Galectin-3 Blockade Inhibits Cardiac Inflammation and Fibrosis in Experimental Hyperaldosteronism and Hypertension


Abstract—Hypertensive cardiac remodeling is accompanied by molecular inflammation and fibrosis, 2 mechanisms that finally affect cardiac function. At cardiac level, aldosterone promotes inflammation and fibrosis, although the precise mechanisms are still unclear. Galectin-3 (Gal-3), a β-galactoside–binding lectin, is associated with inflammation and fibrosis in the cardiovascular system. We herein investigated whether Gal-3 inhibition could block aldosterone-induced cardiac inflammation and fibrosis and its potential role in cardiac damage associated with hypertension. Aldosterone-salt–treated rats presented hypertension, cardiac inflammation, and fibrosis that were prevented by the pharmacological inhibition of Gal-3 with modified citrus pectin. Cardiac inflammation and fibrosis presented in spontaneously hypertensive rats were prevented by modified citrus pectin treatment, whereas Gal-3 blockade did not modify blood pressure levels. In the absence of blood pressure modifications, Gal-3 knockout mice were resistant to aldosterone-induced cardiac inflammation. In human cardiac fibroblasts, aldosterone increased Gal-3 expression via its mineralocorticoid receptor. Gal-3 and aldosterone enhanced proinflammatory and profibrotic markers, as well as metalloproteinase activities in human cardiac fibroblasts, effects that were not observed in Gal-3–silenced cells treated with aldosterone. In experimental hyperaldosteronism, the increase in Gal-3 expression was associated with cardiac inflammation and fibrosis, alterations that were prevented by Gal-3 blockade independently of blood pressure levels. These data suggest that Gal-3 could be a new molecular mechanism linking cardiac inflammation and fibrosis in situations with high-aldosterone levels, such as hypertension. (Hypertension. 2015;66:767-775. DOI: 10.1161/HYPERTENSIONAHA.115.05876.)

Key Words: aldosterone ■ fibrosis ■ galectin 3 ■ hypertension ■ inflammation

Hypertensive cardiac remodeling, characterized by cardiac inflammation and fibrosis, is a major risk factor for cardiovascular morbidity and mortality, and it is a leading cause of chronic heart failure.1 Cardiac remodeling is characterized by an initial inflammatory phase, which facilitates extracellular matrix (ECM) changes resulting in myocardial fibrosis.2 Aberrant remodeling of the ECM during fibrosis is also brought about by the deregulated expression of matrix metalloproteases (MMPs). Activation of MMPs results not only in the degradation of structural components of the ECM but also in the activation of growth factors sequestered in the ECM, facilitating inflammation.3 However, the molecular mechanisms of hypertensive cardiac remodeling remain incomplete. A better knowledge of the underlying mechanisms may highlight novel mediators of cardiac remodeling that could identify biotargets for novel pharmacological therapies.

Aldosterone acts classically via mineralocorticoid receptors (MRs).4 Aldosterone levels are increased in hypertensive patients and in spontaneously hypertensive rats (SHR)5,6 being considered as an inducer of hypertensive organ damage.7 Chronic hyperaldosteronism concurs with high blood pressure (BP) in causing cardiovascular complications, including left ventricular hypertrophy, arterial wall stiffening, myocardial infarction, and atrial fibrillation.8 MR activation and high-salt intake cause hypertension and inflammation, leading to cardiac inflammation and fibrosis.9 Large randomized controlled...
trials (Randomized Aldactone Evaluation Study [RALES], Eplerenone Post-Acute Myocardial Infarction Heart Failure Efficacy and Survival Study [EPHESUS], and Eplerenone in Mild Patients Hospitalization and Survival Study in Heart Failure [EMPHASIS]) have demonstrated that in patients with severe or mild heart failure, MR antagonists reduce mortality and morbidity.\textsuperscript{10–12}

Galectin-3 (Gal-3) is a 29- to 35-kDa protein, member of a β-galactoside-binding lectin family expressed in fibroblasts,\textsuperscript{13} endothelial cells,\textsuperscript{14} and inflammatory cells,\textsuperscript{15} which seems to be involved in most models of injury. We and others have observed in clinical studies increased levels of Gal-3 in patients with heart failure, which were correlated with serum ECM markers.\textsuperscript{16} In addition, Gal-3 was recently reported to have a prognostic value in patients with coronary artery disease, possibly related to its role in plaque destabilization.\textsuperscript{17}

