 0.2 µm, Bio-Rad). The bands were detected using a chemiluminescence assay (ECL Plus, Amershams). Primary antibodies used recognize: eNOS (1:2500, Transduction Laboratories, 610296), phosphorylated eNOS at Ser1177 (1:2000, Transduction Laboratories, 612392), Akt (1:2000, Cell Signaling Technology, 9272), phosphorylated Akt at Ser (1:2000, Cell Signaling Technology, 9271), ACE (1:1000, Abcam, ab11734), p22phox (1:1000, Santa Cruz Biotechnology, sc-11712), MnSOD (1:50000, Abcam, ab13533) and GAPDH (1: 50000, Abcam, ab8245).

**Immunohistochemical Analysis**

Immunohistochemical localization of phosphorylated eNOS and ACE were determined in frozen or paraffin included aortic 5-µm sections, respectively. Immunohistochemical staining was performed using the same antibodies mentioned above. Briefly, deparaffinization and hydration of paraffin sections or fixation of frozen sections in cold acetone for 5 minutes were followed by pretreatment with 0.3% hydrogen peroxide for 20 minutes to inhibit endogenous peroxidase activity. Subsequently, sections were blocked with 2% horse serum for 30 minutes and incubated with the primary antibody for 1 hour at room temperature. After rinsing with PBS, the sections were incubated for 30 minutes with a biotinylated secondary antibody, followed by incubation with avidin-biotinylated horseradish peroxidase complex (Vectastain ABC, Vector). Serial sections treated with nonimmune IgG did not show any staining. Peroxidase activity positive staining was detected using the DAB detection system (Vector). Sections were counterstained with hematoxylin.

**Statistics**

Values are presented as means±SEM. Relaxant responses were given as percentage relaxation relative to the preconstriction level. Statistical analysis was performed by 1-way analysis of variance (ANOVA) followed by Fisher post hoc test. Statistical significance was assumed at P<0.05.

**Results**

**Global Parameters and Hemodynamics**

Infarct size and body weight were comparable among the experimental groups (Table 1). At 7 days, eplerenone therapy versus placebo significantly reduced LVEDP and improved LV function (Table 1). Circulating aldosterone levels were expressed as counts per minute per milligram dry weight of tissue (cpm/mg). Moreover, whether eplerenone acts as an antioxidant was evaluated using potassium superoxide (3 mmol/L) in the presence of lucigenin (5 µmol/L). Eplerenone (10–3 to 10–6 mol/L) had no free radical scavenging and antioxidant activities.

The oxidative fluorescent dye hydroethidine was used to evaluate in situ production of reactive oxygen species as described.31 Unfixed frozen ring segments were cut into 5-µm-thick sections and placed on a glass slide. Hydroethidine (2 µmol/L) was topically applied to each tissue section and coverslipped. Slides were incubated in a light-protected humidified chamber at 37°C for 30 minutes. Stained sections were investigated using a Nikon Eclipse E600 microscope equipped with a C1 confocal scanning head and a 20-fold oil immersion objective. Pictures were acquired and prepared for presentation using the EZ-C1 3.00 software from Nikon. Aortic rings from sham, placebo MI, and eplerenone-treated MI rats were processed and imaged in parallel.
Table 1. Global Parameters of Placebo Sham-Operated Rats (Sham Pla) and Rats With MI Treated With Placebo (MI Pla) or With Eplerenone (MI Eple) for 7 Days

<table>
<thead>
<tr>
<th>Global Parameters</th>
<th>Sham Pla</th>
<th>MI Pla</th>
<th>MI Eple</th>
</tr>
</thead>
<tbody>
<tr>
<td>LVSP, mm Hg</td>
<td>137±3</td>
<td>99±4*</td>
<td>106±4*</td>
</tr>
<tr>
<td>LVEDP, mm Hg</td>
<td>5.4±0.4</td>
<td>23.9±2*</td>
<td>14.9±2*†</td>
</tr>
<tr>
<td>MAP, mm Hg</td>
<td>127±3</td>
<td>94±5*</td>
<td>105±4*</td>
</tr>
<tr>
<td>LV dP/dtmax, mm Hg/s</td>
<td>5856±355</td>
<td>3423±339*</td>
<td>4508±273†</td>
</tr>
<tr>
<td>LV dP/dtmin, mm Hg/s</td>
<td>4553±238</td>
<td>2566±178*</td>
<td>3475±178†</td>
</tr>
<tr>
<td>BW, g</td>
<td>290±5</td>
<td>278±6</td>
<td>277±4</td>
</tr>
<tr>
<td>Aldosterone, pg/mL</td>
<td>175±15</td>
<td>522±128*</td>
<td>921±139†</td>
</tr>
</tbody>
</table>

LVSP indicates left ventricular systolic pressure; LVEDP, LV end-diastolic pressures; MAP, mean arterial pressure; BW, body weight. Mean±SEM (n=9–12).

