Deletion of Cardiomyocyte Mineralocorticoid Receptor Ameliorates Adverse Remodeling After Myocardial Infarction

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Background—Mineralocorticoid receptor (MR) blockade improves morbidity and mortality among patients with heart failure; however, the underlying mechanisms are still under investigation. We studied left ventricular remodeling after myocardial infarction in mice with cardiomyocyte-specific inactivation of the MR gene (MR<sup>MLCre</sup>) that were generated with a conditional MR allele (MR<sup>flow</sup>) in combination with a transgene expressing Cre recombinase under control of the myosin light-chain (MLC2a) gene promoter.

Methods and Results—Control (MR<sup>flow/flow</sup>, MR<sup>flow/wt</sup>) and MR<sup>MLCre</sup> mice underwent coronary artery ligation. MR ablation had no detectable baseline effect on cardiac morphology and function. The progressive left ventricular chamber enlargement and functional deterioration in infarcted control mice, detected by echocardiography and conductance catheter analysis during the 8-week observation period, were substantially attenuated in MR<sup>MLCre</sup> mice. Chronically infarcted MR<sup>MLCre</sup> mice displayed attenuated pulmonary edema, reduced cardiac hypertrophy, increased capillary density, and reduced accumulation of extracellular matrix proteins in the surviving left ventricular myocardium. Moreover, cardiomyocyte-specific MR ablation prevented the increases in myocardial and mitochondrial O<sub>2</sub><sup>-</sup> production and upregulation of the NADPH oxidase subunits Nox2 and Nox4. At 7 days, MR<sup>MLCre</sup> mice exhibited enhanced infarct neovessel formation and collagen structural organization associated with reduced infarct expansion. Mechanistically, cardiomyocytes lacking MR displayed accelerated stress-induced activation and subsequent suppression of nuclear factor-xB and reduced apoptosis early after myocardial infarction.

Conclusion—Cardiomyocyte-specific MR deficiency improved infarct healing and prevented progressive adverse cardiac remodeling, contractile dysfunction, and molecular alterations in ischemic heart failure, highlighting the importance of cardiomyocyte MR for heart failure development and progression. (Circulation. 2011;123:400-408.)

Key Words: acute myocardial infarction ■ aldosterone ■ heart failure ■ mineralocorticoid receptor ■ remodeling

Mineralocorticoid receptor (MR) blockade reduces morbidity and mortality in patients with heart failure. However, it remains unclear whether the cardioprotective effects of MR antagonists can be attributed to inhibition of the cardiomyocyte MR. While attenuation of left ventricular (LV) dilation and excessive extracellular matrix turnover<sup>4-9</sup> and prevention of electric remodeling appear to be essential mechanisms of MR antagonism,<sup>10-11</sup> extracellular effects such as renal sodium excretion and potassium sparing, restoration of autonomic balance, and improvement in vascular endothelial dysfunction may be of particular importance.<sup>12-14</sup>

Clinical Perspective on p 408

To investigate the pathophysiological role of the cardiomyocyte MR in ischemic heart failure, we investigated cardiac remodeling after myocardial infarction (MI) in mice with cardiac myocyte–specific inactivation of the MR gene. LV remodeling after MI is a dynamic time-dependent process involving the infarcted region and the residual viable myocardium.<sup>15</sup> Thinning and dilatation of the infarcted region (infarct expansion) are events in the early phase of healing, followed by progressive cardiomyocyte hypertrophy and extracellular matrix remodeling remote from the infarct site.<sup>15</sup> Accordingly, we studied cellular and molecular alterations in the surviving LV myocardium in the late post-MI phase (8 weeks) and infarct wound healing, expansion, and neovascularization during the early MI phase (1 to 7 days).

Methods

All procedures were approved by the institutional animal research committee.

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Generation of MR Mutant Mice and MI

A murine model of MR ablation in cardiomyocytes was generated with a conditional MR allele (MR<sup>flox</sup>)<sub>16</sub> and a mouse line expressing Cre recombinase under control of the atrial myosin light chain gene promoter (C57Bl6-Tg-MLC-Cre<sup>+</sup>/0; Figure IA in the online-only Data Supplement).<sup>17</sup> MR<sup>flox</sup> allele and Cre transgene were backcrossed 8 times in C57Bl6; then, MR<sup>flox/wt</sup> and MR<sup>flox/flo</sup> mice, as well as MR<sup>flox/wt</sup> and MR<sup>flox/flo</sup>MLCCre mice, were crossed to generate breeding pairs; MR<sup>flox/wt</sup> and MR<sup>flox/wt</sup>MLCCre were crossed to generate mutant mice (MR<sup>flox/wt</sup>MLCCre<sup>-/+</sup>MR<sup>flox/cre</sup>) and littermate controls (MR<sup>flox/wt</sup> and MR<sup>flox/wt</sup> mice).

MI was induced by permanent left coronary artery ligations in male and female mice that were 8 to 12 weeks of age.<sup>18</sup> A total of 207 mice (control, n=103; MR<sup>MLCCre</sup>, n=104) were subjected to MI (Figure II in the online-only Data Supplement). Twenty-four hours after surgery, surviving mice were randomly selected for the early MI phase (1 to 7 days) or the late post-MI phase (56 days). Mice with MI <40% were excluded from the analyses. The 56-day survival was significantly higher in infarcted MR<sup>MLCCre</sup> mice (88%) compared with control mice (61%; P<0.05). All mice (control, n=12; MR<sup>MLCCre</sup>, n=12) that had undergone sham operation (the suture around the coronary artery was not tied) survived.

Isolation of Cardiomyocytes

Isolation of cardiomyocytes from hearts of control and MR<sup>MLCCre</sup> mice was performed exactly according to AFCS Procedure Protocol PP00000125 (http://www.signaling-gateway.org/reports/v1/CM0005/CM0005.htm).

An expanded Methods section is available in the online-only Data Supplement.

Statistical Analysis

The results are reported as mean±SEM or percentile. The Shapiro–Wilk and Levene tests were used to analyze normality and variance homogeneity of residuals, respectively. Statistical analysis was performed by 1-way ANOVA, 2-way ANOVA, or the Kruskal–Wallis test for comparisons among groups or unpaired the t test and Mann–Whitney U test for comparisons between 2 groups as appropriate. The Holm posthoc test was used to adjust for multiple comparisons except as otherwise indicated. Linear (Pearson) correlations were estimated. Statistical analysis was performed with StatView 5.0.1 and R, Software Environment for Statistical Computations (R version 2.10.1, SAS Institute Inc., Cary, NC). Linear mixed-effects models (MIXED) procedure was performed with the SPSS 16.0 statistical software package (SPSS Inc., Chicago, IL). Two-sided P values were used. Values of P<0.05 were considered statistically significant.

