Mineralocorticoid Receptor Antagonists Modulate Galectin-3 and Interleukin-33/ST2 Signaling in Left Ventricular Systolic Dysfunction After Acute Myocardial Infarction

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ABSTRACT

OBJECTIVES This study aimed to evaluate the specific role of the 2 available mineralocorticoid receptor antagonists (MRAs), eplerenone and spironolactone, on the modulation of galectin-3 (Gal-3) and interleukin (IL)-33/ST2 signaling in an experimental model of left ventricular systolic dysfunction after acute myocardial infarction (MI).

BACKGROUND The molecular mechanisms of benefits of MRAs in patients with left ventricular systolic dysfunction after MI not well understood.

METHODS MI and left ventricular systolic dysfunction were induced by permanent ligation of the anterior coronary artery in 45 male Wistar rats, randomly assigned to no therapy (MI group, n = 15) or to receive MRAs (100 mg/kg/day) for 4 weeks; either eplerenone (n = 15) or spironolactone (n = 15) was used. A sham group was used as a control (n = 8). Elements of the pathway for Gal-3 including transforming growth factor (TGF)-β and SMAD3, as well as that for IL-33/ST2 (including IL-33 and soluble ST2 [sST2]) were analyzed in the infarcted and noninfarcted myocardium by quantitative real-time reverse transcription polymerase chain reaction. Expression of markers of fibrosis (collagen types I and III, tissue inhibitor of metalloproteinase-1) and inflammation (IL-6, tumor necrosis factor-α, monocyte chemotactic protein-1) was also examined.

RESULTS In the infarcted myocardium, compared with sham animals, the MI group had higher concentrations of Gal-3, TGF-β, SMAD3, IL-33, and sST2, as well as higher concentrations of markers of fibrosis and inflammation. Treatment with MRAs down-regulated Gal-3, TGF-β, and SMAD3 and enhanced IL-33/ST2 signaling with lower expression of sST2; protective IL-33 up-regulation was unaffected by MRAs. Modulation of Gal-3 and IL-33/ST2 signaling induced by MRAs correlated with lower expression levels of fibrosis and inflammatory markers. No differences were found between eplerenone and spironolactone. In the noninfarcted myocardium, compared with sham animals, the MI group exhibited a higher expression of Gal-3 and IL-33, but no signs of inflammation or fibrosis were observed; in the presence of MRAs, IL-33 expression was significantly up-regulated, but Gal-3 was unaffected.

CONCLUSIONS MRAs play a pivotal role in the Gal-3 and IL-33/ST2 modulation in post-MI cardiac remodeling. (J Am Coll Cardiol HF 2015;3:50–8) © 2015 by the American College of Cardiology Foundation.
mineralocorticoid receptor antagonists (MRAs), represented by the nonselective agents spironolactone (1) and the selective eplerenone (2,3), have been shown to improve survival in patients with symptomatic chronic heart failure (HF) and acute myocardial infarction (AMI) associated with left ventricular systolic dysfunction. These clinical benefits have been related to the improvement of left ventricular (LV) remodeling and the reduction of cardiac fibrosis (4,5). Activation of mineralocorticoid receptor promotes myocardial fibrosis, inflammation, cardiomyocyte death, and LV hypertrophy (6), but the molecular mechanisms that specifically underlie their clinical benefits are not completely elucidated. In addition, spironolactone and eplerenone differ in their molecular structure, pharmacodynamics, and pleiotropic effects (7,8); however, meaningful differences between the 2 agents are not clearly present, and clinical practice guidelines do not discriminate between agents when recommending the use of an MRA in this setting (9,10).

Galectin-3 (Gal-3) and soluble ST2 (sST2) are promising prognostic biomarkers that are involved in myocardial remodeling, fibrosis, and inflammation (11-14); both are prognostically meaningful after AMI and in patients with established HF. Gal-3, a soluble β-galactoside-binding lectin, has been associated with activation of fibroblasts and macrophages (12). In patient cohorts, higher concentrations of plasma Gal-3 are associated with LV remodeling along with an increased risk of incident HF and mortality (15,16). ST2 is an interleukin (IL)-1 receptor family member with transmembrane (ST2L) and soluble (sST2) isoforms. Through interaction between IL-33 and ST2L, myocardial fibrosis and hypertrophy are prevented (13); in contrast, sST2 acts as a decoy receptor that neutralizes IL-33, and the cardioprotective role of IL-33/ST2 signaling pathway is lost (14), resulting in cardiomyocyte hypertrophy, apoptosis, and fibrosis. In this context, serum sST2 levels are strongly predictive of adverse outcomes in patients with AMI or HF and significantly predict LV remodeling (17-20).