Several inhibitors of Gal-3 have been described, such as the modified citrus pectin (MCP; a complex water soluble indigestible polysaccharide riche in β-galactose) being able to block the lectin’s activity.\textsuperscript{18} We have recently demonstrated that Gal-3 mediates the vascular remodeling and the cardiac fibrosis induced by aldosterone.\textsuperscript{19,20} In addition, Azibani et al\textsuperscript{21} have demonstrated that hyperaldosteronism worsens hypertension-induced fibrosis through an increase of inflammatory molecules such as Gal-3. However, the precise mechanism responsible for aldosterone-induced cardiac inflammation and the role of Gal-3 in the inflammation induced by aldosterone are still unclear. We aimed to evaluate the effects of

![Figure 1](http://hyper.ahajournals.org/)
Inflammation and Fibrosis in Aldo-Salt–Treated Rats and SHR

Table 1. Associations Found Between Myocardial Gal-3 Expression and Parameters Assessing Myocardial Inflammation and Fibrosis in Aldo-Salt–Treated Rats and SHR

<table>
<thead>
<tr>
<th>Parameter</th>
<th>SHR+MCP</th>
<th>SHR</th>
<th>WKY</th>
<th>r</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gal-3 (mRNA)</td>
<td>0.6532</td>
<td>0.5192</td>
<td>0.6641</td>
<td>0.6526</td>
<td>0.0019</td>
</tr>
<tr>
<td>Gal-3 (protein)</td>
<td>0.0001</td>
<td>0.0175</td>
<td>0.0001</td>
<td>0.0045</td>
<td></td>
</tr>
<tr>
<td>OPN (mRNA)</td>
<td>0.7338</td>
<td>0.0155</td>
<td>0.7280</td>
<td>0.6641</td>
<td>0.0019</td>
</tr>
<tr>
<td>Collagen type I (mRNA)</td>
<td>0.5522</td>
<td>0.0175</td>
<td>0.6641</td>
<td>0.6641</td>
<td>0.0019</td>
</tr>
<tr>
<td>TGF-β (mRNA)</td>
<td>0.4989</td>
<td>0.0351</td>
<td>0.6973</td>
<td>0.6526</td>
<td>0.0045</td>
</tr>
<tr>
<td>CCL2 (mRNA)</td>
<td>0.8038</td>
<td>0.0001</td>
<td>0.7441</td>
<td>0.6641</td>
<td>0.0019</td>
</tr>
<tr>
<td>OPN (protein)</td>
<td>0.7338</td>
<td>0.0005</td>
<td>0.7280</td>
<td>0.6641</td>
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<td>0.6526</td>
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</tr>
</tbody>
</table>

Gal-3 inhibition on cardiac inflammation and fibrosis in 3 different animal models of hyperaldosteronism: (1) hypertensive rat model induced by aldosterone-salt treatment, (2) SHR, and (3) normotensive wild-type (WT) and Gal-3 knockout mice treated with aldosterone without salt. In addition, we evaluate the effects of Gal-3 inhibition on cardiac inflammation and fibrosis mediators in human cardiac fibroblasts.

Methods

Detailed methods are available in the online-only Data Supplement.

Animals

Adult male Wistar rats were treated for 3 weeks with vehicle (n=10), aldosterone-salt (Sigma, 1 mg/kg per day diluted in sunflower oil and administered by subcutaneous injection and 1% NaCl as drinking water; n=10), aldosterone-salt plus spironolactone (Sigma, 200 mg/kg per day; n=10), aldosterone-salt plus Gal-3 activity inhibitor, MCP (Enconugenics, 100 mg/kg per day; n=9), spironolactone (n=7), or MCP (n=5) alone. 20

Male normotensive Wistar-Kyoto (n=8) and SHR (n=16) were obtained from Harlan Iberica. Half of the animals of SHR group received MCP (100 mg/kg per day) for 6 weeks in the drinking water. For the Gal-3 knockout model, adult male C57BJ6 WT mice and Gal-3 knockout mice 22 were infused with aldosterone (1 mg/kg per day, osmotic minipump) or vehicle (n=7, each group) for 3 weeks.