The authors had full access to and take full responsibility for the integrity of the data. All authors have read and agree to the manuscript as written.

Results

Cardiac Morphology, Function, and Gene Expression in MR<sup>MLCCre</sup> Mice

As expected, in isolated cardiomyocytes from MR<sup>MLCCre</sup> mice, MR protein expression was barely detectable (Figure IB in the online-only Data Supplement). We did not detect differences between control and MR-deficient mice regarding hemodynamics, LV structure, and LV function (Figure IC through IE and Table in the online-only Data Supplement). Microarray analysis revealed that myocardial gene expression of the MR target gene serum/glucocorticoid regulated kinase 1 (SGK1) was downregulated in MR<sup>MLCCre</sup> mice (2.1-fold versus control; P<0.001).

| Table. Global Characteristics of Control and MR<sup>MLCCre</sup> Mice 8 Weeks After MI |
|---|---|---|
| Control MI | MR<sup>MLCCre</sup> MI |
| n | 14 | 14 |
| BW, g | 26.3±1.0 | 28.9±1.2 |
| Lung fluid weight, g | 0.173±0.017 | 0.115±0.005* |
| LVSP, mm Hg | 69±4 | 75±6 |
| τ, ms | 33±2 | 23±2* |
| Heart rate, bpm | 524±21 | 484±20 |

BW indicates body weight; LVSP, left ventricular systolic pressure; and τ, LV pressure isovolumic decay. Values are mean±SEM. *P<0.05 vs control MI.

Infarct Size

We used the model of left coronary artery ligation to assess the effect of MR ablation on LV healing and remodeling. Mean infarct sizes were similar among the experimental groups (1 day: control, 51±3%, n=8; MR<sup>MLCCre</sup>, 50±2%, n=7; 3 days: control, 46±2%, n=7; MR<sup>MLCCre</sup>, 46±1%, n=6; 7 days: control, 52±2%, n=12; MR<sup>MLCCre</sup>, 50±2%, n=12), see also the Table.

Aldosterone and Corticosterone Plasma Levels After Infarction

Plasma aldosterone and corticosterone levels were markedly increased after MI (Figure III in the online-only Data Supplement). A significant positive correlation was observed between aldosterone and corticosterone plasma levels (r=0.84, P<0.0001). In MR<sup>MLCCre</sup> mice, corticosterone levels tended to be lower 8 weeks after infarction compared with control mice (Figure III in the online-only Data Supplement).

Myocardial gene expression and protein expression of the glucocorticoid receptor and 11β-hydroxysteroid dehydrogenase type 2 (11beta-HSD2) were similar among the experimental groups (Figure IV in the online-only Data Supplement).

Cardiomyocyte-Specific MR Ablation Attenuates Progressive Ventricular Dilation and Improves Cardiac Function After MI

The rightward shift of the pressure-volume curve was prevented in the MR<sup>MLCCre</sup> mice 8 weeks after MI (Figure 1A). LV filling pressure, end-diastolic volume, and end-systolic volume were significantly decreased compared with control mice. Amelioration of LV dilation in MR<sup>MLCCre</sup> mice was associated with significantly improved LV ejection fraction (Figure 1A). The progressive increase in LV end-systolic and end-diastolic areas and diameters observed by serial echocardiography during the 8-week observation period in control mice was attenuated in MR<sup>MLCCre</sup> mice (Figure VA in the online-only Data Supplement). Correspondingly, MR<sup>MLCCre</sup> mice exhibited significantly reduced infarct expansion index and increased scar thickness 8 weeks after MI (Figure 1B).

Right ventricular weight/body weight and pulmonary fluid accumulation were increased in control MI mice compared with sham-operated mice and were significantly reduced in MR<sup>MLCCre-MI</sup> mice 8 weeks after infarction (the Table and Figure VB in the online-only Data Supplement), suggesting that lowered LV filling pressure in MR<sup>MLCCre-MI</sup> mice led
to reduced pressure load on the right ventricle and accumulation of pulmonary fluid. These results show that cardiomyocyte-specific MR ablation prevents the progression of postinfarction cardiac dilation, functional deterioration, and failure.

Cardiomyocyte-Specific MR Ablation Prevents Cardiac Hypertrophy and Extracellular Matrix Accumulation After Infarction

Attenuation of LV dilation and failure in chronically infarcted MR$^{\text{MLCCre}}$ mice was associated with reduced hypertrophy (LV weight and cardiomyocyte size), increased capillary density (Figure 2A and Figure VB in the online-only Data Supplement), and reduced accumulation of extracellular matrix proteins (Figure 2B through 2D) in the surviving LV myocardium 8 weeks after infarction. Microarray analysis of total RNA isolated from the surviving LV myocardium (Figure VI in the online-only Data Supplement) revealed that genes associated with pathological hypertrophy (β-myosin heavy chain, angiotensin-converting enzyme [ACE]), stiffness, and fibrosis (ACE, connective tissue growth factor [CTGF], collagen, peristin, fibronectin, vimentin) were significantly decreased in MR$^{\text{MLCCre}}$ mice compared with control. We confirmed reduced expression of ACE, CTGF,
periostin, fibronectin, and vimentin by Western blot analysis/immunohistochemical staining (Figure 2C and 2D).

**Cardiomyocyte-Specific MR Ablation Prevents Myocardial and Mitochondrial O$_2^-$ Production After Infarction**

Oxidant stress plays a key role in the development of adverse cardiac remodeling and contractile dysfunction after MI. MYocardial O$_2^-$ production was increased substantially in control mice 8 weeks after infarction and nearly normalized in chronically infarcted MRMLCCre mice (Figure 3A). Protein expression of the NADPH oxidase subunits Nox2 and Nox4 was significantly lower in the surviving LV myocardium of MRMLCCre-MI mice (Figure 3C). We additionally measured O$_2^-$ production in mitochondria isolated from the surviving LV myocardium (Figure 3B). Chronic MI led to a significant increase in O$_2^-$ production in mitochondria in control mice, which was suppressed in MRMLCCre mice. Furthermore, the role of MR in aldosterone-induced mitochondrial oxidative stress was assessed in isolated adult cardiomyocytes. Aldosterone stimulation significantly increased mitochondrial O$_2^-$ production and Nox4 upregulation in cardiomyocytes from control but not MRMLCCre mice (Figure VII in the online-only Data Supplement).

