Cardioprotective Effects of Eplerenone in the Rat Heart Interaction With Locally Synthesized or Blood-Derived Aldosterone?

Wenxia Chai, Ingrid M. Garrelds, René de Vries, A.H. Jan Danser

Abstract—Mineralocorticoid receptor antagonism with eplerenone reduces mortality in heart failure, possibly because of blockade of the deleterious effects of aldosterone. To investigate these effects, rat Langendorff hearts were exposed to aldosterone and/or eplerenone. Under normal conditions, aldosterone increased left ventricular pressure and decreased coronary flow. Eplerenone did not block these effects. Eplerenone reduced infarct size (from 68±2% to 53±4%; P<0.05) and increased left ventricular pressure recovery (from 44±2% to 60±5%; P<0.05) after 45 minutes of coronary artery occlusion and 3 hours of reperfusion, whereas aldosterone did not affect these parameters. To verify the origin of cardiac aldosterone, hearts were perfused with 3 to 30 nmol/L aldosterone and either frozen immediately or exposed to washout. Without washout, cardiac aldosterone was 1.5 times aldosterone in coronary effluent (CE), that is, too high to be explained on the basis of its presence in extracellular fluid. The cardiac levels of aldosterone correlated with its CE levels (r=0.81; P<0.01), and both were unaffected by eplerenone. During washout, tissue aldosterone disappeared monophasically (half life, 9±1 minutes), and CE aldosterone disappeared biphasically (half life 1±0 and 8±1 minutes, respectively). During buffer perfusion, cardiac aldosterone was at or below the detection limit. In conclusion, eplerenone improves the condition of the heart after ischemia and reperfusion. This does not relate to interference with the inotropic and vasoconstrictor effects of aldosterone. The majority of cardiac aldosterone, if not all, is derived from the circulation. The rapid, mineralocorticoid receptor–independent kinetics of aldosterone suggest that its accumulation in the heart involves cell surface binding rather than internalization. (Hypertension. 2006; 47:665-670.)

Key Words: mineralocorticoids ■ blood flow ■ arrhythmia ■ ischemia

Two large clinical trials in patients with heart failure have recently shown that the mineralocorticoid receptor (MR) antagonists spironolactone and eplerenone improve morbidity and mortality on top of angiotensin-converting enzyme inhibition.1,2 In particular, a reduction in the rate of sudden death and mortality on top of angiotensin-converting enzyme inhibitor fosinopril, reduced the arrhythmic score (from 68±2% to 53±4%; P<0.05) after 45 minutes of coronary artery occlusion and 3 hours of reperfusion, whereas aldosterone did not affect these parameters. To verify the origin of cardiac aldosterone, hearts were perfused with 3 to 30 nmol/L aldosterone and either frozen immediately or exposed to washout. Without washout, cardiac aldosterone was 1.5 times aldosterone in coronary effluent (CE), that is, too high to be explained on the basis of its presence in extracellular fluid. The cardiac levels of aldosterone correlated with its CE levels (r=0.81; P<0.01), and both were unaffected by eplerenone. During washout, tissue aldosterone disappeared monophasically (half life, 9±1 minutes), and CE aldosterone disappeared biphasically (half life 1±0 and 8±1 minutes, respectively). During buffer perfusion, cardiac aldosterone was at or below the detection limit. In conclusion, eplerenone improves the condition of the heart after ischemia and reperfusion. This does not relate to interference with the inotropic and vasoconstrictor effects of aldosterone. The majority of cardiac aldosterone, if not all, is derived from the circulation. The rapid, mineralocorticoid receptor–independent kinetics of aldosterone suggest that its accumulation in the heart involves cell surface binding rather than internalization.

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Methods

Drugs
Aldosterone was purchased from Sigma-Aldrich Chemie BV. Eplerenone was a kind gift of Pfizer (Capelle a/d IJssel, the Netherlands). Stock solutions of aldosterone (10 mmol/L) and eplerenone (10 mmol/L) were prepared in ethanol.