The investigation was performed in accordance with the Guide for Care and Use of Laboratory Animals published by the US National Institutes of Health (publication number, 82-23, revised in 1996). Body weight was measured once a week. Food and water intakes were determined throughout the experimental period. Systolic BP was estimated basally, at midstudy, and end-of-study through use of a tail-cuff plethysmograph (Narco Bio-Systems) in unrestrained animals. In all experimental models, blood and urines were collected, and hemodynamic parameters were evaluated.

Cell Culture

Human cardiac fibroblasts were obtained from Promocell and maintained in medium fibroblasts media 3. Cells were cultured according to the manufacturer’s instructions. Cells were used between passages 5 and 7. Cells were stimulated with aldosterone (10−7 mol/L, Sigma), Gal-3 (10−4 mol/L; R&D Systems), and spironolactone (10−6 mol/L; Sigma) for 6 hours for mRNA determinations and for 24 hours for protein analysis. The doses were chosen based on previous studies. 19

Statistical Analyses

Data are expressed as mean±SD. Normality of distributions was verified by means of the Kolmogorov–Smirnov test. Data were analyzed using a 1-way ANOVA, followed by a Newman–Keuls test to assess specific differences among groups or conditions using GraphPad Software Inc. The predetermined significance level was P<0.05.

Results

Gal-3 Inhibition Improved Cardiac Inflammation and Fibrosis in 3 Experimental Models of Hyperaldosteronism

We investigated whether Gal-3 could be involved in cardiac inflammation additionally of fibrosis in 3 animal models in which aldosterone levels were abnormally increased. First, the effects of the pharmacological inhibition of Gal-3 with MCP in hypertensive aldosterone-salt–treated rats were analyzed. Then, the effect of Gal-3 blockade was studied in SHR. Finally, normotensive Gal-3 knockout mice were challenged with aldosterone without salt to avoid an increase in BP and its potential confounding effects.

We previously showed that aldosterone-salt treatment induced an increase in BP, cardiac fibrosis (assessed by collagen and transforming growth factor-β mRNA and protein expression), and Gal-3 expression (P<0.05). All these effects were prevented (P<0.05) by cotreatment with spironolactone or MCP. 20 Aldosterone-salt treatment induced an increase in C-reactive protein (7.0-fold; P<0.05) and tumor necrosis factor-α (3.0-fold; P<0.05) without modification in interleukin-1β (IL-1β) plasma levels, effects that were blocked by spironolactone or MCP cotreatment (P<0.05; Figure 1A).

Table 2. Characteristics of SHR Treated With MCP

<table>
<thead>
<tr>
<th>Parameter</th>
<th>SHR</th>
<th>SHR+MCP</th>
</tr>
</thead>
<tbody>
<tr>
<td>BW, g</td>
<td>327.0±11.6*</td>
<td>324.9±17.8*</td>
</tr>
<tr>
<td>HW/BW, mg/g</td>
<td>3.9±0.2*</td>
<td>3.9±0.2*</td>
</tr>
<tr>
<td>SBP, mmHg</td>
<td>221.1±19.8*</td>
<td>221.1±19.8*</td>
</tr>
<tr>
<td>Cardiomyocyte size, μm²</td>
<td>569.1±34.3*</td>
<td>569.1±34.3*</td>
</tr>
<tr>
<td>Aldosterone, pg/mL</td>
<td>291.3±95.6</td>
<td>291.3±95.6</td>
</tr>
<tr>
<td>CRP, pg/mL</td>
<td>34.6±5.7†</td>
<td>34.6±5.7†</td>
</tr>
<tr>
<td>IL-1β, pg/mL</td>
<td>0.3±0.1†</td>
<td>0.3±0.1†</td>
</tr>
</tbody>
</table>

Data values represent mean±SD of 8 animals. BW indicates body weight; CRP, C-reactive protein; HW, heart weight; IL-1β, interleukin-1β; MCP, modified citrus pectin; SHR, spontaneously hypertensive rats; SBP, systolic blood pressure; and WKY, Wistar-Kyoto.