**Enhanced Infarct Collagen Structural Organization and Reduced Infarct Expansion in MRMLCCre Mice 7 Days After MI**

Cardiomyocyte-specific MR ablation also prevented the rightward shift of the pressure-volume curve in the early MI phase (Figure 4A). LV filling pressure, LV end-diastolic volume, and LV ejection fraction were significantly improved 7 days after infarction. MRMLCCre mice exhibited significantly increased scar thickness and reduced infarct expansion index at 7 days (Figure 4B). It is noteworthy that, although total scar collagen was unchanged, collagen organization was improved. Sirius Red polarization microscopy revealed mainly a well-organized collagen matrix with uniform, sharply delineated, well-aligned collagen fibers in MRMLCCre-MI mice, in contrast to a disorganized matrix with a predominance of smaller and fragmented collagen fibers in control MI mice (Figure 4B). Plasma levels of type I collagen C-terminal telopeptide were significantly lower in MRMLCCre (5.22 ± 0.7 ng/mL; n = 7) compared with control mice (8.04 ± 0.6 ng/mL; n = 7; P < 0.05), suggesting less collagen breakdown in MRMLCCre mice 7 days after MI. Collagen fibers are degraded by matrix metalloproteinases (MMPs), leading to thinning and dilatation of the infarcted wall. At 7 days, MMP-9 and MMP-2 activity was more intense in the infarcted wall of control mice compared with MRMLCCre mice (Figure 4B).

**Enhanced Neovascularization in the Healing Myocardium of MRMLCCre Mice**

We observed increased neovascularization in the healing myocardium of MRMLCCre mice 7 days after MI. Immunohistochemical studies revealed increased capillaries and small-lumen vessels in the infarct scar and infarct borders (Figure 5A). Vascular endothelial growth factor (VEGF) protein was substantially higher in the infarct area from MRMLCCre compared with control mice 3 days after infarction (Figure 5B and 5C). Immunohistochemical staining showed

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**Figure 3.** Determination of oxidation products, by isocratic ion-pair high performance liquid chromatography with electrochemical detection method, of hydroethidine in the surviving LV myocardium (A), and of MitoSOX Red in isolated mitochondria (B), from sham-operated, control, and MRMLCCre mice 8 weeks after MI. IS indicates internal standard; HE, hydroethidine; and Mito-HE, MitoSOX Red. C, Protein expression of Nox2 and Nox4 in the surviving LV myocardium as revealed by Western blot analysis from sham-operated, control, and MRMLCCre mice 8 weeks after MI. Statistical analysis was performed by 1-way ANOVA or Kruskal-Wallis test followed by the Holm posthoc test to adjust for multiple comparisons. Mean ± SEM (n = 6 to 10). *P < 0.05 vs sham; †P < 0.05 vs control MI.
more VEGF-positive endothelial cells in the infarcted myocardium in MRMLCre mice (Figure 5B), suggesting that endothelial cells may represent the source of VEGF. Importantly, more CD31+/H11001 cells were found in the healing myocardium of MRMLCre mice 3 days after infarction (Figure 5D).

Targeted Ablation of the MR Allows Cardiomyocytes to Respond Rapidly to Ischemia Through Nuclear Factor-κB Activation

To identify a cardiomyocyte-specific mechanism underlying improved healing response to ischemia in infarcted MRMLCre mice, we focused on nuclear factor-κB (NF-κB), a key signaling component for early inflammatory activation and healing after MI.23 Recent data showed that aldosterone via the MR abrogates IκB degradation and NF-κB activation.24 In isolated cardiomyocytes from MRMLCre mice 90 minutes after coronary artery ligation, we found substantially increased NF-κB DNA binding activity and lower protein levels of the inhibitory protein IκB compared with control (Figure 6A).

To test the hypothesis that the cardiomyocyte MR interferes with NF-κB activation, we examined regulation of NF-κB on tumor necrosis factor-α stimulation in cardiomyocytes isolated from adult control and MRMLCre mice. We observed accelerated activation of NF-κB followed by faster suppression in MR knockout cardiomyocytes, accompanied by inverse effects on IκB protein (Figure VIII in the online-only Data Supplement). In cardiomyocytes from control mice, stimulation with tumor necrosis factor-α at 30 minutes induced IκB protein degradation and maximal induction of NF-κB DNA binding activity, which was sustained for up to 60 minutes. In contrast, in MR knockout cardiomyocytes, tumor necrosis factor-α induced a decrease in IκB protein level and maximal NF-κB activation already after 15 minutes, which returned to normal after 60 minutes. These findings indicate that MR ablation accelerates stress-induced NF-κB activation and subsequent suppression in cardiomyocytes.

Targeted Ablation of the MR Protects Cardiomyocytes Against Apoptosis

NF-κB protects cardiomyocyte from ischemia-induced apoptosis during the first hours after permanent coronary artery ligation,25 whereas MR activation induces cardiomyocyte apoptosis.26,27 Accordingly, we studied whether MR ablation protects ischemic cardiomyocytes from apoptosis. Terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) analyses were performed 24 hours after MI. The rate of apoptosis in the peri-infarct region was decreased in MRMLCre mice compared with control (Figure 6B). These results reveal protection against apoptosis by cardiomyocyte-specific MR ablation early after MI.

Accelerated Recruitment of Inflammatory Cells in the Healing Myocardium of MRMLCre Mice

NF-κB plays a critical role in the induction of RANTES,28 a potent chemoattractant for monocytes and neutrophils.29,30 Of note, at 1 day after MI, we found significantly higher RANTES protein expression in the injured myocardium of MRMLCre mice (158±12 pg/mg protein) compared with control mice (86±7 pg/mg protein; P<0.05), associated with more infiltrating neutrophils and monocytes (Figure 6C).
Importantly, at day 3, the number of neutrophils was clearly reduced in the infarcted myocardium of MR<sub>MLCCre</sub> mice (Figure IXA in the online-only Data Supplement). While infiltration of CD68<sup>+</sup> monocytes/macrophages was confined to the border zone in control mice, these cells were found throughout the entire infarct zone in MR<sub>MLCCre</sub> mice (Figure IXB in the online-only Data Supplement).

**Discussion**

MR antagonism beneficially modulates heart failure progression and survival, but the contribution of renal, vascular, or cardiac MR blockade remained obscure. By cell-specific ablation, the present study proves a pathogenetic role of the cardiomyocyte MR for ventricular remodeling after MI. Selective cardiomyocyte MR ablation in vivo improved infarct healing and protected against progressive adverse cardiac remodeling and molecular alterations in ischemic heart failure, thus highlighting the importance of the cardiomyocyte MR for heart failure development and progression after MI.