Ethical Approval
All of the experiments were performed under the regulations of the Animal Care Committee of the Erasmus MC, in accordance with the “Guiding Principles in the Care and Use of Laboratory Animals” as approved by the American Physiological Society.

Experiments in Langendorff Hearts
Male Wistar rats (n=80, weight 300 to 420 g) obtained from Harlan (Zeist, the Netherlands) were anesthetized with sodium pentobarbital (60 mg/kg, IP). Hearts were rapidly excised and cooled in ice-cold Krebs-Henseleit solution (composition in mmol/L: NaCl 125, KCl 4.7, NaHCO₃ 20, NaH₂PO₄ 0.43, MgCl₂ 1.0, CaCl₂ 1.3, and D-glucose 9.1; pH 7.4) until contractions stopped and prepared for Langendorff perfusion. Continuously carbogen-gassed (95% O₂/5% CO₂) Krebs-Henseleit solution at 37°C was perfused immediately after cannulation of the aorta at a constant perfusion pressure of 80 mm Hg. A water-filled latex balloon was placed in the left ventricle via the left atrium to measure LVP. The volume of the balloon was adjusted to achieve a stable left ventricular end-diastolic pressure (LVEDP) of 5 mm Hg during initial equilibration, and this volume was maintained throughout the experiment. Hearts were paced at 350 bpm. Coronary flow (CF) was measured by an in-line flow probe (Transonic Systems).

After a stabilization period of 15 minutes, 100-μL bolus injections were applied to construct dose-response curves to aldosterone, vehicle (ethanol), and/or eplerenone. In a second series of experiments, we evaluated the effects of aldosterone and eplerenone during ischemia and reperfusion. Hearts were subjected to 45 minutes of left anterior descending coronary artery occlusion followed by 3 hours of reperfusion. Occlusion was preceded by no treatment (control) or a 15-minute exposure 100 nmol/L aldosterone, 1 μmol/L eplerenone, or 100 nmol/L aldosterone+1 μmol/L eplerenone. Aldosterone and/or eplerenone remained present in the perfusion buffer throughout the remainder of the experiment. After the 3-hour reperfusion period, area at risk and infarct size were determined as described before. In a third series of experiments, we determined the uptake and disappearance of aldosterone in the heart. Blood (~0.5 mL) was collected from the rats used in these experiments to measure the endogenous plasma levels of aldosterone. Hearts were perfused with 3, 10, or 30 nmol/L of aldosterone or vehicle for 30 minutes in the absence or presence of 1 μmol/L eplerenone. After the 30-minute perfusion period, the hearts were either frozen in liquid nitrogen (LN₂) or subjected to a washout period. One-minute samples of coronary effluent were collected before the perfusion; at 20 and 25 minutes after the start of the perfusion; and at 1, 2, 3, 4, 5, 10, 20, and 30 minutes during the washout period. Aldosterone-perfused hearts that had been washed for 5 or 30 minutes were also frozen in LN₂.

Aldosterone Measurements
Aldosterone was measured by solid-phase radioimmunoassay (Diagnostic Products Corporation) in rat plasma, coronary effluent, and cardiac tissue. To extract aldosterone from cardiac tissue, hearts were homogenized 1:2 in methanol. The homogenate was centrifuged at 3000 rpm for 15 minutes. Supernatants were then collected, vacuum dried, and dissolved in water before the assay. The detection limit was 25 pg/mL in plasma and coronary effluent and 10 pg/g wet weight.