*P<0.05 vs WKY.
†P<0.05 vs SHR.
Aldosterone-salt–induced hypertensive rats presented an increase in cardiac proinflammatory markers, such as IL-6 (2.4-fold; \(P < 0.05\)), chemokine ligand 2 (CCL2; 2.2-fold; \(P < 0.05\)), and osteopontin (3.8-fold; \(P < 0.05\)) mRNA levels (Figure 1B). This inflammatory response was characterized by CCL2, osteopontin, and macrophage infiltration in myocardium as shown in the representative pictures immunostained for proinflammatory markers (Figure 1C). Cotreatment with spironolactone or MCP abolished \((P < 0.05)\) all of modifications in proinflammatory molecules induced by aldosterone-salt treatment. Strong and positive correlations were found between Gal-3 and osteopontin at mRNA and protein levels (Table 1). No modifications were found between any group in MMP-2, MMP-9, and MMP-13 activities (Figure 1D). Spironolactone or MCP alone did not exert modifications in any of the parameters studied (data not shown).
In the second hypertensive animal model, SHR rats presented an increase in systolic BP when compared with control rats (Table 2). At cardiac level, hypertensive animals showed cardiac hypertrophy presenting an increase in relative heart weight and myocyte size (Table 2). The pharmacological inhibition of Gal-3 with MCP was not able to prevent the increase in neither systolic BP levels nor the cardiac hypertrophy (Table 2). Cardiac Gal-3 expression was upregulated in SHR rats at mRNA (2.5-fold; *P < 0.001) and protein levels (1.8-fold; *P < 0.01) when compared with normotensive rats (Figure 2A). The increase in Gal-3 levels in SHR was accompanied by enhanced levels of circulating aldosterone (1.5-fold; *P < 0.05; Table 2), as it has been previously described, as well as increased circulating proinflammatory markers, such as C-reactive protein and IL-1β (Table 2). SHR group presented an increase in CCL2 (1.7-fold, *P < 0.05; 1.7-fold, *P < 0.05) and osteopontin (2.6-fold, *P < 0.05; 1.6-fold, *P < 0.05) mRNA and protein levels, respectively (Figure 2B) located in the interstitial area and in CD68 cardiac immunohistochemistry (Figure 2C). Hearts from SHR presented an increase in interstitial fibrosis (1.8-fold; *P < 0.05) accompanied by an increase in collagen type I cardiac immunohistochemistry (Figure 2D). Accordingly, with these observations, cardiac levels of collagen type I (1.8-fold, *P < 0.001; 1.6-fold, *P < 0.05) and profibrotic mediators transforming growth factor-β (1.6-fold, *P < 0.001; 1.3-fold, *P < 0.05) and connective tissue growth factor (2.6-fold, *P < 0.001; 1.4-fold, *P < 0.05) were higher in SHR than in control ones at mRNA and protein expressions, respectively (Figure 2E). Strong and positive correlations were found between Gal-3 mRNA levels and proinflammatory

Figure 3. Knockout (KO) galectin-3 (Gal-3) mice were resistant to aldosterone (Aldo)-induced cardiac inflammation. A, Cardiac protein expressions of inflammatory markers in wild-type (WT) and Gal-3 KO mice treated with Aldo. B, Representative photographs of myocardial sections stained with chemokine ligand 2 (CCL2) or osteopontin (OPN). C, matrix metalloprotease-2 (MMP-2) activity in WT and Gal-3 KO mice treated with Aldo. All conditions were performed at least in triplicate. Histogram bars represent the means SD of each group of animals (WT mice, n=7; WT mice treated with Aldo, n=7; Gal-3 KO mice, n=7; and Gal-3 KO mice treated with Aldo, n=7) in arbitrary units (A.U.) or normalized to β-actin. Magnification, ×40. *P < 0.05 vs WT. †P < 0.05 vs WT+Aldo.
and profibrotic markers, such as CCL2, osteopontin, collagen type I, transforming growth factor-β, and connective tissue growth factor (Table 1). MCP cotreatment was able to reduce the increase in inflammation and cardiac fibrosis in SHR. In addition, strong and positive correlations were found between cardiac inflammatory markers and fibrotic mediators showing the relationship between inflammation and fibrosis that occurs during cardiac remodeling (Table 1).