Chronic ventricular remodeling after MI involves cardiac hypertrophy and extracellular matrix accumulation remote from the infarct site, leading to chamber dilation, contractile dysfunction, and heart failure. Targeted overexpression of the human MR in the heart resulted in dilated cardiomyopathy, emphasizing a role for MR in mediating pathological hypertrophy. On the other hand, MR antagonism reduced cardiac hypertrophy and maladaptive remodeling after MI, but this may be mediated by extracardial effects. In the present study, cardiomyocyte MR deficiency prevented cardiac hypertrophy, as evidenced by the reduction in heart weight, myocyte cross-sectional area, and expression of fetal genes such as β-myosin heavy chain in chronically infarcted MR<sub>MLCCre</sub> mice, thus proving the involvement of cardiomyocyte MR-dependent pathways in the hypertrophic response after MI. In addition to biomechanical stress such as chronic volume and pressure overload, several neurohumoral factors regulate cardiomyocyte hypertrophy. Cardiomyocyte MR ablation prevented ACE upregulation, which may explain not only the reduced cardiomyocyte hypertrophy and fibrosis but also the attenuation of oxidative stress in the surviving LV myocardium of chronically infarcted MR<sub>MLCCre</sub> mice. Aldosterone, via MR activation, upregulates ACE expression in...
cardiomyocytes, suggesting the existence of a positive feedback pathway from MR to ACE within the cardiomyocyte renin-angiotensin-aldosterone system.\textsuperscript{32–34} Using mice with conditional cardiomyocyte-restricted human MR overexpression, Di Zhang et al\textsuperscript{33} demonstrated interaction of angiotensin II with cardiomyocyte MR to induce cardiac fibrosis and remodeling through generation of reactive oxygen species.

Angiotensin II–induced myocardial hypertrophy critically involves mitochondrial reactive oxygen species production,\textsuperscript{35} which was also diminished in MR\textsuperscript{MLCCre} mice after MI. Mitochondrial oxidative stress is sufficient to promote cardiac remodeling and failure postinfarction.\textsuperscript{20} Aldosterone has been shown to stimulate myocardial and vascular reactive oxygen species generation.\textsuperscript{14,26,36} Our study provides evidence that cardiomyocyte MR ablation prevents myocardial oxidative stress in vivo and attenuates superoxide anion production in mitochondria, likely secondary to diminished upregulation of the NADPH oxidase subunits Nox2 and Nox4.\textsuperscript{37} Moreover, our studies performed on cardiomyocytes isolated from adult control and MR\textsuperscript{MLCCre} mice are the first directly linking aldosterone action in cardiomyocytes to induction of mitochondrial oxidative stress and Nox4 expression via the MR.

In the context of MI and oxidative stress,\textsuperscript{27,38} glucocorticoids may activate cardiomyocyte MR and mimic the pathophysiological effects of aldosterone. The highly significant positive correlation between aldosterone and corticosterone plasma levels suggests a mechanistic interdependence. Supporting this notion, cortisol levels, as useful predictors of cardiac events in patients with CHF, correlated with plasma aldosterone levels.\textsuperscript{39} Nevertheless, higher serum levels of both cortisol and aldosterone are independent, complementary, and incremental predictors of all-cause mortality risk in patients with chronic heart failure.\textsuperscript{40} Conceivably, improved remodeling in MR\textsuperscript{MLCCre} mice may also be related to the absence of cardiomyocyte MR activation by glucocorticoids.

The direct MR target gene SGK1 was identified as downregulated in the heart of MR\textsuperscript{MLCCre} mice through the use of microarray analysis. SGK1 is dynamically regulated in cardiomyocytes and enhances the cardiomyocyte hypertrophic response.\textsuperscript{32} SGK1 deficiency abrogated mineralocorticoid-induced cardiac hypertrophy and fibrosis.\textsuperscript{42} Mineralocorticoid-induced cardiac fibrosis involves SGK1-dependent upregulation of CTGF,\textsuperscript{42} a key mediator of extracellular matrix protein formation. CTGF expression was substantially reduced in the surviving LV myocardium of chronically infarcted MR\textsuperscript{MLCCre} mice. Thus, lower SGK1 levels in MR\textsuperscript{MLCCre} mice beneficially affect postinfarction ventricular remodeling by dual mechanisms: inhibition of cardiomyocyte hypertrophic response and prevention of CTGF profibrotic effects.

SGK1 has also been implicated in cardiomyocyte survival in vitro.\textsuperscript{41} However, the reduction in apoptotic cardiomyocytes in MR\textsuperscript{MLCCre} mice indicates that, despite SGK1 downregulation, MR-deficient cardiomyocytes are protected against apoptosis. These data are in line with several reports demonstrating that MR activation induces cardiomyocyte apoptosis.\textsuperscript{26,27} Because apoptotic cardiomyocyte loss early after MI contributes to LV dilation and failure,\textsuperscript{43} inhibition of cardiomyocyte apoptosis by MR ablation likely restrained postinfarction remodeling in MR\textsuperscript{MLCCre} mice. Protection of cardiomyocytes against apoptosis in MR-deficient mice appears to be mediated through an NF-κB pathway. Using an elegant transgenic mouse model with cardiac-restricted expression of a mutated IκBα protein, Misra et al\textsuperscript{44} showed that activation of NF-κB prevents cardiomyocyte apoptosis during the first hours after MI. While aldosterone via MR activation abrogates IκBα degradation and NF-κB activation,\textsuperscript{44} we observed substantially lower IκBα protein levels and increased NF-κB DNA binding activity in cardiomyocytes from MR\textsuperscript{MLCCre} mice after 90 minutes after coronary ligation.

The role of NF-κB after MI is controversial and appears to be time dependent. While NF-κB protects cardiomyocytes against apoptosis during the first hours after ischemia,\textsuperscript{25,44} persistent activation of NF-κB mediates maladaptive LV remodeling and functional deterioration.\textsuperscript{23} Noteworthy, our studies performed on cardiomyocytes isolated from adult MR\textsuperscript{MLCCre} mice suggest that both stress-induced NF-κB activation and subsequent suppression were faster in cardiomyocytes lacking the MR.

