Xanthine Oxidoreductase Inhibition Causes Reverse Remodeling in Rats With Dilated Cardiomyopathy

Khalid M. Minhas,* Roberto M. Saraiva,* Karl H. Schuleri, Stephanie Lehrke, Meizi Zheng, Anastasios P. Saliaris, Cristine E. Berry, Konrad M. Vandegaer, Dechun Li, Joshua M. Hare

Abstract—Increased reactive oxygen species (ROS) generation is implicated in cardiac remodeling in heart failure (HF). As xanthine oxidoreductase (XOR) is 1 of the major sources of ROS, we tested whether XOR inhibition could improve cardiac performance and induce reverse remodeling in a model of established HF, the spontaneously hypertensive/HF (SHHF) rat. We randomized Wistar Kyoto (WKY, controls, 18 to 21 months) and SHHF (19 to 21 months) rats to oxypurinol (1 mmol/L; n = 4 and n = 15, respectively) or placebo (n = 3 and n = 10, respectively) orally for 4 weeks. At baseline, SHHF rats had decreased fractional shortening (FS) (31 ± 3% versus 67 ± 3% in WKY, P < 0.0001) and increased left-ventricular (LV) end-diastolic dimension (9.7 ± 0.2 mm versus 7.0 ± 0.4 mm in WKY, P < 0.0001). Whereas placebo and oxypurinol did not change cardiac architecture in WKY, oxypurinol attenuated decreased FS and elevated LV end-diastolic dimension, LV end-systolic dimension, and LV mass in SHHF. Increased myocyte width in SHHF was reduced by oxypurinol. Additionally, fetal gene activation, altered calcium cycling proteins, and upregulated phospho–extracellular signal–regulated kinase were restored toward normal by oxypurinol (P < 0.05 versus placebo-SHHF). Importantly, SHHF rats exhibited increased XOR mRNA expression and activity, and oxypurinol treatment reduced XOR activity and superoxide production toward normal, but not expression. On the other hand, NADPH oxidase activity remained unchanged, despite elevated subunit protein abundance in treated and untreated SHHF rats. Together these data demonstrate that chronic XOR inhibition restores cardiac structure and function and offsets alterations in fetal gene expression/Ca2+ handling pathways, supporting the idea that inhibiting XOR-derived oxidative stress substantially improves the HF phenotype. (Circ Res. 2006;98:271-279.)

Key Words: xanthine oxidoreductase ■ remodeling ■ gene expression ■ heart failure

Emerging data implicates oxidative stress (OS) in heart failure (HF) pathophysiology, contributing to cardiac remodeling, mechanoenetic uncoupling, and depressed myofilament calcium sensitivity. The major enzymatic sources of reactive oxygen species (ROS) in HF are xanthine oxidoreductase (XOR) and nicotinamide adenine dinucleotide 2'-phosphate (NADPH) oxidase. Several studies demonstrate XOR upregulation in animal models and in human dilated cardiomyopathy. Functionally, XOR inhibition (XOI) acutely enhances myocardial mechanical efficiency in both animals and humans with HF. However, whereas NADPH oxidase is implicated in α1-adrenoreceptor stimulated hypertrophic signaling and contributes to OS in reperfused hearts, playing a major role in post–myocardial infarction (MI) microvascular obstruction (“no-reflow” phenomenon) and, like XOR, is increased in human HF, the relative contribution of XOR and NADPH oxidase to HF pathophysiology requires further clarification.

A recent series of studies has begun to examine the role of XOR in the cardiac remodeling process. These data contribute to the growing argument implicating XOR as a key source of ROS in evolving HF. Whether inhibition of XOR can elicit reverse remodeling in established dilated cardiomyopathy remains unknown.

Here we tested the hypothesis that cardiac XOR adversely affects cardiac remodeling in established cardiomyopathy in spontaneously hypertensive/HF (SHHF) rats. We show that chronic XOI reverses maladaptive cardiac remodeling through effects on cardiac structure, function, and fetal gene activation in SHHF rats, and that this process occurs independently of NADPH oxidase.