To complete the understanding of Gal-3 in aldosterone-induced cardiac inflammation and fibrosis, normotensive WT and Gal-3 knockout mice were infused with aldosterone in the absence of salt to avoid an increase in BP and its potential confounding effects. Any of the groups presented modifications in neither hemodynamic nor cardiac parameters. Aldosterone treatment increased CCL2 (1.4-fold; $P<0.05$) and osteopontin (1.4-fold; $P<0.05$) protein levels in WT mice (Figure 3A and 3B), whereas Gal-3 knockout mice were specifically resistant to aldosterone-induced proinflammatory marker increases ($P<0.05$; Figure 3A and 3B). In any of the groups studied, MMP-2 activity was not modified (Figure 3C).

As previously published, all of these modifications induced by aldosterone were accompanied in WT mice by increased Gal-3 expression, interstitial fibrosis, and enhanced ECM components such as collagen I and collagen III, whereas Gal-3 knockout mice were specifically resistant to aldosterone-induced cardiac remodeling, as we already showed.20

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**Figure 4.** Comparative effects between aldosterone (Aldo) and galectin-3 (Gal-3) on proinflammatory and profibrotic markers in human cardiac fibroblasts. **A**, Effects of Aldo ($10^{-8}$ mol/L) and spironolactone (Spiro; $10^{-6}$ mol/L) on Gal-3 intracellular expression in human cardiac fibroblasts. **B**, Effects of Aldo ($10^{-8}$ mol/L) and Gal-3 ($10^{-8}$ mol/L) on tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), IL-6, and chemokine ligand 2 (CCL2) secretion. **C**, Effects of Aldo ($10^{-8}$ mol/L) and Gal-3 ($10^{-8}$ mol/L) on collagen type I, collagen type III, fibronectin, and transforming growth factor-β (TGF-β) expressions. **D**, Effects of Aldo ($10^{-8}$ mol/L) and Gal-3 ($10^{-8}$ mol/L) on matrix metalloprotease-1 (MMP-1), MMP-2, and MMP-9 activities. All conditions were performed at least in triplicate. Histogram bars represent the mean±SD of 4 assays, in arbitrary units (A.U.) normalized to stain free gel or β-actin for supernatant or protein lysate, respectively. *$P<0.05$; **$P<0.01$; ***$P<0.001$ vs control. †$P<0.05$ vs Aldo.
Comparative Effects Between Aldosterone and Gal-3 on Proinflammatory and Profibrotic Markers in Human Cardiac Fibroblasts In Vitro

Treatment with aldosterone (10^{-8} \text{ mol/L}) was able to increase intracellular Gal-3 protein expression (1.5-fold; P<0.05) at 24 hours (Figure 4A). The presence of the MR antagonist spironolactone was able to prevent the increase in Gal-3 protein levels induced by aldosterone (Figure 4A) in human cardiac fibroblasts.

We next compared the effects of aldosterone and Gal-3 on inflammatory markers and ECM production in human cardiac fibroblasts. Cells treated with aldosterone presented an increase in the secretion of proinflammatory markers such as IL-6 (2.0-fold; P<0.01) and CCL2 (1.4-fold; P<0.05; Figure 4B). Gal-3–treated cells enhanced the secretion of IL-1β (1.9-fold; P<0.01), IL-6 (2.2-fold; P<0.001), and CCL2 (1.7-fold; P<0.01; Figure 4B). Complementary, aldosterone was able to increase ECM components such as collagen type I (2.1-fold; P<0.01), collagen type III (2.3-fold; P<0.01), and fibronectin (1.5-fold; P<0.05) but not transforming growth factor-β (Figure 4C). Gal-3–treated cells increased similarly collagen type I (1.9-fold; P<0.01), collagen type III (4.0-fold; P<0.001), and fibronectin (2.0-fold; P<0.01) protein levels (Figure 4C). Furthermore, aldosterone induced an increase in MMP-1 (1.6-fold; P<0.05), MMP-2 (1.5-fold; P<0.01), and MMP-9 (1.4-fold; P<0.05) activities (Figure 4D). Gal-3 induced similar
increases in MMP-1 (1.7-fold, \( P<0.01 \)), MMP-2 (1.4-fold; \( P<0.05 \)), and MMP-9 (1.6-fold; \( P<0.01 \)) activities (Figure 4D).

