Efficacy of mineralocorticoid receptor antagonism in the acute myocardial infarction phase: eplerenone versus spironolactone

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Abstract

Aims The selective mineralocorticoid receptor (MR) antagonist eplerenone given early in patients with acute myocardial infarction (MI) improves clinical outcome, whereas little is known about the effectiveness of early spironolactone therapy. We aimed to compare the ability of eplerenone and spironolactone to promote cardiac repair after experimental MI.

Methods and results Starting immediately after coronary artery ligation, C57BL/6J mice were treated with placebo, eplerenone, or spironolactone. At 7 days, treatment with eplerenone or spironolactone reduced thinning and expansion of healing infarct and improved early left ventricular chamber enlargement. Remarkably, eplerenone therapy resulted in significantly greater improvement than spironolactone of left ventricular contractile function and relaxation, associated with a more considerable leftward and downward shift of the pressure volume curve. Seven-day survival rate was significantly increased only in eplerenone treated mice. Moreover, eplerenone was superior to spironolactone in ameliorating neovessel formation in the injured myocardium. Optimized flow cytometry analysis of the monocyte differentiation marker Ly6C revealed predominant accumulation of Ly6Chigh monocytes/macrophages at the site of ischemic injury during the early inflammatory phase in placebo-treated mice. In contrast, MR antagonism, especially by eplerenone, led to a skewing of the monocyte/macrophage population toward a higher frequency of healing promoting Ly6Clow cells.

Conclusion The MR antagonist eplerenone versus spironolactone showed superior efficacy during the acute MI phase with more beneficial effects on survival, early cardiac dilation, and functional decline. Modulation of monocyte maturation and enhanced infarct neovessel formation appears to play a pivotal role.

Keywords Acute myocardial infarction; Remodelling; Mineralocorticoid receptor; Monocyte subsets

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Introduction

Acute myocardial infarction (MI) and ensuing heart failure are leading causes of death.¹ Pharmacological strategies targeting the pathophysiological mechanisms of cardiac repair after acute MI, hence able to prevent progressive ventricular dilation, functional deterioration, and heart failure, are currently focus of intense investigation. However, effective drugs are still urgently needed.²

Clinical trials have firmly established that mineralocorticoid receptor (MR) blocking therapy with spironolactone and eplerenone provides considerable improvements in cardiovascular mortality and morbidity in patients with severe heart failure (RALES)³, left ventricular (LV) systolic dysfunction after acute MI (EPHESUS),⁴ as well as in patients with mild chronic heart failure (EMPHASIS-HF).⁵,⁶ Current guidelines recommend MR antagonists for patients with worsening chronic systolic heart failure and LV dysfunction after MI but do not discriminate between spironolactone and eplerenone.⁷

Emerging data from clinical trials provide evidence that MR inhibition with eplerenone given early in the course of acute MI improves clinical outcome,⁸,⁹ but the underlying
mechanisms are still under investigation, and to date, no clinical trials have evaluated the effectiveness of an early initiation of spironolactone therapy. Studies involving mice with myeloid-specific MR deletion identified the myeloid MR to be a critical regulator of macrophage polarization.\(^\text{10}\) In addition, experimental data showed improved cardiac healing through the modulation of macrophage recruitment at the site of ischemic injury by immediate MR inhibition postinfarction,\(^\text{11}\) suggesting a crucial role of the MR signalling specifically in monocytes/macrophages during the healing phase postinfarction.

Accordingly, this study compared the efficacy of eplerenone and spironolactone to promote cardiac repair when given early after experimental MI, with special emphasis on monocyte subsets dynamics.

**Methods**

All animal experiments were in accordance with the *Guide for the Care and Use of Laboratory Animals* published by the National Institutes of Health (Publication No. 85–23, revised 1985). All procedures were approved by the Regierung von Unterfranken (Würzburg, Germany; Permit Number: 54–2531.01-15/07) and by the Niedersächsisches Landesamt für Verbraucherschutz und Lebensmittelsicherheit, (Oldenburg, Germany; Permit Number: 33.9-42502-04-13/1124).

**Myocardial infarction and study protocols**

Myocardial infarction was induced by permanent left coronary artery ligation in female C57BL/6J mice that were 8 to 12 weeks of age. Starting immediately after surgery, MI mice were randomly treated with 100 mg/kg of body weight eplerenone or 20 mg/kg of body weight spironolactone, the commonly used doses of these drugs in rodents with MI.\(^\text{11–13}\) Moreover, we tested the efficacy of spironolactone given at the same dose of eplerenone, i.e. 100 mg/kg. Treatments were administered by oral gavage once daily. Placebo-MI and sham-operated animals received vehicle (5% arabic gum).