Given the links between Gal-3 and sST2 with myocardial fibrosis and HF progression, it has been frequently speculated that an intersection between MRA therapy and these 2 pathways may exist. However, links between Gal-3 and sST2 with the benefit of the treatment with MRAs have not been clinically elucidated (21). Accordingly, the present study aimed to evaluate the specific role of the 2 available MRAs, eplerenone and spironolactone, in the modulation of Gal-3 and IL-33/ST2 signaling in an experimental model of left ventricular systolic dysfunction after AMI.

**METHODS**

This study was approved by the Ethics Committee of the University of Murcia and the Hospital Virgen de la Arrixaca. Details of the methods are provided in the Online Appendix.

**EXPERIMENTAL PROTOCOL.** Male Wistar rats (weighing 200 to 250 g, 6 to 8 weeks old) were purchased from the Animal House, Laboratory Animal Service, University of Murcia, Murcia, Spain. The animals were housed in a pathogen-free facility under strict veterinary supervision and maintained in controlled rooms with 12-h light/dark cycles. The animals received commercial rat diet and water ad libitum.

Acute myocardial infarction (AMI) was induced by permanent ligation of the left anterior descending coronary artery (LAD), as described in the Online Appendix. Electrocardiography was used to demonstrate ST-segment elevation and thereby confirm the success of surgery.

The sham group underwent the same surgical procedure except that the LAD was not occluded. Surviving rats were randomly divided into 4 groups: rats operated on but without LAD ligation (sham group, n = 8); rats with ligation of the LAD and without treatment (MI group, n = 15); rats with ligation of LAD and treated with either eplerenone (MI + eplerenone, n = 15) or spironolactone (MI + spironolactone, n = 15) at a dose of 100 mg/kg/day mixed in rat chow. The dose of MRAs was adjusted each week according to the body weight of each animal. Daily amounts of food intake were checked at regular intervals to confirm the dose of administered drug. MRAs were obtained from Sigma-Aldrich Corp. (St. Louis, Missouri). The treatment regimen was started on the day of surgery and maintained until the animals were killed at 4 weeks after the procedure of MI. At this time, the infarcted and noninfarcted areas from left ventricle were carefully removed and processed for the analysis. LV function variables were assessed using a transthoracic echocardiographic examination (4- to 12-MHz phased-array sectorial transducer, HD7 XE, Philips, Andover, Massachusetts) (Online Appendix).

**HISTOLOGICAL STAINING.** Infarct size was quantified by planimetry at death. The left ventricle was cut into 3 transverse sections: apex, middle ring, and...
Echocardiographic parameters of the respective groups of rats are shown in Table 1. The presence of MI was confirmed by echocardiography performed on the day after the LAD ligation procedure, and no differences were found between groups. Four weeks after ligation, MRA treatment was associated with a significant recovery of wall motion score index, left ventricular ejection fraction, and left ventricular end-diastolic volume values. The infarct size at death, measured as a percentage of the infarcted perimeter by histochemical staining, was similar among groups with MI regardless of therapy. No differences were found in serum levels of urea, creatinine, or potassium nor body weight gain (Online Table 2).