Original received July 24, 2005; revision received November 3, 2005; accepted December 5, 2005.

From the Department of Medicine, Cardiology Division and Institute for Cell Engineering (K.M.M., R.M.S., K.H.S., S.L., M.Z., A.P.S., C.E.B., K.M.V., J.M.H.), and Department of Anesthesiology and Critical Care Medicine (D.L.), Johns Hopkins Medical Institutions, Baltimore, Md; and Department of Medicine (R.M.S.), Cardiology Division, Federal University of Sao Paulo, Brazil.

This manuscript was sent to Joseph Loscalzo, Consulting Editor, for review by expert referees, editorial decision, and final disposition.

J.M.H. is a paid consultant for Cardiome Pharma Corp. The terms of this arrangement are being managed by the Johns Hopkins University in accordance with its conflict of interest policies.

*Both authors contributed equally to this study.

Correspondence to Joshua M. Hare, MD, The Johns Hopkins Medical Institutions, Cardiology Division, 733 Rutland Ave, Broadway Research Building 659, Baltimore, MD 21212. E-mail jhare@mail.jhmi.edu

© 2006 American Heart Association, Inc.

Circulation Research is available at http://circres.ahajournals.org

DOI: 10.1161/01.RES.0000200181.59551.71
Materials and Methods
An expanded Materials and Methods section is available in the online data supplement at http://circres.ahajournals.org.

Animals and Experimental Protocol
We studied SHHF (n=25) rats and their controls, Wistar Kyoto (WKY, n=8) rats (Charles River Laboratories Inc, Wilmington, Mass). The SHHF rat is a dilated cardiomyopathy model with hypertension progressing to HF. This model shares common phenotypic features with human HF including activated fetal gene program and elevated XOR activity. We treated both SHHF and WKY rats with the XOR inhibitor oxypurinol. SHHF and WKY rats were randomly assigned to placebo or treatment with oxypurinol for 4 weeks. Echocardiographic measurements were taken at baseline, 2 weeks, and at the end of the study. In vivo assessment of left-ventricular (LV) hemodynamics was performed at the end of treatment and animals were euthanized. The Institutional Animal Care and Use Committee of The Johns Hopkins University School of Medicine approved all protocols and experimental procedures.

Echocardiographic Measurements
Echocardiographic assessments were performed in WKY and SHHF anesthetized (1% to 2% isoflurane inhalation) rats using a Sonos 5500 Echocardiogram (Philips, Andover, Mass) equipped with a 15-MHz linear transducer. LV anterior wall thickness (AWT), posterior wall thickness (PWT), and end-diastolic (LVEDd) and end-systolic (LVESd) diameters were recorded from M-mode images using averaged measurements from 3 to 5 consecutive cardiac cycles.

LV Hemodynamics
Rats were anesthetized by intraperitoneal injection of ketamine (50 mg/kg) and acepromazine (2 mg/kg). A 2-F micromanometer tipped catheter (SPR-838, Millar Instruments, Houston, Tex) was inserted into the right carotid artery and retrogradely advanced into the left ventricle.

Histopathology
Excised hearts were processed using routine histological procedures. Five-micrometer sections were sliced and stained with hematoxylin/eosin (H&E). Myocyte width was measured at the level of the nucleus in longitudinally sectioned myocytes. All measurements were determined using NIH Image version 1.30v for Windows.

Measurement of mRNA Expression by Quantitative PCR
Fluorescence based real time quantitative PCR (qPCR) was used to determine the mRNA expression of the following genes: XOR, atrial natriuretic peptide (ANP), B-type natriuretic peptide (BNP), α-myosin heavy chain (α-MHC), β-MHC, and α-skeletal actin (α-SA).