**Hemodynamic and volume measurements**

Hemodynamic and volume measurements were performed 7 days after coronary artery ligation, under light isoflurane anesthesia and spontaneous respiration, using conductance catheter (SPR-839, Millar Instruments). Pressure–volume signals were acquired by BioBench software (National Instruments). Pvan software (Millar) was used to analyse all pressure–volume loop data recorded at steady-state and during injection of hypertonic saline for the calibration of parallel conductance volume (\(V_p\)). LV volume was calculated for each mouse from conductance volume corrected by the relative \(V_p\).\(^\text{14}\)

**Infarct size, infarct expansion, and scar collagen content**

The hearts were arrested by intravenous KCl injection. Five-micrometer thin sections were serially cut from apex to base and stained with 0.1% sirius red F3B in saturated picric acid. The transverse section representing the middle of LV and with the most marked cavity dilatation was used for expansion index determination. Five evenly spaced radians were passed through the infarct with the centre of the LV section as a reference, and the average infarct thickness was calculated. Non-infarcted LV septal thickness was measured similarly. The expansion index was calculated with the formula: expansion Index = (LV cavity area/total LV area) \(\div\) (septum thickness/scar thickness). Infarct size (fraction of the infarcted left ventricle) was quantified histologically by planimetry and expressed as a percentage of length. Only rats with extensive infarcts (>40%) were included in the study. For scar collagen content LV sections were examined using a Nikon ECLIPSE 50i microscope equipped with filters to provide circularly polarized illuminated tissue images were recorded with a cooled digital camera (DS-5Mc, Nikon) with a ×200 and analysed using SigmaScan Pro 5.0 image analysis software (Systat Software Inc.). Collagen content was expressed as a percentage of the area of each image.

**Immunohistochemistry**

For immunohistochemical analysis LV frozen 5 μm sections were stained using primary antibodies against CD31 (MCA2388, AbD Serotec) and \(\alpha\)-smooth muscle actin (VPS281, Vector Laboratories), a biotinylated rabbit anti-rat antibody, mouse adsorbed (BA-4001 Vector Laboratories), and the Vector™ M.O.M.™ Peroxidase Kit (PK-2200). Dual immunohistochemical staining was performed using DAB Substrate Kit (550880, BD Biosciences) for CD31 and the Histogreen HRP Substrate kit (E109, Linaris) for \(\alpha\)-smooth muscle actin. Sections were counterstained with Vector® Hematoxylin QS (H-3404).

**Flow cytometry**

A fast and gentle method preserving antigens and morphology was developed to obtained a single-cell suspension from mouse heart after MI. Briefly, the hearts were perfused (6 min) and digested (10 min) using a modified Langendorff...
perfusion system according to AfCS Procedure Protocol PP00000125. The cell suspension was carefully centrifuged at 20 g for 4 min, and the supernatant was filtered through a 40 μm nylon mesh (BD Falcon) followed by centrifugation at 300 g for 10 min. The pelleted cells were washed and resuspended in staining buffer (PBS, 2% fetal calf serum, 1 mM EDTA). After pre-selection (side scatter and forward scatter) monocytes/macrophages were identified as Lineage (CD49b, NK1.1, CD45R/B220, CD90, Ly6G)low, CD11bhigh, and (F4/80, I-Ab, CD11c)low/high and distinguished on the basis of the presence of the Ly6C antigen. Data were acquired on an Gallios™ flow cytometer and analysed with Gallios™ software (Beckman Coulter). The following antibodies were used: anti-CD90.2-PE (BD Biosciences, 553005), NK1.1-PE (BD Biosciences, 553165), Ly-6G-PE (BD Biosciences, 551461), CD49b-PE (BD Biosciences, 553858), CD45R/B220-PE (BD Biosciences, 553090), CD11c-Biotin (BD Biosciences, 553800), F4/80-Biotin (Serotec, MCA497B), I-Ab-Biotin (BD Biosciences, 553550), Ly-6C-FITC (BD Biosciences, 553104), Streptavidin-PerCP (BD Biosciences, 554064), and CD11b-APC (BD Biosciences, 553312).

