Mice Lacking Osteopontin Exhibit Increased Left Ventricular Dilation and Reduced Fibrosis After Aldosterone Infusion

Flora Sam, Zhonglin Xie, Henry Ooi, David L. Kerstetter, Wilson S. Colucci, Mahipal Singh, and Krishna Singh

Background: Osteopontin, also known as cytokine Eta-1, plays an important role in postmyocardial infarction remodeling by regulating collagen accumulation. Aldosterone promotes collagen synthesis and structural remodeling of the heart. The role of osteopontin in aldosterone-induced fibrosis and myocardial remodeling is unknown. Osteopontin expression and left ventricular structural and functional remodeling were determined in wild-type and osteopontin knockout mice after aldosterone infusion.

Methods and Results: Immunohistochemical analyses showed increased interstitial osteopontin protein in the wild-type left ventricle after 7 days of aldosterone infusion. After 4 weeks of aldosterone infusion, heart rate was unchanged, and there were similar increases in blood pressure (BP) and heart-to-body weight ratio in both wild-type and knockout mice. Left ventricular end-diastolic diameter was significantly higher, whereas percent fractional shortening was significantly lower ($P < .05$) in knockout versus wild-type mice after 4 weeks of aldosterone infusion. Aldosterone infusion increased fibrosis and apoptosis (TUNEL-positive) in both wild-type and knockout mice. However, the increase in the extent of fibrosis and apoptosis was significantly lower in knockout hearts.

Conclusions: Increased osteopontin plays an important role in the regulation of aldosterone-induced remodeling with effects on left ventricular dilation, fibrosis, and apoptosis. Am J Hypertens 2004;17:188–193 © 2004 American Journal of Hypertension, Ltd.

Key Words: Heart, aldosterone, osteopontin, fibrosis, apoptosis.

The dynamic synthesis and breakdown of extracellular matrix (ECM) is considered as an essential process during myocardial remodeling. The myocardial remodeling in hypertensive cardiac hypertrophy, after myocardial infarction (MI), and in dilated cardiomyopathy is associated with increased deposition of ECM proteins such as collagens. Cardiac fibrosis is considered as a major determinant of cardiac function.1–3 However, the mechanisms underlying the deposition of ECM in the heart are unclear. The levels of aldosterone are increased in the heart after MI.4 Aldosterone has been suggested to play a pivotal role in the development of myocardial fibrosis. Brilla and colleagues have demonstrated that aldosterone infusion promotes fibrosis in rat hearts.5 The effects of aldosterone on cardiac fibrosis were prevented by spironolactone, a nonspecific aldosterone receptor blocker.6 Importantly, spironolactone significantly reduced the risk of morbidity and death among patients with severe congestive heart failure (CHF) due to left ventricular systolic dysfunction in the Randomized Aldactone Evaluation Study (RALES).7 Serum markers of cardiac fibrosis were found to be increased in CHF patients. Spironolactone significantly reduced the levels of these markers.8

Osteopontin (OPN), a cell secreted adhesive glycoprotein with arginine-glycine-aspartic acid (RGD) cell-binding sequence, interacts with integrins (αvβ3, αvβ5, αvβ1) and CD44 receptors.9,10 OPN is a multifunctional protein.9,11 It has been shown to interact with fibronectin and collagen, suggesting a possible role in...
matrix organization or stability. Transgenic mice studies indicate disorganization of matrix and alterations in collagen fibrillogenesis in the absence of OPN. OPN is expressed in the heart. Previously, using spontaneously hypertensive and aortic-banded rats, we have shown that expression of OPN coincides with the development of heart failure. Recently, we demonstrated that OPN expression increases in the heart after MI and increased expression of OPN plays an important role in post-MI remodeling by promoting collagen synthesis and accumulation.

Aldosterone infusion has recently been shown to increase OPN expression in rat heart. However, the role of OPN in aldosterone-induced fibrosis and myocardial remodeling has not yet been studied. This study was undertaken to determine whether aldosterone infusion increases OPN in mouse hearts, and whether increased expression of OPN plays a role in aldosterone-induced myocardial remodeling with respect to physiologic function, fibrosis, and apoptosis.