There are several potential mechanisms for improved infarct healing in MR\textsuperscript{MLCCre} mice. Rapidly induced NF-κB activation in MR-deficient cardiomyocytes during the first minutes after ischemia may have triggered the manifold cellular activities that underscore the processes of inflammation and healing. NF-κB is a key signaling component for early inflammatory activation and recruitment of inflammatory cells after MI.\textsuperscript{43} Exogenous glucocorticoids, which inhibit NF-κB activation and inflammatory cell infiltration, impair infarct wound healing, resulting in adverse early LV dilation.\textsuperscript{23}

Optimal infarct healing is closely linked to rapid recruitment of inflammatory cells, timely inflammatory pathway suppression, neovessel formation, and deposition of collagen-based matrix.\textsuperscript{8,22,23,44} Thus, accelerated recruitment of neutrophils and monocytes, associated with upregulation of the angiogenic cytokine VEGF, likely contributed to enhanced neovascularization, resulting in reduced infarct expansion and improved early LV function in MR-deficient mice. Increased recruitment of neutrophils and macrophages within the first days after MI has recently been shown to lead to enhanced neovascularization, a reduction in infarct thinning, and functional improvement in 11βHSD1-deficient mice.\textsuperscript{46}

The improved collagen structural organization in the infarct scar in MR\textsuperscript{MLCCre} mice may also be of particular importance, most likely secondary to MMP downregulation.\textsuperscript{44} Disorganized collagen matrix renders the scar less resistant to distortion during overload and predisposes to thinning and dilatation of the infarcted wall, paving the way for progressive global ventricular dilation, LV dysfunction, and heart failure.\textsuperscript{21–23}

Although the sequence of events cannot easily be elucidated in vivo in the animal model, mechanisms such as reduced infarct expansion and early dilatation of the LV cavity, associated with a decrease in myocardial wall stress, likely contributed to the improvement in cardiac remodeling, contractile dysfunction, and heart failure. Increased wall stress in the noninfarcted myocardium triggers a cascade of
molecular, cellular, and physiological responses that can lead to further cardiac dilation and pathological remodeling, including hypertrophic cardiac growth and fibrosis.\textsuperscript{15}

We cannot exclude that MR-mediated effects on fibroblasts and endothelial cells may also be involved in heart failure progression after MI. While MR activation in cardiac fibroblasts stimulates collagen synthesis and proliferation,\textsuperscript{47} in endothelial cells, MR activation enhances generation of reactive oxygen species and impairs vascular reactivity.\textsuperscript{14} However, the present study provides evidence that the cardiomyocyte MR plays a central role in healing and remodeling after MI.

From a clinical viewpoint, our results provide important new insights into the mechanisms underlying cardioprotection by MR blockade and add further evidence that more favorable effects on LV remodeling can be achieved by immediate initiation of MR blockade after MI. This should be tested in a randomized prospective trial in patients with acute coronary syndromes. Of note, in a retrospective analysis of the Eplerenone Post–Acute Myocardial Infarction Heart Failure Efficacy and Survival Study (EPHESUS),\textsuperscript{3,4} earlier MR antagonism with eplerenone (3 to 7 days after MI) was associated with more favorable outcomes compared with later initiation (7 to 14 days after MI).

Conclusions

Cardiomyocyte-specific inactivation of the MR gene protects against adverse cardiac remodeling and contractile dysfunction in ischemic heart failure. The beneficial cardiac effects are likely attributable to improved infarct healing and prevention of cardiomyocyte hypertrophy, extracellular matrix accumulation, and myocardial oxidative stress. These data may indicate that clinical benefits of MR blockade in patients with acute or chronic heart failure are mediated largely via a cardiomyocyte-dependent mechanism.

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Disclosures

None.

References


CLINICAL PERSPECTIVE

Mineralocorticoid receptor (MR) antagonism beneficially modulates heart failure progression and survival after myocardial infarction, but the contribution of renal, vascular, or cardiac MR blockade remained obscure. The present study highlights the importance of the cardiomyocyte MR for left ventricular remodeling and failure after myocardial infarction. Cardiomyocyte-specific inactivation of the MR gene improved infarct healing and prevented progressive adverse cardiac remodeling and contractile dysfunction, with the likely contribution of both early and late effects. In the early phase after myocardial infarction, cardiomyocyte-specific MR deletion reduced cardiomyocyte apoptosis, enhanced infarct neovessel formation, and attenuated infarct expansion, whereas in the late post–myocardial infarction phase, cardiomyocyte hypertrophy, reactive fibrosis, and oxidative stress in the surviving left ventricular myocardium were prevented. Our results suggest that the clinical benefits of MR blockade in patients with acute or chronic ischemic heart failure are mediated largely via a cardiomyocyte-dependent mechanism. Furthermore, these results provide strong evidence that more favorable effects on cardiac healing and remodeling after myocardial infarction can be achieved by immediate initiation of MR blockade.
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Deletion of Cardiomyocyte Mineralocorticoid Receptor Ameliorates Adverse Remodeling After Myocardial Infarction

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Supplemental Methods

Echocardiographic Analysis
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Tissue sampling and infarct size determination
The right (RV) and left ventricles (LV), including septum were separated in ice-cold saline. The LV was cut into three transverse sections: apex, middle ring (~2 mm), and base. From the middle ring, 5µm-sections were cut and hematoxylin/eosin and picrosirius red stains were performed. Four lengths were derived from each digital image of the sections, including epicardial and endocardial infarct lengths and epicardial and endocardial circumferences, using Sigma Scan Pro 5.0 program. Epicardial infarct ratio was obtained by dividing epicardial infarct length by epicardial circumference. Endocardial infarct ratio was calculated.
similarly. Infarct size derived from this approach was calculated as \[
\frac{[(\text{epicardial infarct ratio} + \text{endocardial infarct ratio})/2]}{2} \times 100.
\]
For the biochemical analysis (cytokines, VEGF, MMP) in the infarcted myocardium after MI size estimation (the LV was pressed flat on glass plates and the boundary lengths of the infarcted and noninfarcted epicardial and endocardial surfaces were traced and digitized), the LV was divided into infarcted area and noninfarcted myocardium.\(^2\)

**Infarct expansion**

A separate group of animals was used for infarct expansion analysis.\(^2\) The hearts were arrested by intravenous KCl injection and perfusion-fixed with 4% phosphate-buffered formalin. 5µm thin sections were serially cut from apex to base and stained with picrosirius red. 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 center 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 = \[(\text{LV Cavity Area}/\text{Total LV Area}) \times (\text{Septum Thickness}/\text{Scar Thickness})\].

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Mouse hearts were fixed in 4% buffered formalin and embedded in paraffin. 5µm picrosirius red sections were examined using a Nikon ECLIPSE 50i microscope equipped with filters to provide circularly polarized illumination.\(^3\) Tissue images were recorded with a cooled digital camera (DS-5Mc, Nikon) with a magnification 200X, and analyzed using SigmaScan Pro 5.0 image analysis software (SPSS Inc., USA). Collagen content was expressed as a percentage of the area of each image.