**Statistical analysis**

The results are reported as mean ± SEM. Normality and variance homogeneity of residuals were checked by Shapiro–Wilk and Levene test, respectively. Statistical analysis was performed by one-way ANOVA or Kruskal–Wallis test as appropriate followed by Holm post hoc test. Survival distributions were estimated by the Kaplan–Meier method and compared by the log-rank test. Statistical analysis was performed with R, Software Environment for Statistical Computing and Graphics, Version 3.0.0 and StatView 5.0.1 software (Abacus Concepts, Inc.). Two-sided *P* values <0.05 were considered statistically significant.

Figure 1 Effects of immediate treatment with eplerenone (Eple) and spironolactone (Spiro) on survival and hemodynamics in mice 7 days after myocardial infarction (MI). (A) Kaplan-Meier survival curve. Log-rank, *P* < 0.05. (B) LV filling pressure (LVEDP), LV end-diastolic volume (LVEDV), and representative LV pressure–volume loops measured in vivo with conductance catheter in sham-operated rats (Sham) and mice after MI. Mean ± SEM (n = 6–9). *P* < 0.05 vs. Sham; †P < 0.05 vs. Placebo (Pla)-MI.
Results
Survival, hemodynamics, and cardiac dilation

Kaplan–Meier analysis revealed significantly increased survival 7 days postinfarction only in eplerenone treated mice (Figure 1a). Infarct size was similar among the experimental groups (Table 1). At 7 days post-MI, mice on placebo developed elevated LV filling pressure (LVEDP), LV end-systolic and end-diastolic volumes, and marked LV dysfunction, as assessed by LV ejection performance, $dP/dt_{max}$, $dP/dt_{min}$, and LV $dP/dt_{max}$ divided by instantaneous pressure, a load-independent measure of contractile function (Figures 1 and 2 and Table 1). Spironolactone versus placebo significantly decreased LVEDP and LV end-systolic and end-diastolic volumes. Remarkably, eplerenone therapy resulted in significantly greater improvement than spironolactone of LV contractile function and relaxation, associated with a more considerable downward and leftward shift of the pressure volume curve.

Table 1 Global parameters of sham-operated (Sham) mice and of placebo, eplerenone, and spironolactone mice 7 days after myocardial infarction

|                | Sham | Placebo MI | Eplerenone MI | Spironolactone MI |
|----------------|------|------------|---------------|-------------------|
| $N$            | 6    | 9          | 9             | 9                 |
| MI (%)         | -    | 52 ± 2     | 53 ± 1        | 51 ± 1            |
| BW (g)         | 20 ± 1.9 | 18.4 ± 0.8 | 18.5 ± 0.7    | 18.2 ± 0.6        |
| Heart rate (bpm) | 473 ± 67 | 404 ± 13   | 437 ± 26      | 409 ± 26          |
| LVSP (mmHg)    | 111 ± 3 | 75 ± 1*    | 80 ± 3*       | 73 ± 4*           |
| LVESV (μL)     | 12 ± 4 | 53 ± 3*    | 33 ± 2*‡      | 39 ± 3*†          |

BW, body weight; LVSP, left ventricular systolic pressure; LVESV, left ventricular end-systolic volume. Values are mean ± SEM.

*P < 0.05 vs. Sham
†P < 0.05
‡P < 0.01 vs. placebo MI.

Figure 2 Eplerenone (Eple) therapy led to greater improvement of left ventricular (LV) contractile function and relaxation than spironolactone (Spiro) 7 days after myocardial infarction (MI). LV maximal rate of pressure rise (LV $dP/dt_{max}$), LV maximal rate of pressure decline (LV $dP/dt_{min}$), LV $dP/dt_{max}$ normalized by instantaneous pressure (LV $dP/dt_{max}$/IP), and LV ejection fraction. Mean ± SEM (n = 6–9). *P < 0.05 vs. Sham; †P < 0.05 vs. Placebo (Pla)-MI; §P < 0.05 vs. Eple-MI.
Of note, increasing the dosage of spironolactone to 100 mg/kg slightly exacerbated early LV dysfunction compared with spironolactone at 20 mg/kg (LV filling pressure 18 ± 1 mmHg; dP/dt\textsubscript{max} 3413 ± 295; dP/dt\textsubscript{min} 2258 ± 198 mmHg/s; n = 5). Moreover, the mortality rate was significantly higher in the 100 mg/kg spironolactone group compared with mice treated with 20 mg/kg spironolactone (Figure 1a). The deaths resulted from acute heart failure as revealed by the presence of pleural effusion and lung congestion at necropsy. Therefore, we used the treatment with 20 mg/kg spironolactone for further analyses.

Infarct expansion and neovascularization

Early LV chamber enlargement is a consequence of thinning and dilatation of the infarct segment.\textsuperscript{2} Consistently, infarcted hearts treated with eplerenone or spironolactone showed significant reduction in wall thinning and infarct expansion index vs. placebo, although the MR antagonist eplerenone led to superior improvement (Figure 3).