*p<0.05 vs Sham Pla; †p<0.05 vs MI Pla.

markedly enhanced after MI and further increased by MR inhibition (Table 1).

Vascular Reactivity, Western Blot Analysis, and Immunohistochemistry

Acetylcholine-induced endothelium-dependent relaxation of aortic rings preconstricted with phenylephrine was blunted in aortae from MI rats 7 days postinfarction (Figure 1A; Table 2). Eplerenone treatment restored the maximum relaxation (E<sub>max</sub>) to acetylcholine. The endothelium-independent vasorelaxation induced by DEA NONOate was similar among the experimental groups (data not shown). ACE protein levels and immunohistochemical staining were significantly enhanced in aortae of MI placebo and reduced with eplerenone therapy (Figure 3B and 3C).

Vascular Reactive Oxygen Species

Superoxide anion formation was significantly increased in the aortae from placebo MI rats, and completely normalized by eplerenone treatment 7 days postinfarction (Figure 4A). Consistently, confocal scanning microscopy images, which used hydroethidine to visualize reactive oxygen species formation, demonstrated enhanced signal intensity throughout the vascular wall in aortic rings from placebo MI rats versus sham, and reduced signals in eplerenone-treated MI rats (Figure 4B). Vascular NAD(P)H oxidase subunit p22<sup>lox/lox</sup> protein levels were elevated in placebo MI compared with sham-operated rats 7 days postinfarction, and normalized by eplerenone (Figure 5A). Moreover, eplerenone therapy significantly increased vascular MnSOD protein levels 7 days postinfarction (Figure 5B).

Discussion

Our study demonstrates that immediate MR blockade improves early vasomotor dysfunction and oxidative stress in rats with heart failure after MI. Eplerenone normalized endothelium-dependent maximum relaxation, restored eNOS phosphorylation, and diminished reactive oxygen species formation. Furthermore, MR blockade attenuated the increases in ACE vascular expression and in angiotensin I–induced vasoconstriction.

Impaired endothelium-dependent NO-mediated vasorelaxation contributes to elevated vascular resistance in patients with heart failure and is considered an important predictor of subsequent cardiac events.1,32,33 Aldosterone correlates inversely with arterial compliance, which may be partially related to NO.34 The reduction of endothelium-dependent vasodilatation in response to acetylcholine, through aldosterone-mediated attenuation of NO bioavailability, has been proposed as an important mechanism of endothelial dysfunction.18,35 Clinical trials demonstrated improvement of endo-
thelial vasodilator dysfunction and NO bioactivity by MR blockade in patients with chronic heart failure. In the current study, eplerenone ameliorated the blunted maximum endothelium-dependent vasorelaxation 7 days after MI, thus underscoring the importance of MR activation for the cardiovascular alterations early after MI. MR blockade restored eNOS phosphorylation at Ser\(^{1177}\), a major regulator of NO production which may explain, at least in part, the improved endothelial function early after myocardial infarction. Our study thus provides in vivo evidence of the recently reported aldosterone-mediated downregulation of eNOS phosphorylation in cultured endothelial cells.

NAD(P)H-dependent vascular oxidase in the aortic wall has been identified as a major source of increased vascular reactive oxygen species and impaired endothelial function in chronic heart failure. MR antagonism improved impaired endothelial function by decreasing NAD(P)H oxidase subunit p22\(^{\text{phox}}\) expression, NAD(P)H oxidase activity and superoxide anion generation in chronic heart failure and atherosclerosis. The present study demonstrated that MR blockade normalized increased reactive oxygen species production and prevented vascular p22\(^{\text{phox}}\) upregulation early after MI. Although Nagata et al. reported enhanced generation of reactive oxygen species through activation of NADPH oxidase by aldosterone in cultured human endothelial cells, reactive oxygen species formation appears to be increased throughout the whole vascular wall after MI, suggesting that also smooth muscle cell NADPH oxidase is involved. Together, these findings underline the central role of MR activation in stimulating vascular reactive oxygen species generation early after MI. Downregulation of ACE by eplerenone therapy may reduce local angiotensin II, which is known to upregulate vascular NADPH oxidase activity. Moreover, angiotensin II induction of oxidative stress in the vasculature is at least partially mediated by aldosterone. Thus, MR antagonism could improve endothelial function also by a reduction of angiotensin II–dependent reactive oxygen species generation.