TABLE 1. Baseline Echocardiographic Characteristics

<table>
<thead>
<tr>
<th></th>
<th>WKY (n=8)</th>
<th>SHHF (n=17)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, months</td>
<td>18–21</td>
<td>19–21</td>
</tr>
<tr>
<td>Weight, g</td>
<td>453±13</td>
<td>467±12</td>
</tr>
<tr>
<td>HR, beats/min</td>
<td>389±14</td>
<td>347±10†</td>
</tr>
<tr>
<td>LVM, mg</td>
<td>1006±144</td>
<td>2012±149*</td>
</tr>
<tr>
<td>FS, %</td>
<td>66.6±2.8</td>
<td>31.4±2.5*</td>
</tr>
<tr>
<td>LVESd, mm</td>
<td>7.03±0.38</td>
<td>9.73±0.17</td>
</tr>
<tr>
<td>LVEDd, mm</td>
<td>2.37±0.26</td>
<td>6.70±0.31†</td>
</tr>
<tr>
<td>AWT, mm</td>
<td>1.67±0.13</td>
<td>2.04±0.08†</td>
</tr>
<tr>
<td>PWT, mm</td>
<td>2.13±0.21</td>
<td>2.32±0.10</td>
</tr>
</tbody>
</table>

HR indicates heart rate. *P<0.0001, †P<0.05.

Measurement of XOR Activity
XOR activity was measured using the horseradish peroxidase-linked Amplex Red fluorescence assay (Molecular Probes, Invitrogen Detection Technologies), as described previously.

Oxidative Fluorescent Microtopography Using the Fluorescent Dihydroethidium Probe
Fresh, unfixed heart segments from WKY, WKY+oxypurinol, SHHF, and SHHF+oxypurinol rats were frozen and oxidative...
fluorescent microtopography was performed using the fluorescent dihydroethidium (DHE) probe, as described previously.21

**GSH/GSSG Ratio**

Determination of the reduced glutathione/glutathione disulfide (GSH/GSSG) ratio was performed by using the glutathione assay kit (Cayman chemical, Ann Arbor, Mich).

**Measurement of NAPDH Oxidase Activity**

NAPDH-dependent superoxide (O$_2^-$) production was measured in LV homogenates (mentioned in XOR activity) using lucigenin-enhanced chemiluminescence (β-NADPH 300 μmol/L; at room temperature) on a microplate luminometer (Veritas, Turner Biosystems, Sunnyvale, Calif).

**Western Blotting**

Whole heart proteins were prepared and Western blots analysis was performed as described.22 The blots were incubated with primary anti-p47$^{phox}$ antibody, anti-p22$^{phox}$ antibody, anti-p67$^{phox}$ antibody, anti-gp91$^{phox}$ antibody, anti–sarcoplasmic reticulum Ca$^{2+}$ ATPase (SERCA2a) antibody, anti–Na$^+$/Ca$^{2+}$ exchanger (NCX) antibody, anti-phospholamban (PLB) antibody, anti–extracellular signal-regulated kinase (ERK) antibody, or anti-pERK antibody.

**Statistical Analysis**

Data are reported as mean ± SEM. Statistical significance was determined by 1-way or 2-way ANOVA where appropriate, followed by Student–Newman–Keuls post hoc analysis (GraphPad, Instat, and STATA statistical software). The null hypothesis was rejected at $P<0.05$.

**Results**

**Reverse LV Remodeling**

Baseline echocardiography revealed that SHHF rats were characterized by decreased FS, with increased LV mass (LVM) and LV internal diameters, as compared with aged matched WKY controls (Table 1). The higher LVM present in SHHF rats was attributable to both increased LVEDd and AWT in comparison to WKY controls (Table 1).

At the end of 4 weeks of oxypurinol treatment, SHHF treated rats had improved FS (45.7% versus 27.5% in untreated SHHF, $P<0.01$), smaller LVEDd (9.7 ± 0.7 mm versus 11.6 ± 0.4 mm in untreated SHHF, $P<0.01$) and LVESd (5.6 ± 0.9 mm versus 8.5 ± 0.3 mm in untreated SHHF, $P<0.01$), and lower LVM (1790 ± 217 mg versus 2731 ± 225 mg in untreated SHHF, $P<0.01$) as compared with untreated SHHF rats (Figure 1A through 1D). Placebo-treated SHHF rats displayed progressive increases in LVEDd, LVESd, and LVM, with decreased FS. Echocardiographic parameters remained unchanged throughout the period of treatment (placebo or oxypurinol) in WKY.