**Table 1.** Echocardiographic Parameters

<table>
<thead>
<tr>
<th></th>
<th>Sham</th>
<th>MI</th>
<th>Eple</th>
<th>Spiro</th>
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<tr>
<td><strong>24 h post-MI</strong></td>
<td></td>
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<tr>
<td>WMSI</td>
<td>1.0</td>
<td>1.6 ± 0.28*</td>
<td>1.7 ± 0.35*</td>
<td>1.6 ± 0.17†</td>
</tr>
<tr>
<td>LVEF, %</td>
<td>50.0 ± 10.0</td>
<td>33.0 ± 11.0*</td>
<td>34 ± 10*</td>
<td>34 ± 11*</td>
</tr>
<tr>
<td>LVEDV, ml/g²</td>
<td>0.9 ± 0.08</td>
<td>1.1 ± 0.32</td>
<td>1.0 ± 0.31</td>
<td>1.0 ± 0.27</td>
</tr>
<tr>
<td><strong>4 weeks post-MI</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WMSI</td>
<td>1.0</td>
<td>1.7 ± 0.16*</td>
<td>1.2 ± 0.12</td>
<td>1.3 ± 0.18</td>
</tr>
<tr>
<td>LVEF, %</td>
<td>50 ± 14</td>
<td>31 ± 14*</td>
<td>39 ± 9†</td>
<td>37 ± 13†</td>
</tr>
<tr>
<td>LVEDV, ml/g²</td>
<td>1.0 ± 0.03</td>
<td>1.4 ± 0.37*</td>
<td>1.1 ± 0.07†</td>
<td>1.1 ± 0.12‡</td>
</tr>
<tr>
<td>Infarct size, %</td>
<td>36.1 ± 2.1</td>
<td>30.1 ± 2.3</td>
<td>34.1 ± 3.5</td>
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</tr>
</tbody>
</table>

Values are mean ± SD. WMSI, LVEF, and LVEDV were determined 24 h post-MI and 4 weeks post-MI. Infarct size was determined at death. *p < 0.01 vs. the sham group. †p < 0.05 vs. the MI group.

Eple = eplerenone; LVEF = left ventricular ejection fraction; MI = myocardial infarction; Spiro = spironolactone; WMSI = wall motion score index.

base. From the middle ring, formalin-fixed transverse myocardial sections (5 μm) were stained with Masson trichrome using standard techniques. High-resolution images were obtained through a slide scanner Leica SN400F (Leica Microsystems Inc., Buffalo Grove, Illinois). Infarct size was calculated as the average of all slices and expressed as a percentage of the infarcted perimeter. Collagen volume was calculated as the average of all slices and expressed as the ratio of Masson trichrome-stained collagen area to total myocardium area.

**RNA extraction and quantitative reverse-transcription polymerase chain reaction.** RNA was isolated from frozen tissue, and quantitative polymerase chain reaction was performed according to the manufacturer’s protocol with minor modification (Online Table 1).

**Statistical analysis.** Data were expressed as mean ± SEM or SD as stated and were analyzed using SPSS software version 19 (SPSS Inc., Chicago, Illinois). Paired-sample comparisons and 1-way analysis of variance were used as appropriate. Graphing was performed using SigmaPlot 11.0 software (Systat Software Inc., San Jose, California). Statistical significance was assumed at p < 0.05.

**Results**

Echocardiographic parameters of the respective groups of rats are shown in Table 1. The presence of MI was confirmed by echocardiography performed on the day after the LAD ligation procedure, and no differences were found between groups. Four weeks after ligation, MRA treatment was associated with a significant recovery of wall motion score index, left ventricular ejection fraction, and left ventricular end-diastolic volume values. The infarct size at death, measured as a percentage of the infarcted perimeter by histochemical staining, was similar among groups with MI regardless of therapy. No differences were found in serum levels of urea, creatinine, or potassium nor body weight gain (Online Table 2).

**Gal-3 signaling pathway and MRAs.** Gal-3 expression levels and molecules involved in the Gal-3 signaling pathway (transforming growth factor [TGF]-β and SMAD-3) were analyzed in the infarcted and noninfarcted myocardium by quantitative reverse transcription polymerase chain reaction for each experimental group. In the infarcted myocardium (Figure 1), Gal-3 gene expression was induced (p < 0.001) as well as TGF-β (p < 0.001) and SMAD-3 (p = 0.001) compared with the sham group. Treatment with either eplerenone or spironolactone did not reduce Gal-3 expression in the noninfarcted myocardium (p > 0.50). The expression of TGF-β and SMAD-3 in the noninfarcted myocardium was similar to that of the sham group. As expected, in the infarcted myocardium, Gal-3 expression correlated with that of TGF-β (rs = 0.68, p < 0.001) and SMAD-3 (rs = 0.26, p = 0.047). This correlation was not found in the noninfarcted myocardium.