Optimal cardiac repair after ischemia is closely linked to formation of new blood vessels. Acquisition of a muscular coat is crucial for maturation/stabilization of infarct neovessels.\textsuperscript{2} We observed a substantially greater number of capillaries, identified as small lumen vessels positively staining for CD31, as well as microvessels with α-smooth muscle actin-positive pericyte coverage in the infarcted myocardium after MR-blocking therapy with eplerenone compared with spironolactone (Figure 4).

Monocyte/macrophage homeostasis in the injured myocardium

Earlier studies of our group showed that eplerenone improved LV dilation by mechanisms/effects induced within the first 3 days after ischemia.\textsuperscript{11} Accordingly, at day 3 post-MI we analysed the monocytes/macrophage infiltrative response, recognized as playing a pivotal role in the regulation of infarct wound healing and tissue repair.\textsuperscript{2} Optimized flow cytometry analysis of the monocyte differentiation marker Ly6C clearly revealed predominant infiltration of Ly6C\textsuperscript{high} monocytes/macrophages in the healing myocardium of placebo-treated mice (Figure 5). By contrast, MR antagonism led to a skewing of the monocyte/macrophage population toward a higher frequency of healing promoting Ly6C\textsuperscript{low} cells (Figure 5). Of note, eplerenone significantly enhanced the ratio of healing-promoting Ly6C\textsuperscript{low} to pro-inflammatory Ly6C\textsuperscript{high} monocytes/macrophages in the infarcted myocardium (Ly6C\textsuperscript{low}/Ly6C\textsuperscript{high}).

Figure 3 Reduction in infarct wall thinning and expansion in mice treated with either eplerenone (Eple) or spironolactone (Spiro). Representative sections from infarcted hearts, infarct expansion, scar thickness, and collagen content in mice 7 days after myocardial infarction (MI). Mean ± SEM (n = 9). †P < 0.05 vs. Placebo (Pla)-MI.

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Figure 4 (A) Immunohistochemical staining showing greater number of capillaries, identified as small lumen vessels positively staining for CD31 (brown), and microvessels with α-smooth muscle actin-positive pericyte coverage (green) in the injured myocardium after MR-blocking therapy with eplerenone compared with spironolactone (scale bar, 100 μm). (B) Magnification of insets (scale bar, 50 μm). Mean ± SEM (n = 9). †P < 0.05 vs. Placebo (Pla)-MI.

Pla-MI, 0.297 ± 0.09; Eple-MI, 0.858 ± 0.13; Spiro-MI, 0.524 ± 0.08; n = 5, P < 0.05 Eple-MI vs. Pla-MI).

Discussion

In the current study, we found superior efficacy of the selective MR antagonists eplerenone compared with spironolactone during the acute MI phase with more beneficial effects on monocyte subsets dynamics, infarct neovascularisation, early LV dilatation, and functional deterioration.

Several potential mechanisms might underlie the superiority of selective MR blockade during the acute MI phase. The two MR antagonists have different pharmacokinetic and pharmacological properties.15 Spironolactone, structurally similar to progesterone, also binds to glucocorticoid, progesterone, and androgen receptors. In contrast, eplerenone selectively blocks the MR and has only little affinity for other steroid receptors.15 We cannot exclude that non-specific binding of spironolactone and/or its metabolites to steroid receptors could have affected monocyte/macrophage homeostasis and that steroid-related side effects could have exacerbated the process of cardiac repair during the acute phase of MI. Another untoward side effect that might influence infarct wound healing attributed to spironolactone, unlike eplerenone, include elevation of blood glucose levels.16 Recent findings indicated that spironolactone, but not eplerenone, impaired glucose intolerance in metabolic syndrome. In patients with chronic heart failure eplerenone showed superior metabolic effect especially on HbA1c compared with spironolactone.16,17 Furthermore, spironolactone has been reported to increase blood glucose levels in patients with resistant hypertension and with type 2 diabetes mellitus.18,19 In the Candesartan in Heart Failure Assessment of Reduction in Mortality and Morbidity study spironolactone therapy was associated with the development of diabetes mellitus.20

Particularly noteworthy is our finding that administration of MR antagonists, especially eplerenone, led to a skewing of the monocyte/macrophage population toward a higher frequency of Ly6C<sup>low</sup> cells at the site of ischemic injury already during the early inflammatory phase. Down-regulation of the monocyte differentiation marker Ly6C characterizes the maturation of mouse monocytes.21 Accumulation of monocytes/macrophages in the infarcted myocardium is biphasic, involving an early Ly6C<sup>high</sup> inflammatory phase and a later Ly6C<sup>low</sup> reparative phase.22 Ly6C<sup>high</sup> cells elicited a predominantly inflammatory (M1-type) response, while the more mature Ly6C<sup>low</sup> a M2 type response linked to inflammation resolution, angiogenesis and wound repair.23 Thus, stimulating maturation/differentiation of these cells toward a Ly-6C<sup>low</sup> phenotype by MR inhibition resulted in improved neoangiogenic response and scar tissue formation.