In vivo hemodynamic analysis revealed increased LV volumes in untreated SHHF rats ($P<0.05$) in comparison to WKY that regressed toward normal after 4 weeks of oxypurinol treatment (Table 2). Ejection fraction was smaller ($P<0.05$) in comparison to untreated SHHF rats than in WKY and treated SHHF rats. Oxypurinol induced an increase in the slope of the end-systolic pressure-volume relation (Ees) ($P=0.01$; Table 2 and Figure 1E). There was no significant difference in LV end-systolic pressure or LV end-diastolic pressure across the groups (Table 2).

Reverse remodeling was demonstrated at the histological level as oxypurinol treatment regressed the cellular hypertrophy in SHHF rats. The cell width of cardiac myocytes from SHHF untreated rats (10.8 ± 0.3 μm, n = 35 cells, $P<0.05$) was higher than in both treated (9.8 ± 0.2 μm, n = 39) and untreated (9.4 ± 0.2 μm, n = 39) WKY rats. Oxypurinol treatment led to a significant regression in cardiac myocytes width in SHHF rats (9.8 ± 0.2 μm, n = 34, $P<0.05$) as compared with untreated SHHF rats (Figure 2). Additionally, the mitogen-activated protein kinase pathway was studied by

---

**TABLE 2. Hemodynamic Measurements**

<table>
<thead>
<tr>
<th></th>
<th>WKY (n=3)</th>
<th>WKY+O (n=3)</th>
<th>SHHF (n=4)</th>
<th>SHHF+O (n=5)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Preload</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LVEDP (mm Hg)</td>
<td>9.9 ± 2.5</td>
<td>7.0 ± 0.31</td>
<td>10.5 ± 0.8</td>
<td>9.8 ± 2.5</td>
</tr>
<tr>
<td>LVEDV (RVU)</td>
<td>8.4 ± 2.7</td>
<td>8.6 ± 1.6</td>
<td>25.8 ± 4.9†</td>
<td>9.5 ± 1.5†</td>
</tr>
<tr>
<td><strong>Afterload</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LVESP (mm Hg)</td>
<td>125.0 ± 17.5</td>
<td>89.2 ± 1.2</td>
<td>134.6 ± 14.7</td>
<td>123.9 ± 11.2</td>
</tr>
<tr>
<td>LVESV (RVU)</td>
<td>1.9 ± 1.2</td>
<td>0.9 ± 0.4</td>
<td>19.7 ± 4.9†</td>
<td>5.1 ± 1.4‡</td>
</tr>
<tr>
<td>Ea</td>
<td>18.9 ± 7.5</td>
<td>12.1 ± 1.8</td>
<td>21.8 ± 1.2 †</td>
<td>30.3 ± 4.1 †</td>
</tr>
<tr>
<td><strong>Contractility</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EF (%)</td>
<td>80.2 ± 6.1</td>
<td>90.2 ± 3.7</td>
<td>27.2 ± 6.5†</td>
<td>50.6 ± 9.0 †‡</td>
</tr>
<tr>
<td>dP/dt-IP (1/s)</td>
<td>118.3 ± 7.4</td>
<td>143.0 ± 16.9</td>
<td>81.7 ± 9.8 *</td>
<td>69.9 ± 3.7 *</td>
</tr>
<tr>
<td>Ea</td>
<td>11.7 ± 1.4</td>
<td>22.1 ± 2.7 †</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Diastolic function</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>dP/dt$_{max}$ (mm Hg/s)</td>
<td>−6883 ± 1308</td>
<td>−4092 ± 454</td>
<td>−6424 ± 1021</td>
<td>−5181 ± 469</td>
</tr>
<tr>
<td>$\tau$ (ms)</td>
<td>13.8 ± 0.4</td>
<td>11.0 ± 2.2</td>
<td>14.4 ± 0.9</td>
<td>15.0 ± 1.1</td>
</tr>
</tbody>
</table>