**Gal-3 Inhibition Blocked Aldosterone Prolinflammatory and Profibrotic Effects in Human Cardiac Fibroblasts In Vitro**

To investigate the role of Gal-3 in aldosterone effects in human cardiac fibroblasts, Gal-3 silencing was used. Gal-3 siRNA reduced significantly Gal-3 mRNA (0.9-fold; \( P<0.001 \)) and protein expressions (0.5-fold; \( P<0.05 \)) when compared with controls (Figure 5A). Aldosterone treatment did not modify Gal-3 levels in Gal-3–knocked down cells (Figure 5A). In Gal-3–silenced cells, aldosterone did not increase CCL2 and osteopontin at mRNA levels and IL-6 and CCL2 at protein levels (Figure 5B). Moreover, aldosterone did not modify collagen type I and collagen type III mRNA and protein levels, as well as fibronectin protein levels (Figure 5C) in Gal-3–knocked down cells. Furthermore, Gal-3 inhibition was able to block the increase in MMP-1 and MMP-2 activities induced by aldosterone (Figure 5D).

**Discussion**

The purpose of this study was to investigate the role of Gal-3 in cardiac inflammation and fibrosis and its role as a mediator of aldosterone-induced cardiac damage in vitro and in vivo. In animal models, Gal-3 pharmacological inhibition prevented cardiac inflammation and fibrosis associated with an excess of aldosterone levels independently of BP levels. In human cardiac fibroblasts, aldosterone increased Gal-3 expression via MR. Gal-3 enhanced the production and secretion of proinflammatory and profibrotic mediators. Moreover, Gal-3 inhibition blocked aldosterone-induced proinflammatory and profibrotic molecules. Thus, Gal-3 emerges as a key mediator of inflammatory response and cardiac fibrosis associated with high-aldosterone situations.

Previous studies by our group have demonstrated that Gal-3 inhibition prevented cardiac fibrosis induced by aldosterone.\(^{20}\) Similar beneficial effects of Gal-3 inhibition have been reported on cardiac fibrosis, remodeling, and dysfunction in angiotensin II–treated animals independently of BP levels.\(^{21}\) In aldosterone-salt–treated rats, Gal-3 inhibition with MCP blocked hypertension, normalizing BP levels.\(^{20}\) Independently of hypertension, aldosterone per se has been demonstrated to promote cardiac inflammation and fibrosis, characterized by perivascular mononuclear infiltrate cells and increased adhesion markers with chemokine and cytokine expression as it has been reported.\(^{24}\) The pharmacological blockade of Gal-3 and genetic disruption of Gal-3 were able to prevent the increase in inflammatory markers and in MMP activities induced by aldosterone, suggesting that Gal-3 is a novel mediator of cardiac inflammation. This effect of Gal-3 on aldosterone actions was confirmed in human cardiac fibroblasts where aldosterone and Gal-3 showed proinflammatory and profibrotic properties in cardiac fibroblasts, increasing inflammatory markers, MMP activities, and ECM components, as it has been previously published in other cell types.\(^{19,25}\) Moreover, in cardiac fibroblasts, Gal-3 silencing was able to prevent the increase in inflammatory and fibrotic markers induced by aldosterone.

SHR is characterized by structural and functional changes in the heart,\(^{26}\) such as cardiac hypertrophy that is accompanied by an inflammatory process.\(^{27,28}\) However, the role of aldosterone excess in the inflammatory and fibrotic responses in this model remains poorly understood. Aldosterone and Gal-3 levels, as well as cardiac inflammation and fibrosis, were increased in SHR when compared with normotensive controls. The increase in cardiac Gal-3 levels in this experimental hypertension was also observed in a double transgenic animal model of cardiac hyperaldosteronism and systemic hypertension.\(^{21}\) Gal-3 pharmacological inhibition did not affect hypertension in SHR because MCP-treated rats present similar BP levels compared with nontreated SHR. However, under hypertensive conditions, Gal-3 blockade exerted beneficial effects, diminishing cardiac inflammation and fibrosis. Thus, these results show the key role of Gal-3 in the cardiac remodeling associated with hypertension and the beneficial effects of Gal-3 pharmacological inhibition on cardiac inflammation and fibrosis, which finally could affect cardiac function and heart failure development.

In summary, Gal-3 emerges as a new molecule for regulating cardiac inflammation and fibrosis progression associated with high-aldosterone levels. This study demonstrates the beneficial effects of Gal-3 blockade in situations with high-aldosterone levels, such as hypertension, representing a novel therapeutic target in cardiac inflammation and fibrosis.