**IL-33/ST2 signaling pathway and MRAs.** Modulation of IL-33/ST2 signaling pathway by MRAs was also evaluated by analyzing the mRNA expression of cardioprotective IL-33 and its soluble decoy receptor sST2, as shown in Figure 1. In the infarcted myocardium, there were higher levels of IL-33 (p < 0.001) and sST2 (p < 0.001) compared with the sham group, which were positively correlated to each other (rs = 0.55, p < 0.001). Interestingly, the high levels of the cardioprotective cytokine IL-33 were not affected by MRA administration (eplerenone, p = 0.24; spironolactone, p = 0.54); in contrast, the sST2 levels were reduced by treatment with both MRAs (eplerenone, p = 0.005; spironolactone, p = 0.008). In the noninfarcted myocardium of MI group (Figure 2B), IL-33 levels were higher compared with the sham group (p = 0.009), and treatment with either eplerenone or spironolactone was associated with a significantly higher IL-33 expression (eplerenone, p = 0.002; spironolactone, p < 0.001). No significant differences were observed in sST2 levels (p = 0.49) in the noninfarcted area.

**Fibrosis.** As shown in Figure 3, in the infarcted area, the expression of collagen type I (Col I) (p <
0.014) and tissue inhibitor of metalloproteinase (TIMP)-1 (p < 0.001) was increased. Treatment with either eplerenone or spironolactone was associated with a lower expression of Col I, Col III, and TIMP-1. Modulation of Gal-3 and IL-33/sST2 by MRAs was correlated with myocardial fibrosis, by measuring RNA expression of Col I, Col III, and TIMP-1 (Table 2). Masson trichrome staining for interstitial fibrosis in the infarcted area is shown in Figure 3D; collagen volume fraction was increased (p < 0.001) compared with the sham group. The treatment with either eplerenone or spironolactone was associated with a reduction in collagen volume fraction in the infarcted myocardium (eplerenone, p = 0.01; spironolactone, p = 0.02). In the noninfarcted area, the mRNA levels of Col I or Col III did not differ between the MI group and the sham group (p > 0.10 for all analyses), and no significant changes in collagen volume fraction were found in the noninfarcted area of MI group (Online Figure 1).

**INFLAMMATION.** As shown in Figure 4, the MI group exhibited signs of inflammation in the infarcted area as indicated by increased levels of tumor necrosis factor-α (p < 0.001), IL-6 (p = 0.003), and monocyte chemotactic protein 1 (p = 0.001). The expression levels of tumor necrosis factor-α were significantly lower in the presence of eplerenone (p = 0.006), whereas levels of IL-6 were lower in the presence of spironolactone (p = 0.03). The increased levels of monocyte chemotactic protein-1 were unaffected by the treatment with any MRA. In a correlation analysis (Table 2), the expression levels in the infarcted area of molecules involved in both Gal-3 and IL33/sST2 signaling were significantly correlated with the markers of inflammation. No significant changes were observed.
in the noninfarcted area of the MI group (p > 0.10 for all analyses).

**DISCUSSION**

There has been a growing interest in elucidating the mechanisms involved in the clinical benefit of MRAs. In a similar manner, an increase in the understanding of Gal-3 and sST2 has led to interest in explaining their prognostic value and to explore their potential as therapeutic targets. The principal finding of the present study was the demonstration that MRAs modulate the myocardial expression profiles involved in the signaling of Gal-3 and IL-33/sST2, which correlated with markers of extracellular matrix turnover and inflammation. Therefore, this modulation could participate in a downstream cardioprotective effect of MRAs, explaining their value in the setting of left ventricular systolic dysfunction after AMI. To our knowledge, this is the first demonstration of such an interaction between therapy with MRAs and both Gal-3 and IL-33/sST2 signaling. In addition, the study shows that either eplerenone and spironolactone exerted similar effects.