LVEDP indicates LV end-diastolic pressure; LVEDV, LV end-diastolic volume; LVESP, LV end-systolic pressure; LVESV, LV end-systolic volume; RVU, relative volume units; Ea, effective arterial elastance; Ees, slope of the end-systolic pressure-volume relation; EF, LV ejection fraction; dP/dt-IP, peak rate of rise in LV pressure divided by instantaneous developed pressure; dP/dt$_{max}$, peak rate of decline in LV pressure; $\tau$, time constant of isovolumetric relaxation. *$P<0.05$ in relation to WKY, †$P<0.05$ in relation to SHHF.
detected the protein abundance of total and phosphorylated ERK. The pERK/ERK ratio was upregulated in SHHF rats and restored toward normal in the oxypurinol-treated group (Figure 3).

**Ca^{2+}** Cycling Proteins

The depressed cardiac performance in SHHF rats was associated with changes in Ca^{2+} handling proteins. SERCA2a was downregulated (−0.68-fold), NCX (1.36-fold) was upregulated, and PLB (data not shown) was unchanged in SHHF relative to WKY untreated controls. In oxypurinol-treated SHHF rats, NCX was restored toward normal, whereas SERCA2a had partial restoration (Figure 3).

**Fetal Gene Program Activity**

Hypertrophied and failing hearts are characterized by altered expression of the prototypical members of the fetal gene program.\(^2^3\) We confirmed upregulated expression of ANP (7.3-fold, \(P<0.001\)), BNP (1.7-fold, \(P<0.001\)), β-MHC (2.4-fold, \(P<0.001\)), and α-SA (3.7-fold, \(P<0.001\)), with downregulation of α-MHC (4.4-fold, \(P<0.001\)), in the left ventricle of SHHF rats\(^1^8\) as compared with WKY (Figure 4). Oxypurinol treatment offset the changes in expression of these genes in SHHF rats, with a complete restoration being achieved for BNP and β-MHC. Oxypurinol did not change the expression of any of these genes in WKY rats (Figure 4).

**Oxidative Stress**

ROS production was higher in SHHF rats compared with WKY subgroups and oxypurinol treatment restored it toward normal. Oxidative fluorescent microtopography using the fluorescent probe DHE (orange staining), demonstrated elevated \(O_2^-\) production in SHHF cardiac myocytes as compared with controls (Figure 5A). Oxypurinol treatment reduced \(O_2^-\) production in SHHF, while having no effect in controls (Figure 5A). The increase in OS in SHHF rats was further characterized by the decrease in GSH/GSSG ratio \((P<0.05\); Figure 5B), an index of intracellular OS, in relation to controls; Oxypurinol treatment restored GSH/GSSG ratio in SHHF toward normal, demonstrating reduction in ROS production.

**Myocardial XOR Expression and Activity**

XOR mRNA was upregulated in SHHF compared with WKY, and oxypurinol treatment did not affect XOR gene expression (Figure 5C). This increased expression translated into increased XOR activity in SHHF (54.9±4.1 mU/μg) as compared with WKY (37.2±1.5 mU/μg, \(P<0.05\); Figure 5D). Oxypurinol treatment reduced XOR activity in SHHF toward normal (SHHF+oxypurinol 35.7±3.7 mU/μg, \(P<0.05\) versus SHHF and \(P=NS\) versus WKY) but did not affect XOR activity in WKY (WKY+oxypurinol 35.8±2.4 mU/μg, \(P=NS\) versus WKY).