Increasing evidence suggests that optimal infarct healing requires coordinated differentiation of monocytes/macrophages
at the site of ischemic injury.\textsuperscript{2,24} Modulation of the Ly6Chigh/Ly6C\textsuperscript{low} ratio by silencing of the chemokine-receptors CCR2 reduced the number of inflammatory Ly6C\textsuperscript{high} monocytes and ischemia reperfusion injury.\textsuperscript{25} Conversely, dendritic cell ablation sustained enhanced Ly6C\textsuperscript{high} monocytes but decreased Ly6C\textsuperscript{low} monocyte accumulation into the infarcted heart, leading to impaired neoangiogenesis and exacerbated infarct expansion and LV dysfunction.\textsuperscript{26} Hofmann et al. described higher proportion of pro-inflammatory Ly6C\textsuperscript{high} monocytes in the healing myocardium, increased LV dilation, and impaired scar formation in CD4 KO mice after MI.\textsuperscript{27} Interestingly, in patients with primary acute MI classical CD14\textsuperscript{++}CD16\textsuperscript{−} (Ly6C\textsuperscript{high} analogs) and CD14\textsuperscript{+}CD16\textsuperscript{+} (most resemble Ly6C\textsuperscript{low} cells) were sequentially mobilized, and the peak levels of CD14\textsuperscript{+}CD16\textsuperscript{+} monocytes were negatively associated with the extent of myocardial salvage and the recovery of cardiac function.\textsuperscript{28}

Eplerenone promoting in the early inflammatory phase of healing the switch of monocytes/macrophages toward a Ly6C\textsuperscript{low} M2 phenotype, more effectively than spironolactone, substantially enhanced the angiogenic response to ischemic injury, finally culminating in greater improvement of cardiac remodelling and dysfunction. Patients might accordingly derive the greatest benefit by immediate initiation of MR-blocking therapy by eplerenone after acute MI.

Current international guidelines recommend MR antagonist therapy for patients with chronic systolic heart failure and LV dysfunction after MI but do not discriminate\textsuperscript{5,7} between eplerenone and spironolactone (or its metabolite...
cannren oste). Accumulating data provided evidence that selective MR inhibition with eplerenone given early in the course of acute MI improves clinical outcome.\(^5\)\(^6\)\(^7\) The landmark EPHESUS trial showed that earlier MR antagonism with eplerenone (3–7 days) was associated with more beneficial outcomes compared with later (7–14 days) initiation after acute MI. In the recent double-blind REMINDER study,\(^9\) patients presenting with acute ST-segment elevation MI without heart failure were randomized to eplerenone within 24 h of symptoms. Eplerenone therapy during the acute MI phase was safe and well tolerated and associated with improvement in the primary outcome, mainly driven by a significant reduction of BNP/NT-proBNP levels. Noteworthy, subgroup analyses showed a trend towards an even greater benefit with very early treatment (6 h). A prospective randomized study with 134 patients showed that spironolactone therapy for 1 month started immediately after acute MI improved cardiac outcome, mainly driven by a significant reduction of BNP/NT-proBNP levels. Moreover, the main trials, EPHESUS, EMPHASIS-HF, and RALES, showed a trend towards an even greater benefit with very early treatment (6 h). A prospective randomized study with 134 patients showed that spironolactone therapy for 1 month started immediately after acute MI improved cardiac outcome.

In the context of our present study, it has to be noted that in patients with/after acute MI, to date, there is only evidence for treatment with eplerenone from large randomized clinical studies.

In conclusion, the present study showed that MR inhibition targeting monocyte subsets dynamics at the site of ischemic injury promoted cardiac repair and highlighted the superiority of selective MR blockade during the acute MI phase.

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Conflict of Interest

D.F. and J.B. received research grant support from Pfizer related to eplerenone. J.B. received honoraria for lectures from Pfizer. The other authors have no conflicts to declare.

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