Data Analysis
Data are expressed as mean±SEM. Dose-response curves were analyzed using the logistic function described by de Lean et al to obtain PEC₅₀ (~10⁻⁴ log EC₅₀) values. The maximum effect (Eₘₐₓ) was determined by repeated-measures ANOVA. Aldosterone levels below the detection limit were taken to be equal to the detection limit. Washout kinetics were analyzed according to a 1- or 2-compartment model. Statistical comparison between various conditions was by ANOVA, followed by post hoc evaluation according to Tukey. Univariate linear regression between the aldosterone levels in cardiac tissue and coronary effluent was assessed by calculation of Pearson’s coefficient of correlation. P values <0.05 were considered significant.

Results

Hemodynamic Studies
Baseline values of LVP and CF were 80±1.6 mm Hg (n=54) and 11±0.2 mL/min, respectively. Aldosterone (n=6) increased LVP by maximally 9±2% (P<0.01; PEC₅₀ 9.8±0.4) and reduced CF by maximally 16±4% (P<0.01; PEC₅₀ 8.7±0.6; Figure 1). Eplerenone (n=6) also increased LVP (Eₘₐₓ 7±3%; PEC₅₀ 8.9±1.3; P<0.05) without affecting CF (Figure 1). Vehicle (ethanol; n=6) did not affect LVP or CF. Eplerenone (0.1 or 1 μmol/L; n=6 and 5, respectively) did not abolish the effects of aldosterone on LVP or CF.

Occlusion of the left anterior descending coronary artery reduced LVP to ~30 mm Hg and decreased CF by ~50%.

Figure 1. (left) Effect of eplerenone (eple), spironolactone (spiro), and vehicle on CF and LVP. Eplerenone increased LVP (P<0.05) without affecting CF. (right) Effect of aldosterone on CF and LVP in the absence (control) or presence of 0.1 μmol/L eplerenone, 1 μmol/L eplerenone, or 1 μmol/L spironolactone. Aldosterone increased LVP (P<0.01) and decreased CF (P<0.01), and these effects were unaltered by either eplerenone or aldosterone. Values (mean±SEM, n=6) are expressed as percentage change from baseline. Spironolactone data have been obtained from Chai et al and were corrected for vehicle effect.
Hemodynamic Parameters, Area at Risk, and Fibrillation Incidence at Baseline After 45 Minutes of Coronary Artery Occlusion (Ischemia) and During Reperfusion

<table>
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<td>LVP, mL/min</td>
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<td>CF, mL/min</td>
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<td>Area at risk, %</td>
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<td>Fibrillation incidence</td>
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*Aldo indicates aldosterone; Spiro, spironolactone; Eple, eplerenone.

Spironolactone data have been obtained from Chai et al. Data are mean±SEM of 6 to 7 experiments.

In control hearts (n=6), LVP and CF increased rapidly during the reperfusion phase (Table), stabilizing after approximately 20 minutes at 44±3% and 85±13% of preischemia values (Figure 2). LVEDP increased to 23±5 mm Hg. Pretreatment with aldosterone (n=6) did not affect LVP recovery, nor did it prevent the rise in LVEDP. Aldosterone also did not reduce CF recovery (Figure 2). Pretreatment with eplerenone (n=6) significantly enhanced recovery of LVP (P<0.05) but not of CF (Table; Figure 2). Eplerenone did not diminish the rise in LVEDP. Ventricular fibrillation occurred within the first 10 minutes of reperfusion of all control hearts (Table). Neither eplerenone nor aldosterone affected the incidence of fibrillation. The area at risk was identical in all of the hearts (Table). Infarct size (expressed as a percentage of the area at risk) was 68±2% in control hearts (Figure 2). Eplerenone reduced infarct size (P<0.05), whereas the infarct size in aldosterone-treated hearts was not different from control. Combined exposure to eplerenone and aldosterone (n=7) yielded similar results as eplerenone alone (Table; Figure 2).