**NADPH Oxidase Activity and Protein Abundance**

We measured NADPH oxidase activity to determine the relative contribution of NADPH oxidase to the increased \(O_2^-\) production in SHHF rats. NADPH oxidase activity was not increased in SHHF relative to WKY rats (63.2±8.01 light units/mg · min^{-1} in SHHF versus 56.2±10.44 light units/mg · min^{-1} in WKY rats, \(P=NS\); Figure 6A), and oxypurinol did not affect this activity in either group (52.4±4.70 light units/mg · min^{-1} in WKY+oxypurinol and 59.18±7.98 light units/mg · min^{-1} in SHHF+oxypurinol, \(P=NS\); Figure 6A). NADPH oxidase activity was inhibited by diphenyleneiodonium (DPI), but not by allopurinol or \(N^\prime\)-nitro-L-arginine methyl ester hydrochloride (l-NAME) in all 4 groups (Figure 6A). Interestingly, despite unchanged NADPH activity, cardiac protein abundance of NADPH oxidase subunits (Gp91phox and P67phox) were elevated in SHHF rats in relation to WKY, whereas P22phox and P47phox were unchanged (Figure 6B). Oxypurinol did not affect the abundance of these subunits.

**Discussion**

The major findings of this study are that chronic XOIs with accompanying reductions in OS favorably affects the natural history of HF, inducing reverse remodeling with restoration of cardiac structure and function while changing the altered patterns of Ca^{2+} cycling proteins and reversing alterations in...
gene expression in an established model of genetic cardiomyopathy. The beneficial effect of oxypurinol treatment was not secondary to a reduction in afterload between SHHF groups. Furthermore, SHHF rats exhibited increased XOR mRNA expression and activity but unchanged NADPH oxidase activity, and with oxypurinol treatment, XOR activity and O$_2^-$ production, but not expression, were reduced toward normal, whereas NADPH oxidase activity was unaltered, suggesting the improved HF phenotype was attributable to reduction in XOR-mediated OS.

There is accumulating data supporting a role for OS in HF, and previous studies have used ischemic$^2,6$ or pacing-induced HF$^4,9,14$ models to demonstrate the importance of OS in HF. In addition to the development of cardiac hypertrophy$^{23,24}$ and post-MI remodeling,$^2$ OS has been linked to abnormal excitation–contraction coupling,$^{25,26}$ myocyte apoptosis,$^{27,28}$ and β-adrenergic downregulation.$^9$ Recent studies in post-MI models$^{2,6,29}$ and in a troponin I–truncated mouse model$^{30}$ have reported that XOI attenuates LV remodeling and dysfunction,$^9$ reducing myocardial hypertrophy and interstitial fibrosis,$^2$ while improving excitation–contraction coupling, cardiac contractility, β-adrenergic regulation, and survival.$^6,31$ Additionally, using a canine model, our group recently reported improved contractility, reduced systemic vasoconstriction, and improved ventricular vascular coupling, but unaltered preload, by chronic XOI.$^{14}$ In the SHHF rats, oxypurinol also resulted in improved contractility, but, unlike in the pacing dog model,$^{14}$ LV volumes were reduced. This difference is likely attributable to differences in the models and the fact that the stimulus for cardiac injury was given in an ongoing basis in the previous canine study. Whether XOI can reverse established LV dysfunction in nonischemic dilated cardiomyopathy had heretofore remained unknown. Our observation of substantial reverse remodeling, resulting in better cardiac performance and architecture in treated versus untreated animals, offers unique insights into the mechanisms underlying hypertrophy and HF pathophysiology. Notably, the SHHF rat is a hypertensive model that evolves into a phase of frank LV dysfunction$^{15}$ and exhibits symptoms and biochemical changes that parallel those observed in patients with cardiomyopathy and congestive HF.$^{15,18}$

The pattern of NCX upregulation and SERCA2a downregulation observed in SHHF rats is also present in other HF experimental models$^{32–34}$ and in human HF.$^{35–37}$ Unchanged
PLB expression in SHHF is also observed in other HF models, such as post-MI and in human HF. Oxyipurinol restored NCX protein abundance toward normal and partially restored SERCA2a expression. Reduced SERCA2a protein abundance with partial restoration after treatment correlates with depressed systolic cardiac performance in SHHF rats that improves with oxyipurinol treatment. The reduced SERCA2a protein abundance could contribute to diastolic dysfunction, but the concomitant increase in NCX likely contributes to improved diastolic Ca\(^{2+}\) removal, as the relaxation time constant (\(\tau\)) in SHHF rats was close to controls (Table 2).