Methods
Vertebrate Animals and Experimental Method

All experiments were performed in accordance with protocols approved by the Institutional Animal Care and Use Committee. The knockout (KO) and wild-type (WT) mice were of a 129 × black Swiss hybrid background. Uninephrectomized mice (body weight 27 to 30 g) received 0.15 μg/h d-aldosterone (Sigma, St. Louis, MO) through an Alzet mini-osmotic pump (Alza Corporation, Palo Alto, CA) and were kept on 1% NaCl in drinking water for 4 weeks. Sham animals underwent the same intervention except they received saline infusion instead of aldosterone. The dose of aldosterone was calculated from previous studies, where rats (body weight 160 to 180 g) received 0.75 μg/h of aldosterone.

Physiologic Measurements

Tail-cuff systolic blood pressure (BP) and heart rate (HR) were measured at baseline, 2 and 4 weeks of aldosterone infusion using a noninvasive tail-cuff system (BP-2000, Visitech, Apex, NC).

In nonsedated mice, left ventricular (LV) end-diastolic diameter (LVEDD), LV end-systolic diameter (LVESD), and percent fractional shortening (% FS) were measured by two-dimensional M-mode echocardiography using an Acuson Sequoia 256 and a 15-MHz transducer as previously described. The % FS was calculated as described.

Fibrosis and Immunohistochemistry

To measure fibrosis, trichrome-stained sections (5 μm) were visualized by light microscopy and the entire section was quantified using Bioquant Image analysis software (Memphis, TN). The OPN protein expression was studied using monoclonal anti-OPN antibodies (monoclonal antibody, AK2C5, provided by Aaron J. Kowalski and David T. Denhardt, Rutgers University, Piscataway, NJ) as described. The OPN protein staining was quantified using Bioquant Image analysis software.

TUNEL and Hoechst 33258 Staining

To detect apoptosis, terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) followed by Hoechst 33258 staining was carried out in 4-μm thick sections from LV apex, midcavity, and base as described. TUNEL-positive (green fluorescent) and total number (blue fluorescent) of nuclei per unit area was counted.

Statistical Analysis

Data are presented as mean ± SEM. The echocardiographic data was analyzed by repeated measures ANOVA. Comparisons between individual groups were made using two-tailed Student t test. A P value of < .05 was considered significant.

Results
Expression of OPN in Myocardium After Aldosterone Infusion

Immunohistochemical analysis demonstrated low basal staining for OPN in WT-sham hearts (Fig. 1A). There was increased immunoreactivity for OPN in the hearts after 7 days of aldosterone infusion (Fig. 1B). Quantitative image analysis indicated a 1.9 ± 0.1 fold increase in OPN protein staining (n = 3; P < .05 as compared to sham). Most of the increased staining was observed in the interstitium.
### Systolic BP, HR, and Body and Heart Weights

Sham-KO (n = 3) and sham-WT (n = 3) exhibited no differences in the parameters studied at any time point, thus were grouped. Four weeks of aldosterone infusion significantly increased systolic BP in both WT and KO mice as compared to sham (sham, 121 ± 6 mm Hg; n = 6; WT, 159 ± 11 mm Hg [P < .05 v sham], n = 8; KO, 155 ± 8 mm Hg [P < .05 v sham], n = 6; Table 1) with no significant differences between the two aldosterone-infused groups. There were no significant differences in HR (sham, 585 ± 6 beats/min; WT, 611 ± 9 beats/min; KO 591 ± 26 beats/min; P = not significant) and body weight (sham, 24 ± 0.9 g; WT, 27 ± 1.5 g; KO, 28 ± 1.3 g; P = not significant) between the sham and aldosterone-infused mice. Four weeks of aldosterone infusion caused similar increases in total heart weight-to-body weight (HW/BW) ratios (sham, 5 ± 0.8 mg/g; WT, 8.1 ± 0.4 mg/g [P < .05]; KO, 7.7 ± 0.5 mg/g [P < .05]) with no significant difference between the two aldosterone-infused groups. There was a trend toward increased wet/dry lung and liver ratio; however, the data were not found significant.