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Pulmonary edema was assessed as net fluid weights (difference between the wet and dry weights).
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For immunohistochemical analysis, LV frozen or paraffin 5µm sections were stained using primary antibodies against fibronectin (610077, BD Biosciences Pharmingen), vimentin (550513, BD Biosciences Pharmingen), CTGF (sc-14939, Santa Cruz Biotechnology), CD31 (MCA2388, AbD Serotec), VEGF (sc-7269, Santa Cruz Biotechnology), neutrophil (sc-59338, Santa Cruz Biotechnology), CD68 (MCA1957, AbDSerotec). Briefly, deparaffinization and hydration of paraffin sections or fixation of frozen sections in cold acetone for 5 min were followed by pretreatment with 0.3% hydrogen peroxide for 20 min to inhibit endogenous peroxidase activity. Subsequently, sections were blocked with 2% horse serum for 30 min and incubated with the primary antibody for 2 h at room temperature. After rinsing with PBS, the sections were incubated for 30 min with a biotinylated secondary antibody. Staining was performed using VECTASTAIN® Elite ABC kit (PK-6100, Vector Laboratories) and DAB (SK4100, Vector Laboratories). Dual immunohistochemical staining were performed using DAB for CD31 and HistoGreen HRP-Substrate-Kit (E109, Linaris) for VEGF. Sections were counterstained with hematoxylin.

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**Aldosterone, corticosterone, type I collagen C-terminal telopeptide**

Plasma aldosterone (Sorin Biomedica) and corticosterone (MP Biomedicals) levels were measured by radioimmunoassay. Plasma levels of type I collagen C-terminal telopeptide were determined by ELISA (Uscn Life Science).

**Microarray**

Total RNA from LV samples (surviving LV myocardium) was extracted using mirVana™ miRNA isolation kit (Ambion, Applied Biosystems) following the manufacturer’s protocol. RNA quality was assessed with Bioanalyzer 2100 (Agilent). RNA samples were converted to biotinylated cRNA and hybridized to GeneChip Mouse Genome 430 2.0 Array (Affymetrix) according to the manufacturer’s directions. Microarray data analysis was performed using R packages from the Bioconductor project (www.bioconductor.org).

**Determination of \( \text{O}_2^- \) formation**

LV tissues (30 mg) were finely ground in ice-cold Krebs-HEPES buffer, and then suspended in Krebs-HEPES buffer containing 10 µM hydroethidine (HE, Invitrogen) and incubated at 37°C for 20 min. Subsequently, LV suspension was washed and placed in 100 µL of cold methanol containing 3,4-Dihydroxycinnamic acid (5 nM) as internal standard, homogenized, and then centrifuged at 10000g for 10 min. The supernatant was then analyzed by HPLC-electrochemical (EC) as described below. Mitochondria were isolated from the LV using the Qproteome Mitochondria Isolation Kit (QIAGEN). Isolated mitochondria were incubated in
Krebs-HEPES buffer containing 10 µM Mito-HE (MitoSOX™ Red; Invitrogen), incubated at 37°C for 20 min, followed by centrifugation at 6000g for 10 min. Subsequently, mitochondria were washed with Krebs-HEPES buffer, centrifuged at 6000g for 10 min, placed in 80 µL of cold methanol containing 3,4-Dihydroxycinnamic acid (5 nM), homogenized, and centrifuged at 10000g for 10 min. Adult cardiomyocytes were cultured in serum-free MEM for 12 h, stimulated with aldosterone (100 nmol/L) for 8 h and then exposed to the mitochondria-targeted hydroethidine (Mito-HE, 0.7 µmol/L) at 37°C for 20 min. Subsequently, cardiomyocytes were washed 3x with culture medium, harvested in 160 µL of cold methanol containing 3,4-Dihydroxycinnamic acid (5 nM), and centrifuged at 10000g for 10 min.

A rapid and highly sensitive isocratic ion-pair HPLC-electrochemical (EC) method, with the use of an internal standard, was developed for separation of HE, Mito-HE, and its oxidation products. Isocratic elution was performed (flow-rate 0.9 mL/min) using a Synergi4µ Polar-RP80A column (250x460mm, 00G-4336-E0, Phenomenex) and mobile phase (octanesulfonic acid 100 µM, NaH₂PO₄ 50 mM, pH 2.7), containing 35% acetonitrile (v/v) for HE, 2-hydroxy-E⁺, and E⁺ separation. Acetonitrile concentration was adjusted to 40% for separation of Mito-HE, mito-2-hydroxy-E⁺ and mito-E⁺. An ESA CoulochemII with Modell 5011 analytical cell (first electrode, 0.00V; second detecting electrode +0.35V) was used. Our new HPLC-EC method, incorporating an ion-pair reagent in the mobile phase, has the advantage that gradient separation is not necessary and allows more distinct separation of HE/Mito-HE oxidations products, and the separation is achieved within 20 min. Noteworthy, an internal standard (IS, 3,4-Dihydroxycinnamic acid) was used and the ratio of oxidation products peak heights and of the IS was used for quantitative analysis. The use of an internal standard corrects the errors due to extraction of HE/Mito-HE oxidation products from tissue/mitochondria/cells and variations of injection volume and of HPLC-EC system.
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**NF-κB DNA binding activity**

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**RANTES levels**

Infarcted LV myocardium was homogenized in ice-cold RIPA buffer. Protein expression of RANTES, was measured using Quantibody® Mouse Cytokine Array 1 (RayBiotech, Inc.) according to the instructions of the manufacturer.
## Supplemental Table

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<th>Control</th>
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<tr>
<td>$n$</td>
<td>10</td>
<td>10</td>
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<tr>
<td>BW (g)</td>
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<td>LVSP (mmHg)</td>
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<td>LVEDP (mmHg)</td>
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<td>5±0.6</td>
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<td>LVESV (µL)</td>
<td>13±4</td>
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<td>LVEDV (µL)</td>
<td>28±3</td>
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<td>PA ESA (mm$^2$)</td>
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<td>PA EDA (mm$^2$)</td>
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<td>Myocyte cross-sectional area (µm$^2$)</td>
<td>237±16</td>
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<tr>
<td>Capillary density</td>
<td>41.7±3</td>
<td>40.9±3</td>
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LVSP, LV systolic pressure; LVEDP, LV end-diastolic pressure; LVESV, LV end-systolic volume; LVESV, LV end-diastolic volume; EF, LV ejection fraction; PA (mid-papillary) ESA, end- systolic cavity area; PA EDA, end-diastolic cavity area. Values are mean±SEM.
Supplemental Figure 1