The imbalance between reactive oxygen species production and its scavenging is considered the main feature of vascular oxidative stress. Inadequate antioxidant reserves contribute to endothelial dysfunction in heart failure. The key protective role of the diverse superoxide dismutase (SOD) enzymes in vascular oxidative stress has been widely described, as recently reviewed. Addition of exogenous SOD improved endothelium-dependent vasorelaxation. Mn-SOD is 1 of 3 SODs found in mammalian cells which catalyze the dismutation of superoxide to hydrogen peroxide, and considered to be essential for mitochondrial function maintenance and mammalian survival. MnSOD is abundant in endothelial cells relative to other cell types, and may play an important protective role in oxidative stress situations like ischemia and inflammation. MR antagonism promoted MnSOD upregulation early after MI. This effect may account for the reduction in p22\(^{\text{phox}}\) expression, once this subunit can be upregulated in a positive feedback by reactive oxygen

**Figure 2.** A, eNOS phosphorylation at Ser\(^{1177}\) as determined by Western blot analysis in the aorta of placebo treated sham-operated rats (Sham Pla) and rats with MI treated with placebo (MI Pla) or with eplerenone (MI Eple) for 7 days. Mean±SEM (n=8 to 11). †P<0.05 vs MI Pla. B, Immunohistochemistry demonstrated phosphorylated eNOS expression in rat aortae. IgG indicates the negative control. Arrowheads show endothelial cells layer. Magnification ×250.

**Figure 3.** A, Concentration-response curve to angiotensin I (n=9 to 11; A); ACE expression (n=10 to 11; B) and representative immunohistochemical detection of ACE (C) in aortic rings of placebo treated sham-operated rats (Sham Pla) and rats with MI treated with placebo (MI Pla) or with eplerenone (MI Eple) for 7 days. Mean±SEM. *P<0.05, **P<0.01 vs Sham; †P<0.05 vs MI Pla. Photomicrography magnification ×400.
species generation.\textsuperscript{49} The rise of antioxidant protection prevents NO inactivation by $\text{O}_2^-$ and peroxynitrite ($\text{ONOO}^-$) formation and also may break another positive feedback in which $\text{ONOO}^-$ inactivates MnSOD.\textsuperscript{50} Furthermore, aldosterone has recently been shown to downregulate glucose-6-phosphate dehydrogenase activity, resulting in reduced NO levels, increased reactive oxygen species, and impaired vascular reactivity.\textsuperscript{51} Coordinated, all these beneficial effects could account for the positive balance between NO and $\text{O}_2^-$ promoted by eplerenone therapy.

Aldosterone stimulates ACE expression and activity in rat neonatal cardiac myocytes, thus increasing the conversion of angiotensin I to angiotensin II.\textsuperscript{9,52} Furthermore, the induction of ACE by aldosterone was recently described in cultured rat endothelial cells via a MR-dependent pathway.\textsuperscript{10} This mechanism likely contributes to enhanced vasoconstrictor response to angiotensin I and vascular ACE upregulation, as we observed markedly elevated circulating aldosterone levels after MI, and MR inhibition prevented ACE upregulation and partially reduced angiotensin I–induced vasoconstriction. Thus, the positive feedback loop for a local RAAS, namely aldosterone-mediated ACE upregulation, is likely to be involved in the development of endothelial dysfunction and vascular injury induced by aldosterone early after MI. Although there is controversy about the main enzyme responsible for the angiotensin I/angiotensin II conversion, ACE seems to be the main one in rats.\textsuperscript{53,54}

Immediate MR blockade reduced LV filling pressure and ameliorated LV contractile dysfunction 7 days postinfarction. Although the sequence of events cannot easily be elucidated in the animal model, these cardiac effects of MR antagonism are likely to contribute to the beneficial effects on endothelial dysfunction and oxidative stress early postinfarction.

In conclusion, we show for the first time that MR blockade with eplerenone improved endothelial dysfunction in the early phase post-MI. Underlying mechanisms involve inhibition of vascular ACE upregulation and improvement of eNOS-derived NO bioavailability.

**Perspectives**

In the present study, MR blockade with eplerenone improved endothelial dysfunction, NO bioavailability, and oxidative stress early post-MI. Underlying mechanisms involve inhibition of vascular ACE upregulation and improvement of eNOS-derived NO bioavailability.

**Figure 4.** A, Superoxide anion production detected by lucigenin enhanced chemiluminescence in aortic rings of placebo treated sham-operated rats (Sham Pla) and rats with MI treated with placebo (MI Pla) or with eplerenone (MI Eple) for 7 days. Mean±SEM (n=8 to 14). $**P<0.01$ vs Sham Pla; $††P<0.01$ vs MI Pla.

**Figure 5.** p22\textsuperscript{phox} (n=7 to 9; A) and MnSOD (n=8 to 10; B) protein expression as determined by Western blot analysis in the aorta of placebo treated sham-operated rats (Sham Pla) and rats with MI treated with placebo (MI Pla) or with eplerenone (MI Eple) for 7 days. Mean±SEM. $**P<0.01$ vs Sham Pla; $†P<0.05$ vs MI Pla.
stress early after experimental MI. In addition to beneficial modulation of electrical remodeling these vascular effects of MR blockade may contribute to the marked reduction of mortality by eplerenone treatment within 30 days in patients with acute MI complicated by heart failure. Impaired endothelial vasodilator function is an independent predictor of clinical deterioration and death in heart failure. In EPHEMUS the patients randomized early to treatment derived more benefit than that randomized late, however, even better effects may be achieved by instant eplerenone therapy postinfarction.

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Disclosures
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References


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