Kinetic Studies

Aldosterone levels in rat plasma were 85±16 pg/mL (n=5). Aldosterone levels in the perfusate of vehicle-perfused hearts were <58±15 pg/mL. In 5 of 8 hearts, these levels were below the detection limit. The cardiac aldosterone levels in vehicle-perfused hearts were <20±5 pg/g wet weight. In 1 of 3 hearts this level was below the detection limit. The level of aldosterone in the coronary effluent during perfusion with 10 nmol/L aldosterone was 2161±87 pg/mL at 20 minutes and 2132±99 pg/mL at 25 minutes (P value was not significant; n=9), indicating that a steady state had been reached. The steady-state level in coronary effluent was 97±5% of the level in the perfusion buffer. After discontinuation of the aldosterone perfusion, aldosterone disappeared from the coronary effluent in a biphasic manner (n=6; Figure 3). The rapid phase had a half life (t1/2) of 1.1±0.1 minute, and the slow phase had a t1/2 of 7.9±1.1 minute. The cardiac tissue level of aldosterone, immediately after the 10 nmol/L aldosterone perfusion had been switched off, was 2533±327 pg/g wet weight (n=3). During the washout phase, the tissue level decreased in a monophasic manner, with a t1/2 of 9.1±1.0 minutes (Figure 3). Eplerenone, at a concentration of 1 μmol/L, did not affect the steady-state tissue level reached after a 30-minute perfusion with 10 nmol/L aldosterone (3471±197 pg/g wet weight, n=3; P value not significant versus without eplerenone). This suggests that MR do not contribute to the accumulation of aldosterone in the heart. The cardiac tissue levels after a 30-minute perfusion with 3 or 30 nmol/L aldosterone were 982±207 and 6720±941 pg/g wet weight (n=3 for both), respectively. Tissue levels (expressed per gram of wet
weight) closely correlated with coronary effluent levels (expressed per milliliter of effluent; \( r = 0.81; P < 0.01 \); Figure 4).

**Discussion**

The present study reveals no blocking effect of eplerenone toward the inotropic and vasoconstrictor effects of aldosterone in the rat Langendorff heart, thereby confirming that these effects are exerted in a “nongenomic” manner, not involving MR. In fact, eplerenone, like spironolactone, induced a modest inotropic effect of its own. Incorporation of our previously published data on spironolactone in Figure 1 allows a direct comparison of the hemodynamic effects of the 2 drugs under the same experimental conditions. The maximum inotropic effect of spironolactone and eplerenone is identical. However, the eplerenone concentration required to induce this inotropic effect was \( \approx 3 \) orders of magnitude higher than the spironolactone concentration required to induce a similar effect, and thus, because the doses of both drugs are comparable, inotropic effects are less likely to occur during treatment with eplerenone than during treatment with spironolactone. Unlike spironolactone, eplerenone did not affect CF. Possibly, therefore, the spironolactone-induced effects on flow are mediated through a different receptor than its effects on contractility. The latter has been reported to involve increased myosin ATPase calcium sensitivity and diastolic calcium concentration.

Eplerenone protected the heart during ischemia and reperfusion in a similar way as spironolactone, that is, both drugs reduced infarct size and improved LVP recovery (see Table and Figure 2). Therefore, this cardioprotective effect is most likely MR mediated. It could relate to blockade of the proarrhythmogenic actions of aldosterone and/or the aldosterone-induced increase in oxygen radical synthesis. The first possibility is particularly supported by the reduction of the fibrillation incidence by spironolactone (and the tendency for a similar reduction by eplerenone). If so, aldosterone was apparently still present in the isolated perfused rat Langendorff heart. The effect does not relate to the non-MR–mediated coronary constrictor effects of aldosterone.