### Echocardiographic Measurements

At baseline, there was no difference in LVESD, LVEDD, and % FS among the sham, WT, and KO groups (data not shown). This lack of difference persisted at 2 weeks. At 4 weeks of infusion, aldosterone did not affect LVEDD in the WT group. However, LVEDD was significantly increased in the KO group (P < .05 v sham and WT; Fig. 2 and Table 1). Baseline % FS was not different among the three groups. However, there was a significant reduction in the KO group after 4 weeks of aldosterone infusion (P < .05 v sham and WT). Septal wall thickness was significantly increased in both aldosterone-infused groups as compared to sham. However, the values were not significantly different between the two aldosterone-infused groups.

### Myocardial Fibrosis

Quantitative analysis of trichrome-stained sections indicated increased fibrosis in both aldosterone-infused (4 weeks) groups as compared to sham. However, the increased fibrosis was significantly lower in the KO group as compared to WT (sham, 1.6% ± 0.2%; WT, 33% ± 2.3% [P < .05 v sham]; KO, 14.6% ± 5.8% [P < .05 v sham, P = .04 v WT]; Fig. 3 A–C).

### Apoptosis

Aldosterone infusion (4 weeks) increased cardiac cell apoptosis as observed by TUNEL staining in both WT and KO hearts. The magnitude of increase in apoptosis was significantly lower in the KO hearts (sham, 3 ± 0.7 apoptotic cells/10⁵ nuclei; WT, 65 ± 19 apoptotic cells/10⁵ nuclei [P < .05 v sham]; KO, 13.8 ± 4 apoptotic cells/10⁵ nuclei [P < .05 v sham, P < .05 v WT]; Fig. 3D).

**Table 1.** Morphometric and echocardiographic measurements after 4 weeks of aldosterone infusion

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Sham</th>
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<th>KO</th>
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<tbody>
<tr>
<td>n</td>
<td>6</td>
<td>6</td>
<td>8</td>
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<tr>
<td>Body weight (g)</td>
<td>24 ± 0.9</td>
<td>28 ± 1.3</td>
<td>27 ± 1.5</td>
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<tr>
<td>Heart rate (beats/min)</td>
<td>585 ± 6</td>
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<td>611 ± 9</td>
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<td>Systolic BP (mm Hg)</td>
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<td>EDD (cm)</td>
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<td>0.25 ± 0.01</td>
<td>0.30 ± 0.01*†</td>
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<tr>
<td>ESD (cm)</td>
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<td>0.12 ± 0.004</td>
<td>0.18 ± 0.01*†</td>
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<tr>
<td>% FS</td>
<td>54 ± 5.8</td>
<td>57 ± 2</td>
<td>40 ± 4.4*†</td>
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<tr>
<td>Septal wall thickness (mm)</td>
<td>0.74 ± 0.12</td>
<td>1.23 ± 0.09*</td>
<td>1.29 ± 0.13*</td>
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Data are expressed as mean ± SEM.  
EDD = end-diastolic diameter; ESD = end-systolic diameter; % FS = percent fractional shortening.  
* P < .05 v sham; † P < .05 v WT.

**FIG. 2.** Echocardiographic images obtained from wild-type (A) and knockout (B) hearts after 4 weeks of aldosterone infusion. EDD = end-diastolic diameter.
Discussion

The major new finding of this study is that the mice lacking OPN exhibit LV dilation with reduced % FS after aldosterone infusion (compared to WT mice). Aldosterone infusion increases fibrosis and apoptosis in both WT and KO mouse hearts; however, the increase in fibrosis and apoptosis is significantly lower than in the KO group.

Previously, we observed increased expression of OPN in the heart during transition from hypertrophy to failure in spontaneously hypertensive and aortic banded rats18 and in mice after MI. 14 Here, we show increased OPN expression in mouse hearts after aldosterone infusion, predominantly in the interstitial cells. The interstitial localization of OPN is consistent with our previous findings. 14,18 Recently, Rocha and colleagues 19 observed increased expression of OPN in rat hearts, mainly in the areas of perivascular space and myocardial necrosis, in a model of aldosterone-induced myocardial remodeling. Eplerenone, a selective aldosterone inhibitor, inhibited aldosterone-induced OPN expression in the heart. 19 Our findings and those of Rocha and colleagues demonstrate that aldosterone increases OPN expression in the heart.