In recent years, Gal-3 and sST2 have emerged as linked to the pathophysiology of adverse myocardial remodeling and HF (11). Both predict the development of de novo HF in the general population and are prognostic markers among patients with AMI and acute or chronic HF (15-20). As a consequence, they were recently mentioned in HF clinical practice guidelines as biomarkers of myocardial fibrosis, adding prognostic information to clinical variables and other biomarkers (9,10). A recent analysis suggested sST2 to be substantially more prognostic than Gal-3 in patients with HF, but these clinical results do not necessarily inform biological significance of these 2 unrelated pathways (22). Additionally, the expected benefits of MRAs in those with elevated concentrations of these 2 peptides have been elusive. A previous study suggested that eplerenone might reduce LV remodeling in patients with increased sST2 after AMI, whereas Gal-3 concentrations in the same cohort did not (23,24). Similarly, in patients with established HF, Gal-3 did not predict a benefit of MRA therapy (21).

We therefore wished to examine the question more fundamentally in an animal model of AMI evaluating myocardial expression of Gal-3 and sST2 at 4 weeks. At this time, Gal-3 and IL-33/sST2 were significantly up-regulated, which is compatible with findings in other animal models of adverse cardiac remodeling (14,25-27). The up-regulation of Gal-3 and sST2 is consistent with the involvement of both molecules in the setting of early remodeling after AMI and provides a plausible explanation for their measurable elevation in patients clinically affected (17,23,28). In addition, the up-regulation of IL-33 supports its participation as a protective response from cardiomyocytes and fibroblasts in both the infarcted and noninfarcted myocardium, as previously suggested (13,29).

MRA treatment was associated with an inhibition of the observed up-regulation of Gal-3, TGF-β, and SMAD-3. Only 2 studies in experimental models of hypertension have reported this relationship between MRA and Gal-3 regulation (30,31). In addition, MRA treatment decreased expression of sST2 and did not affect the up-regulated level of IL-33; such a finding would yield an overall protective enhancement of IL-33/ST2 signaling. Intriguingly, in the noninfarcted (but presumably hemodynamically stressed) myocardium, IL-33 was up-regulated, and this response was enhanced in the presence of MRAs. This further supports the suggested protective role of IL-33 in response to myocardial stress. On the whole, our findings identify both Gal-3 and IL-33/sST2 systems as putative MRA targets at an early stage after AMI, mainly in the infarcted myocardium but also in the remote area.

AMI initiates a complex, profound, and rapid response in terms of cellular and extracellular remodeling. MRAs have been shown to reduce post-infarction collagen synthesis and progressive cardiac remodeling in experimental models and populations with AMI and HF (32-34). In the present study,
MRAs attenuated the increase in myocardial markers of inflammation and fibrosis in the infarcted myocardium, and both effects correlated with the down-regulation of Gal-3 signaling and the enhancement of IL-33/ST2L signaling. Gal-3 induces cardiac inflammation and fibrosis via the TGF-β/SMAD-3 signaling pathway and mediates aldosterone-induced fibrosis (31), and its genetic disruption or inhibition attenuates cardiac fibrosis, LV dysfunction, and subsequent HF development in animal models of HF (25,35). The antifibrotic role of IL-33/ST2L signaling has been demonstrated in an animal model of pressure overload, and administration of sST2 blocked the favorable effect of IL-33 in a dose-dependent manner (13,29). In the remote area, markers of fibrosis and inflammation were not increased, which
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TABLE 2 Correlation Between Expression of Gal-3 and IL-33/sST2 Molecules and Remodeling Markers in the Infarcted Myocardium

<table>
<thead>
<tr>
<th></th>
<th>Fibrosis</th>
<th>Inflammation</th>
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<tbody>
<tr>
<td></td>
<td>Col I</td>
<td>Col III</td>
</tr>
<tr>
<td>Gal-3</td>
<td>r = 0.58*</td>
<td>r = 0.29</td>
</tr>
<tr>
<td>TGF-β</td>
<td>r = 0.66*</td>
<td>r = 0.47</td>
</tr>
<tr>
<td>SMAD-3</td>
<td>r = 0.72*</td>
<td>r = 0.85*</td>
</tr>
<tr>
<td>IL-33</td>
<td>r = 0.57*</td>
<td>r = 0.39*</td>
</tr>
<tr>
<td>sST2</td>
<td>r = 0.58*</td>
<td>r = 0.39*</td>
</tr>
</tbody>
</table>