XOI is previously shown to improve myocardial energetics. In this regard, HF is associated with decrease of total creatine pool, \([pCr]/[pCr]/[ATP]\) ratio, and myocardial total creatine kinase (CK) activity and a fetal shift in CK isofrom expression, and HF treatment is accompanied by increase in total CK activity and partially restoration of CK isofrom expression. Furthermore, XOR reduces CK activity in vitro, and this effect is reversed by superoxide dismutase and XOI improve ventricular function while normalizing high-energy phosphate ratio in post-MI-induced HF in mice. Therefore, it is possible that improvement in myocardial energetics may be one of the mechanisms for XOI beneficial effects in SHHF rats.

Furthermore, our findings are in line with the known activation of the fetal gene program that occurs in HF. This program consists of a constellation of myocardial genes switched off shortly after birth but selectively reactivated in response to chronic hemodynamic overload, including ANP, BNP, \(\alpha\)-MHC, \(\beta\)-MHC, and \(\alpha\)-SA. Recent studies using gene therapy approaches targeting calcium cycling genes to alter protein transcription in failing hearts have shown promising results. However, this is the first study investigating the effects of XOI on OS in HF which demonstrates that administration of an orally active compound has effects on gene transcription preventing adverse remodeling while preserving cardiac function in genetically programmed cardiomyopathy. The influence of XOI on the fetal gene program is noteworthy and suggests a direct effect of ROS on the transcription of these genes, as angiotensin-converting enzyme inhibitors and aldosterone antagonists, which also attenuate the progression of myocardial remodeling in SHHF rats, do so without having the transcriptional effects we observed with XOI.

Finally, we evaluated the relative role of XOR and NADPH oxidase in OS in HF. Recently discovered interac-
tions between NADPH oxidase and XOR delineate crosstalk regarding ROS generation. NADPH oxidase may maintain endothelial XOR levels, playing a critical role in the conversion of xanthine dehydrogenase to XOR. Also, mice deficient in gp91phox, continue to exhibit NADPH-dependent O$_2^-$ generation and develop pressure overload–induced hypertrophy, suggesting alternative sources of ROS generation.

Our findings demonstrate that XOR is an important source of ROS generation in HF, with probably a more relevant role than NADPH oxidase, a contention supported by our physiological observations with regard to increased XOR activity and O$_2^-$ production, and unaffected NADPH activity in SHHF rats, despite increased NADPH oxidase subunit abundance. This hypothesis is further supported by the profound effects of XOI on reverse remodeling, Ca$^{2+}$ cycling protein abundance and fetal gene activation in rat myocardium, which occur with concomitant decreased XOR activity but maintained NADPH oxidase activity. However, the fact that we have not tested a NADPH oxidase inhibitor in vivo limits our conclusion.

The limitations of the present work include the fact that the LV diameters, mass, and FS measured by echocardiography did not reduce beyond baseline levels in SHHF treated animals. LV remodeling persisted in untreated animals but was halted in treated animals according to echocardiographic parameters. However, the observations that XOI-treated SHHF rats had LV volumes measured by conductance catheter and myocyte size similar to WKY controls does support...
a reverse remodeling effect. Additionally, it remains unclear whether the changes in either the activation of the fetal gene program or calcium cycling protein abundance preceded changes in LV structure and function.

In summary, we have demonstrated that chronic XOI with oxypurinol causes reverse LV remodeling, improves function, alters Ca\(^{2+}\) cycling protein abundance, and restores molecular markers of the fetal gene program toward normal in SHHF rats. Furthermore, we show that improved HF phenotype is attributable to XOR-mediated reduced OS and that the contribution of NADPH oxidase is relatively minimal. These data support the idea that XOR is a primary source of ROS generation in failing hearts and that its upregulation contributes to maladaptive cardiac hypertrophy, directly participating in the progression of LV failure.

Acknowledgments

This work was supported by the Donald W. Reynolds Foundation and NIH grants RO1 HL-65455 and RO1 AG-025017 (to J.M.H.).