**Perspectives**

Gal-3 inhibition blocks cardiac inflammation and fibrosis in experimental hyperaldosteronism independently of the presence of high-BP levels. More in-depth mechanistic studies would be needed to understand the mechanisms by which Gal-3 inhibition blocks aldosterone hypertensive effects. Further clinical studies are required to establish the potential therapeutic benefit of Gal-3 inhibition in hypertensive patients.

**Acknowledgments**

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**Disclosures**

None.

**References**


Galectin-3 Blockade in Inflammation and Fibrosis

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13. Martínez-Martínez et al

14. What Is New?

- Galectin-3 (Gal-3) mediates aldosterone-induced cardiac inflammation and fibrosis.
- Gal-3 blockade protects against cardiac inflammation and fibrosis in hypertension.
- Gal-3 could be one mechanistic connection between cardiac inflammation and fibrosis.

15. What Is Relevant?

- Gal-3 is a new biomarker, recommended for the management of heart failure (American Heart Association 2013 Guidelines), reflecting disease severity and prognosis.


Novelty and Significance

- This study provides a new pharmacological agent that attenuates cardiac inflammation and fibrosis in situations with high-aldosterone levels such as hypertension.

Summary

- Gal-3 blockade inhibits cardiac inflammation and fibrosis in hypertensive rats. Genetic disruption and pharmacological inhibition of galectin-3 effectively block cardiac inflammation and fibrosis induced by aldosterone.
Galectin-3 Blockade Inhibits Cardiac Inflammation and Fibrosis in Experimental Hyperaldosteronism and Hypertension

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GALECTIN-3 BLOCKADE INHIBITS CARDIAC INFLAMMATION AND FIBROSIS IN EXPERIMENTAL HYPERALDOSTERONISM AND HYPERTENSION

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This file includes:

1 supplemental method
3 supplemental references
2 supplemental tables
SUPPLEMENTAL METHODS

Animals
Adult male Wistar rats were treated for 3 weeks with vehicle (n =10), Aldo-salt (Sigma, 1 mg/kg/day diluted in sunflower oil and administered by subcutaneous injection and 1% NaCl as drinking water, n =10), Aldo-salt plus spironolactone (Sigma, 200 mg/kg/day, n=10), Aldo-salt plus Gal-3 activity inhibitor, modified citrus pectin (MCP; Enconugenics, 100 mg/kg/day, n=9), spironolactone (n=7), or MCP (n=5) alone. Normotensive male Wystar-Kyoto (WKY; n=8) and spontaneously hypertensive rats (SHR; n=16) were obtained for Harlan Ibérica. Half of the animals of SHR group received the Gal-3 activity inhibitor, modified citrus pectin (MCP; 100 mg/kg/day) for 6 weeks in the drinking water. For the Gal-3 knockout (KO) model, adult male C57BJ6 WT mice and Gal-3 KO mice were infused with Aldo (1 mg/kg/day, osmotic minipump) or vehicle (n=7, each group) for 3 weeks.

The investigation was performed in accordance with the Guide for Care and Use of Laboratory Animals published by the U.S. National Institutes of Health (publication no. 82-23, revised in 1996). Body weight was measured once a week. Food and water intake were determined throughout the experimental period. Blood pressure (SBP) was estimated basally, at mid-study and end-of-study through use of a tail-cuff plethysmograph (Narco Bio-Systems) in unrestrained animals. In all experimental models, blood and urines were collected and haemodynamic parameters were evaluated.

Cell culture
Human Cardiac Fibroblasts were obtained from Promocell and maintained in medium Fibroblasts Media 3. Cells were cultured according to the manufacturer’s instructions. Cells were used between passages 5-7. Cells were stimulated with Aldosterone (10^{-8} M, Sigma), Gal-3 (10^{-8} M, R&D Systems) and Spironolactone (Spiro, 10^{-6} M, Sigma) for 6h for mRNA determinations and for 24h for protein analysis. The doses were chosen based on previous studies.