*a < 0.001, **p < 0.01, ***p < 0.05

Col I = collagen type I; Col III = collagen type III; Gal-3 = Galectin-3; IL = interleukin; MCP = monocyte chemotactic protein; sST2 = soluble ST2; TGF = transforming growth factor; TIMP = tissue inhibitor of metalloproteinase; TNF = tumor necrosis factor.

suggests that the observed up-regulation of Gal-3 and IL-33 may precede remodeling processes that occur later in the remote area. Before our study, there were no available data regarding the relationship between anti-inflammatory or anti-fibrotic effects of MRAs relative to IL-33 and sST2.

Current guidelines recommend an MRA (either spironolactone or eplerenone) after AMI. Although the indication is based on the benefits obtained with eplerenone in the EPHEBUS (Eplerenone Post-Acute Myocardial Infarction Heart Failure Efficacy and Survival Study) (2), it is generally believed that the 2 agents have the same effects because no clinical trials have directly compared the efficacy of both drugs. However, the 2 molecules differ in their molecular structure and pharmacodynamics, as well as in other not well-known pleiotropic and nongenomic effects (7,36,37). In addition, observational analyses in contemporary populations receiving spironolactone have not replicated the results obtained by eplerenone in clinical trials (38,39). Given the lack of clinical studies, we compared the effects of spironolactone and eplerenone, and the results support that both exert similar favorable myocardial effects after AMI. These benefits are related to the inhibition of the MR and not the modest diuretic effect of these agents (40); in fact, we did not find differences in terms of body weight or potassium sparring between groups.

Although data exist suggesting favorable effects of eplerenone on LV remodeling after AMI among those patients with higher sST2 levels (23) but not higher Gal-3 levels (21,24), our results differ considerably from clinical analyses. Indeed, the present study suggests that MRAs favorably regulate local expression of Gal-3 and IL-33/sST2 in the myocardium after AMI. However, myocardial expression does not always correlate with serum levels (41), and it could explain why clinical studies do not support the use of Gal-3 for guiding the use of MRA therapy (21); it is quite possible that circulating Gal-3 concentrations do not actually reflect myocardial processes, and sST2 concentrations may more accurately reflect the presence of reversible processes than Gal-3. Supporting this suggestion is the finding that use of beta-blockers also improved prognosis when levels of sST2 were elevated in patients with HF (42), whereas no such interaction with Gal-3 was found in this same cohort.

The findings of the present study are limited by the experimental design and that relationships with fibrosis and inflammation markers were based

FIGURE 4 Cardiac mRNA Expression of Inflammatory Markers in the Infarcted Myocardium

Quantitative reverse transcription polymerase chain reaction analysis of TNF-α (A), IL-6 (B), and MCP-1 (C). Data are expressed as fold of sham group. **p < 0.01, ***p < 0.001 compared with the sham group. #p < 0.05, ##p < 0.01 compared with the MI group. MCP = monocyte chemotactic protein; TNF = tumor necrosis factor; other abbreviations as in Figure 1.
on associations, but causality was not demonstrated. Further in vitro assays should be performed in culture cardiomyocytes or cardiac fibroblasts to explore the direct actions of MRAs. Nevertheless, our study appears to identify Gal-3 and IL-33/sST2 as therapeutic targets modulated by MRAs and suggests that the beneficial actions of MRAs after AMI could be mediated by their ability to either block Gal-3 signaling or to enhance IL-33/SST2 signaling. Given results from clinical studies that suggest a lack of associations between circulating Gal-3 and MRAs, our results call into question the measurement of Gal-3 concentrations for the purpose of deciding on therapeutic intervention, as circulating concentrations of the marker may not reflect myocardial processes. Our seminal results expand our knowledge of the cardioprotective role of MRAs and provide information that could be translated to the evaluation of therapeutic strategies focused on modulating these signaling pathways.

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KEY WORDS eplerenone, heart failure, myocardial infarction, remodeling, spironolactone

APPENDIX For details of the methods and supplemental tables and figure, please see the online version of this article.