Aldosterone, at a concentration of 100 pmol/mL, did not additionally deteriorate the condition of the heart during the ischemia plus reperfusion procedure. Thus, the endogenous aldosterone levels in the heart were presumably \(<100\) pmol/g, despite earlier reports by Silvestre et al describing such high aldosterone levels in the isolated perfused rat heart (50 to 500 pg/mg protein, or, because 1 g of tissue corresponds with 65 to 100 mg protein, \( \approx 10 \) to 150 pmol/g). Indeed, the cardiac tissue levels of aldosterone in our study were \(<20\) pg/g. In some of our experiments, aldosterone was detectable in coronary effluent, obtained during the 30 minutes of perfusion with vehicle before the collection of the heart. Although this could reflect local synthesis of aldosterone, it might also represent washout of blood-derived aldosterone that had accumulated in the heart in vivo.

To study the kinetics of uptake and washout of circulating aldosterone in the heart, we perfused the rat Langendorff heart with aldosterone using concentrations corresponding with those in patients with severe heart failure. Aldosterone perfusion resulted in a rapid rise of the cardiac aldosterone levels, independent of MR. After 30 minutes, the tissue
levels (expressed per gram of wet weight) were on average 1.5 times higher than the levels in coronary effluent (expressed per milliliter). Because the extracellular volume in this preparation is \( \frac{0.6}{M_{Lg}} \) the cardiac aldosterone levels are 2 to 3 times higher than expected if the presence of aldosterone was limited to the extracellular fluid. Apparently, therefore, aldosterone accumulates in a compartment other than extracellular fluid, for example, it binds to cell surface receptors and/or reaches intracellular sites. In support of this concept, the washout of aldosterone after stopping the perfusion followed a biphasic pattern: a rapid phase corresponding with its disappearance from extracellular fluid and a slow phase corresponding with its washout from a second compartment. This pattern resembles that of cardiac renin, which accumulates in extracellular fluid and binds to membrane receptors.\(^\text{23,26}\)

After perfusion of the Langendorff heart with aldosterone, the cardiac aldosterone levels correlated with the aldosterone levels in the perfusion buffer over a wide range, indicating a large capacity of the heart to accumulate aldosterone. Extrapolating this relationship to the plasma aldosterone levels in the rat (84 pg/mL) yields an in vivo cardiac tissue level of 1.5x84 \( \approx \) 125 pg/g, in full agreement with the cardiac tissue levels reported by others in rats on a normal salt diet.\(^\text{13,28}\) These levels are \( > \)6 times the level present after buffer perfusion. Apparently, the 15 minutes of equilibration plus 30 minutes of perfusion with aldosterone-free buffer before the collection of the heart were sufficient to wash away \( > \)85% of cardiac aldosterone. This is exactly what one would expect on the basis of a 1/2 of \( \approx \) 8 to 9 minutes for the aldosterone disappearance from tissue sites, and, thus, it can be concluded that, under normal circumstances, the majority of cardiac aldosterone, if not all, is taken up from the circulation. This does not exclude the possibility that local production of aldosterone becomes an important determinant of cardiac aldosterone levels under pathological conditions.\(^\text{29}\)

**Perspectives**

The present study complements previous studies on the disappearance of cardiac aldosterone after adrenalectomy\(^\text{13,28}\) and simultaneously provides an explanation for the reduction in sudden death among patients taking MR antagonists.\(^\text{1,2}\) On the one hand, the heart displays a large capacity to accumulate aldosterone. This explains why cardiac aldosterone in rats overexpressing human renin and human angiotensinogen (\( > \)99% of which is derived from the adrenal gland) can be up to 10-fold higher than in serum.\(^\text{28}\) On the other hand, cardiac aldosterone disappears rapidly during perfusion with aldosterone-free buffer. This provides an explanation for the presence of aldosterone in the effluent of buffer-perfused hearts.\(^\text{12}\) The rapid, MR-independent kinetics of aldosterone observed in this study suggest that its cardiac accumulation involves cell surface binding rather than internalization followed by binding to intracellular MR. The cardioprotective effects of eplerenone during ischemia and reperfusion in the Langendorff preparation are most likely because of blockade of the MR-mediated arrhythmogenic effects\(^\text{3}\) of this cell surface-bound aldosterone.

**References**


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