Real-time reverse transcription PCR
Total RNA was extracted with Trizol Reagent (Euromedex) and purified using the RNeasy kit, according to the manufacturer’s instructions (Qiagen). First strand cDNA was synthesized according to the manufacturer’s instructions (Roche). Quantitative PCR analysis was then performed with SYBR green PCR technology (ABGene) (Table S1 and S2). Relative quantification was achieved with MyiQ (Bio-Rad) software according to the manufacturer’s instructions. Data were normalized by HPRT and β-actin levels and expressed as percentage relative to controls. All PCRs were performed at least in triplicate for each experimental condition.

Western blot analysis
Aliquots of 20 μg of total proteins were prepared from either cell extracts or cardiac homogenates. Also, aliquots of culture media containing 25 μl of supernatant from HCF were electrophoresed on SDS polyacrylamide gels and transferred to Hybond-c Extra nitrocellulose membranes (Amersham Biosciences). Membranes were incubated with primary antibodies for: Gal-3 (Thermo Scientific, dilution 1/500), collagen type I (Santa Cruz; dilution 1:500), collagen type III (Santa Cruz; dilution 1:500), fibronectin (Santa Cruz; dilution 1:500), transforming growth factor-beta (TGF-β; Abcam, dilution 1:1000), connective tissue growth factor (CTGF; Torrey Pines Biolabs Inc., dilution
Chemokine Ligand 2 (CCL2; Santa Cruz; dilution 1/500), Osteopontin (OPN; Santa Cruz; dilution 1/500) and Tumor Necrosis Factor alpha (TNF-α; R&D Systems; dilution 1:500), and β-actin (Sigma; dilution 1:1000) as a loading control. After washing, detection was made through incubation with peroxidase-conjugated secondary antibody, and developed using an ECL chemiluminescence kit (Amersham). After densitometric analyses, optical density values were expressed as arbitrary units. Results are expressed as an n-fold increase over the values of the control group in densitometric arbitrary units. All Western Blots were performed at least in triplicate for each experimental condition.

**ELISA**

C-Reactive Protein (CRP), TNF-α, Interleukin-6 (IL-6), IL-1β and concentrations were measured in the supernatants of the cells or in plasma samples by ELISA according to the manufacturer's instructions (R&D Systems).

**Gelatin zymography**

Aliquots of culture media containing 25 μl of supernatant were resolved on a 10% SDS polyacrylamide gel containing 0.3 % gelatin. The gel was rinsed three times for 15 min with a solution of 2.5% Triton X 100 to remove SDS and renature the proteins, followed by incubation for 48 h at 37°C in 1000 mmol/l Tris-HCl, pH 7.5 with 1000 mmol/l CaCl₂ and 5000 mmol/l NaCl to promote degradation of gelatin. Gels were fixed in 40% methanol and 10% acetic acid, and then stained for 30 min in 0.25% Coomassie blue R-250 to identify proteolytic activity of metalloproteinases (MMPs).

**Transfection of cells with siRNA**

Cells were seeded into 6-well plates at 70% confluence and transfected with a pool of three siRNAs (GeneCust) Gal-3 target-specific and using MATra-si (IBA) according to the manufacturer's recommendations. Cells were allowed to recover for 24h before stimulation. Scramble siRNAs were used as a control.

**Immunohistological evaluation**

Paraffin-embedded adipose tissue sections (5 μm) were used. Slides were treated with H₂O₂ for 10 min to block peroxidase activity. All samples were blocked with 5% normal goat serum in PBS for 1 h and incubated for 1h with Gal-3 (Thermo Scientific; dilution 1/50), CCL2 (Santa Cruz; dilution 1/50), OPN (Santa Cruz; dilution 1/50), CD68 (Santa Cruz; dilution 1/50), Collagen type I (Santa Cruz; dilution 1/50), washed three times, and then incubated for 30 min with the horseradish peroxidase-labeled polymer conjugated to secondary antibodies (Dako Cytomation). The signal was revealed by using DAB Substrate Kit (BD Pharmingen). As negative controls, samples followed the same procedure described above but in the absence of primary antibodies.

**Statistical analyses**

Data are expressed as mean ± SD. Normality of distributions was verified by means of the Kolmogorov–Smirnov test. Data were analyzed using a one-way analysis of variance, followed by a Newman-Keuls to assess specific differences among groups or conditions using GraphPad Software Inc. The predetermined significance level was P < 0.05.
SUPPLEMENTAL REFERENCES


SUPPLEMENTAL TABLES

Table S1: Primers used in rats in real time PCR analysis

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