*Generation and baseline characteristics of MR*$_{MLCCre}$ *mice.* (A) Schematic representation of a conditional murine mineralocorticoid receptor (MR) allele (*Nr3c2*) showing the area of the floxed exon 3 and the neighboring exon 2 and exon 4. Selective deletion of exon 3 in cardiac myocytes (MR*$_{MLCCre}$) was achieved in mice having two conditional MR alleles and a transgene expressing Cre recombinase under control of the atrial myosin light chain promoter (MLCCre). Symbols: open triangles, loxP sites; grey triangles, qRT-PCR primers. (B) MR protein expression in isolated cardiomyocytes, as revealed by Western blot analysis. (C) Left ventricular contractility analysis at baseline and in response to β-adrenergic agonist dobutamine. Mean±SEM (n=3). Statistical analysis was performed by MIXED procedure. Repeated factor: Dobutamine; fixed factors: group (Control, MR*$_{MLCCre}$) and Dobutamine; correlation structure: first-order autoregressive. (D) Representative LV pressure-volume loops measured *in vivo* with conductance catheter. (E) Myocyte cross-sectional area (WGA, red, marks cell membranes) and capillary density as identified by CD31 immunohistochemistry (green-yellow); myocardial fibrosis (Sirius red staining).
Supplemental Figure 2
Flow chart of the study design. A total of 103 control (MR^{flx/flx}, MR^{flx/wt}) and 104 MR^{MLCCre} mice were subjected to myocardial infarction (MI). On the day after MI (24 hours after surgery), surviving mice were randomly selected for the acute MI-phase (1 to 7 days) or the chronic MI-phase (56 days). Mice were excluded from the analyses for two reasons: perioperative death (within the first 24 hours after surgery) and MI size <40%.
Supplemental Figure 3

Aldosterone and corticosterone plasma levels in control and MRMLCre mice, 1 day, 3 days, 7 days and 8 weeks after myocardial infarction (MI) or sham-operation. Mean±SEM (n=4-10). Statistical analysis was performed by 2-way ANOVA. Unpaired t-test was used to compare each time point versus respective sham. *P<0.05 vs respective sham.
Supplemental Figure 4
Myocardial gene expression (A) as well as protein expression (B) of the glucocorticoid receptor and 11-beta-hydroxysteroid dehydrogenase type 2 (11beta-HSD2) in control and MR^{MLCcre} mice, 56 days after myocardial infarction (MI) or sham-operation. Mean±SEM (n=6-10). Statistical analysis was performed by 1-way ANOVA followed by Holm post hoc test to adjust for multiple-comparisons.
Supplemental Figure 5

(A) Serial echocardiography of control and MR$_{MLCCre}$ mice 1 day, 7 days, and 56 days after myocardial infarction. PA, (mid-papillary); ESA, end-systolic area; EDA, end-diastolic area; ESD, end-systolic diameter; EDD end-diastolic diameter. Mean±SEM (n=14). Statistical analysis was performed by MIXED procedure. Repeated factor: time; fixed factors: group (Control, MR$_{MLCCre}$) and time; correlation structure: first-order autoregressive. Contrasts between the groups, Control and MR$_{MLCCre}$ at day 1, day 7 and day 56 were performed with the subcommand TEST in SPSS. *P<0.05, †P<0.01 vs control.

(B) LV/BW and RV/BW in control and MR$_{MLCCre}$ mice, 8 weeks after myocardial infarction (MI). Statistical analysis: Mann-Whitney U test.
### Supplemental Figure 6

Heatmaps of significantly regulated genes in the surviving LV myocardium of MR<sup>MLCCre</sup> vs control mice 8 weeks after myocardial infarction (P<0.05). Gene upregulation with fold change (FC) >1.4 and downregulation with FC > 1.5 are shown.
Supplemental Figure 7
Mitochondrial superoxide anion formation and Nox4 protein expression in cardiomyocytes isolated from adult control and MR\textsuperscript{MLCCre} mice. Cardiomyocytes were stimulated with aldosterone (100 nmol/L) for 8 hours. Mean±SEM (n=3). Statistical analysis was performed by 1-way ANOVA followed by Holm post hoc test to adjust for multiple-comparisons. *P<0.05 Control-Aldo 100nmol/L vs respective vehicle, †P<0.05 MR\textsuperscript{MLCCre}-Aldo 100nmol/L vs Control-Aldo 100nmol/L.
Supplemental Figure 8

Time course of NF-κB activation and IκBα degradation following TNFα stimulation in cardiomyocytes isolated from adult control and MR<sup>MLCCre</sup> mice. Cardiomyocytes were stimulated with TNFα (10ng/mL) for 5, 15, 30 and 60 minutes. (A) NF-κB activation analysed by DNA-binding activity of p65 in nuclear extracts; (B) IκBα protein expression in cytoplasmic extracts. Mean±SEM (n=3). Statistical analysis was performed by 1-way ANOVA. Changes versus baseline and versus maximum of NF-κB activation/IκBα degradation were analyzed using unpaired t-test. *P<0.05 vs respective unstimulated cardiomyocytes, †P<0.05 vs MR<sup>MLCCre</sup> cardiomyocytes stimulated with TNFα for 15 minutes.
Supplemental Figure 9
Immunohistochemical staining showing infiltration of (A) neutrophils and (B) monocytes (anti-CD68) in the healing myocardium of control and MR^{MLCCre} mice, 3 days after myocardial infarction. Statistical analysis was performed by unpaired t-test. Mean±SEM (n=4-6), †P<0.05 vs control. (A, magnification x200; B, magnification x400).
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SUPPLEMENTAL MATERIAL

Deletion of Cardiomyocyte Mineralocorticoid Receptor Ameliorates Adverse Remodeling After Myocardial Infarction

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<tbody>
<tr>
<td><strong>n</strong></td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>BW (g)</td>
<td>28.5±1.5</td>
<td>29.5±1.7</td>
</tr>
<tr>
<td>LV/BW (mg/g)</td>
<td>3.21±0.15</td>
<td>3.31±0.16</td>
</tr>
<tr>
<td>RV/BW (mg/g)</td>
<td>0.74±0.04</td>
<td>0.76±0.04</td>
</tr>
<tr>
<td>LVSP (mmHg)</td>
<td>86±4</td>
<td>84±3</td>
</tr>
<tr>
<td>LVEDP (mmHg)</td>
<td>5±0.5</td>
<td>5±0.6</td>
</tr>
<tr>
<td>LVESV (µL)</td>
<td>13±4</td>
<td>14±3</td>
</tr>
<tr>
<td>LVEDV (µL)</td>
<td>28±3</td>
<td>27±2</td>
</tr>
<tr>
<td>EF (%)</td>
<td>67±7</td>
<td>65±7</td>
</tr>
<tr>
<td>PA ESA (mm&lt;sup&gt;2&lt;/sup&gt;)</td>
<td>3.45±0.2</td>
<td>2.82±0.2</td>
</tr>
<tr>
<td>PA EDA (mm&lt;sup&gt;2&lt;/sup&gt;)</td>
<td>7.96±0.5</td>
<td>8.03±0.3</td>
</tr>
<tr>
<td>Myocyte cross-sectional area (µm&lt;sup&gt;2&lt;/sup&gt;)</td>
<td>237±16</td>
<td>239±20</td>
</tr>
<tr>
<td>Capillary density</td>
<td>41.7±3</td>
<td>40.9±3</td>
</tr>
</tbody>
</table>