The effects of aldosterone on hypertension and myocardial hypertrophy, fibrosis, and necrosis are established in rats. 23,24 Aldosterone has recently been shown to increase myocyte apoptosis in adult rat hearts. 25 Consistent with these findings, we observed that 4 weeks of aldosterone infusion induces hypertension (evidenced by increased systolic BP), and myocardial hypertrophy (evidenced by increased heart weight-to-body weight ratio and septal wall thickness) with increased fibrosis and cardiac cell apoptosis in mice. Aldosterone infusion increases hypertrophy to a similar degree in both KO and WT hearts as seen by increased HW/BW ratio and increased septal wall thickness. Interestingly, increased LV chamber diameter and reduced contractile function were observed only in KO hearts, suggesting a mismatch in cardiac hypertrophy and chamber dilation. The reasons for the mismatch are not yet clear. However, we observed increased LV dilation and reduced collagen deposition in the OPN KO hearts as compared to WT after MI. 14 An important aspect of aldosterone infusion is the increased fibrosis in the heart. The presence of focal fibrosis in the mouse hearts indicates necrosis of cardiac myocytes. OPN has been suggested to regulate the synthesis or turnover of ECM proteins, including collagen. 15,16,26 In this study, we observed increased fibrosis in both WT and KO groups after aldosterone infusion. However, the increase in fibrosis is significantly lower in the KO group. The reduced fibrosis in the KO group raises the possibility of side-to-side slippage of cardiac myocytes in the absence of appropriate increase in fibrosis leading to increased LV dilation.

Osteopontin has been shown to play pro- and anti-apoptotic roles in different cell types. In renal epithelial cells, neutralizing anti-OPN antibody markedly increased apoptosis. 16 OPN KO mice exhibit increased apoptosis in postischemic acute renal failure. 27 In contrast to these studies, Yumoto et al. 28 observed significant reduction in chondrocyte apoptosis in OPN deficient mice in a model of rheumatoid arthritis. In this study, we observed reduced cardiac cell apoptosis in the absence of OPN. Previously, using MI as a model of myocardial remodeling, we observed a trend toward reduced cardiac myocyte apoptosis in OPN KO hearts versus WT. 14 OPN interacts with αvβ3, αvβ5, and αvβ1 integrins, and CD44 family of receptors. 10 Thus, the differential effects of OPN on apoptosis may reflect differences in the kinetics of coupling to dif-
different OPN receptors in different cell types. Of note, spironolactone has been shown to increase β3 integrins in kidney and heart muscle cells.29

The data presented here indicate that increased OPN expression after aldosterone infusion protects against LV dilation, possibly by promoting fibrosis, and thus, plays an important role in the regulation of aldosterone-induced myocardial remodeling. However, the trigger for increased OPN expression in the heart requires further investigation. We have shown that angiotensin II (Ang II) increases OPN expression in cardiac fibroblasts and microvascular endothelial cells.30,31 The observations that high-dose losartan prevents aldosterone-induced cardiac fibrosis, and aldosterone treatment increases AT1 receptor mRNA32 suggest that AT1 receptor may be a target for aldosterone. However, the possibility that aldosterone may directly affect OPN expression cannot be ruled out.

The aldosterone infusion increases LV dilation with reduced fibrosis and apoptosis in mice lacking OPN. After cardiac myocyte death, fibrillar collagen replaces the lost cells. Therefore, it is possible that reduced fibrosis in OPN KO hearts may be, at least in part, due to reduced apoptosis. However, it should be emphasized that our data of fibrosis and apoptosis are obtained after 4 weeks of aldosterone infusion. A thorough time course analysis of components of fibrosis including collagens and matrix metalloproteinases may be necessary to obtain further insights into the regulation of fibrosis. Furthermore, the relationship between cardiac fibrosis and LV dilation may not be simple, as spironolactone reduces fibrosis and improves survival.11,12 An approximate increase in fibrosis may be an important compensatory response during myocardial remodeling.

Acknowledgments
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References