LVSP, LV systolic pressure; LVEDP, LV end-diastolic pressure; LVESV, LV end-systolic volume; LVESV, LV end-diastolic volume; EF, LV ejection fraction; PA (mid-papillary) ESA, end- systolic cavity area; PA EDA, end-diastolic cavity area. Values are mean±SEM.
Generation and baseline characteristics of MR\textsuperscript{MLCCre} mice. (A) Schematic representation of a conditional murine mineralocorticoid receptor (MR) allele (\textit{Nr3c2}) showing the area of the floxed exon 3 and the neighboring exon 2 and exon 4. Selective deletion of exon 3 in cardiac myocytes (MR\textsuperscript{MLCCre}) was achieved in mice harboring two conditional MR alleles and a transgene expressing Cre recombinase under control of the atrial myosin light chain promoter (MLCCre). Symbols: open triangles, loxP sites; grey triangles, qRT-PCR primers. (B) MR protein expression in isolated cardiomyocytes, as revealed by Western blot analysis. (C) Left ventricular contractility analysis at baseline and in response to β-adrenergic agonist dobutamine. Mean±SEM (n=3). Statistical analysis was performed by MIXED procedure. Repeated factor: Dobutamine; fixed factors: group (Control, MR\textsuperscript{MLCCre}) and Dobutamine; correlation structure: first-order autoregressive. (D) Representative LV pressure-volume loops measured \textit{in vivo} with conductance catheter. (E) Myocyte cross-sectional area (WGA, red, marks cell membranes) and capillary density as identified by CD31 immunohistochemistry (green-yellow); myocardial fibrosis (Sirius red staining).
Supplemental Figure 2
Flow chart of the study design. A total of 103 control (MR^{flox/flox}, MR^{flox/wt}) and 104 MR^{MLCCre} mice were subjected to myocardial infarction (MI). On the day after MI (24 hours after surgery), surviving mice were randomly selected for the acute MI-phase (1 to 7 days) or the chronic MI-phase (56 days). Mice were excluded from the analyses for two reasons: perioperative death (within the first 24 hours after surgery) and MI size <40%.
Supplemental Figure 3
Aldosterone and corticosterone plasma levels in control and MR<sup>MLC<sub>C</sub>re</sup> mice, 1 day, 3 days, 7 days and 8 weeks after myocardial infarction (MI) or sham-operation. Mean±SEM (n=4-10). Statistical analysis was performed by 2-way ANOVA. Unpaired t-test was used to compare each time point versus respective sham. *P<0.05 vs respective sham.
Supplemental Figure 4
Myocardial gene expression (A) as well as protein expression (B) of the glucocorticoid receptor and 11-beta-hydroxysteroid dehydrogenase type 2 (11beta-HSD2) in control and MR^MLC^Cre mice, 56 days after myocardial infarction (MI) or sham-operation. Mean±SEM (n=6-10). Statistical analysis was performed by 1-way ANOVA followed by Holm post hoc test to adjust for multiple-comparisons.
Supplemental Figure 5

(A) Serial echocardiography of control and MR^{MLCCre} mice 1 day, 7 days, and 56 days after myocardial infarction. PA, (mid-papillary); ESA, end-systolic area; EDA, end-diastolic area; ESD, end-systolic diameter; EDD end-diastolic diameter. Mean±SEM (n=14). Statistical analysis was performed by MIXED procedure. Repeated factor: time; fixed factors: group (Control, MR^{MLCCre}) and time; correlation structure: first-order autoregressive. Contrasts between the groups, Control and MR^{MLCCre} at day 1, day 7 and day 56 were performed with the subcommand TEST in SPSS. *P<0.05, †P<0.01 vs control.

(B) LV/BW and RV/BW in control and MR^{MLCCre} mice, 8 weeks after myocardial infarction (MI). Statistical analysis: Mann-Whitney U test.
Supplemental Figure 6

Heatmaps of significantly regulated genes in the surviving LV myocardium of MRMLCcre vs control mice 8 weeks after myocardial infarction (P<0.05). Gene upregulation with fold change (FC) >1.4 and downregulation with FC > 1.5 are shown.
Supplemental Figure 7
Mitochondrial superoxide anion formation and Nox4 protein expression in cardiomyocytes isolated from adult control and MR\(^{MLCCre}\) mice. Cardiomyocytes were stimulated with aldosterone (100 nmol/L) for 8 hours. Mean±SEM (n=3). Statistical analysis was performed by 1-way ANOVA followed by Holm post hoc test to adjust for multiple-comparisons. *P<0.05 Control-Aldo 100nmol/L vs respective vehicle, †P<0.05 MR\(^{MLCCre}\)-Aldo 100nmol/L vs Control-Aldo 100nmol/L.
Supplemental Figure 8

Time course of NF-κB activation and IκBα degradation following TNFα stimulation in cardiomyocytes isolated from adult control and MRMLCCre mice. Cardiomyocytes were stimulated with TNFα (10ng/mL) for 5, 15, 30 and 60 minutes. (A) NF-κB activation analysed by DNA-binding activity of p65 in nuclear extracts; (B) IκBα protein expression in cytoplasmic extracts. Mean±SEM (n=3). Statistical analysis was performed by 1-way ANOVA. Changes versus baseline and versus maximum of NF-κB activation/IκBα degradation were analyzed using unpaired t-test. *P<0.05 vs respective unstimulated cardiomyocytes, †P<0.05 vs MRMLCCre cardiomyocytes stimulated with TNFα for 15 minutes.
Supplemental Figure 9

Immunohistochemical staining showing infiltration of (A) neutrophils and (B) monocytes (anti-CD68) in the healing myocardium of control and MR^{MLCCre} mice, 3 days after myocardial infarction. Statistical analysis was performed by unpaired *t*-test. Mean±SEM (n=4-6), †P<0.05 vs control. (A, magnification x200; B, magnification x400).
Supplemental References