References


Xanthine Oxidoreductase Inhibition Causes Reverse Remodeling in Rats With Dilated Cardiomyopathy

Khalid M. Minhas, Roberto M. Saraiva, Karl H. Schuleri, Stephanie Lehrke, Meizi Zheng, Anastasios P. Saliaris, Cristine E. Berry, Konrad M. Vandegaer, Dechun Li and Joshua M. Hare

Circ Res. 2006;98:271-279; originally published online December 15, 2005;
doi: 10.1161/01.RES.0000200181.59551.71

Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2005 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/98/2/271

An erratum has been published regarding this article. Please see the attached page for:
http://circres.ahajournals.org/content/98/9/e70.full.pdf

Data Supplement (unedited) at:
http://circres.ahajournals.org/content/suppl/2005/12/15/01.RES.0000200181.59551.71.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation Research can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Circulation Research is online at:
http://circres.ahajournals.org/subscriptions/
In an article by Minhas et al (Circ Res. 2006;98:271–279), “Xanthine Oxidoreductase Inhibition Causes Reverse Remodeling in Rats With Dilated Cardiomyopathy,” a collaborator who contributed significantly to the work was not listed in the authorship of the manuscript. In a petition to the journal, the authors stated their omission of Lili A. Barouch, MD, Assistant Professor of Medicine, Division of Cardiology, Johns Hopkins Hospital, Baltimore, Md.

The corrected authorship should read: Khalid M. Minhas, Roberto M. Saraiva, Karl H. Schuleri, Stephanie Lehrke, Meizi Zheng, Anastasios P. Saliaris, Cristine E. Berry, Lili A. Barouch, Konrad M. Vandegaer, Dechun Li, Joshua M. Hare.
Animals and Experimental Protocol

We studied SHHF (n=25, 19-21 months old, 467±12 g) rats and their controls, Wistar Kyoto (WKY, n=8, 18-21 months old, 453±13 g) rats (Charles River Laboratories, Inc. Wilmington, MA). The SHHF rat is a genetic model of dilated cardiomyopathy with hypertension progressing to HF. This model shares common phenotypic features with human HF, including activated renin angiotensin aldosterone system and elevated natriuretic peptides, elevated tumor necrosis factor α, increased left ventricle (LV) end-diastolic volume, decreased LV ejection fraction, activated fetal gene program, and elevated XOR activity. We treated both SHHF and WKY rats with the XOR inhibitor oxypurinol. SHHF and WKY rats were randomly assigned to placebo (n=10 and n=3, respectively) or treatment with oxypurinol (1 mM, n=15 and n=4, respectively) in drinking water, for four weeks. Echocardiographic measurements were taken at baseline, two weeks, and at the end of the study. In vivo assessment of LV hemodynamics was performed at the end of treatment. Animals were sacrificed and gross anatomical pictures were taken and the hearts were either fixed in paraformaldehyde 4% for histological studies or Animals were sacrificed and hearts were snap-frozen immediately for molecular studies. The Institutional Animal Care and Use Committee of The Johns Hopkins University School of Medicine approved all protocols and experimental procedures.

Echocardiographic Measurements

LV mass (LVM) was calculated as LVM (mg) = 1.055 x [(AWT + PWT + LVEDd)³ – (LVEDd)]. LV fractional shortening (FS) was calculated as FS (%) = (LVESd / LVEDd) x 100.

LV hemodynamics
Cardiac preload was indexed as the left ventricular end-diastolic volume (LVEDV) and pressure (LVEDP); cardiac afterload was evaluated as effective arterial elastance (Ea, ratio of LV end systolic pressure to stroke volume), the LV end-systolic volume (LVESV) and pressure (LVESP); and myocardial contractility was indexed by the LV ejection fraction (EF), the slope of the end-systolic pressure-volume relation (Ees), and the peak rate of rise in LV pressure (dP/dt$_{max}$) divided by instantaneous developed pressure (dP/dt-IP). Ventricular relaxation was measured as the time constant $\tau$ for isovolumetric relaxation and peak rate of decline in LV pressure (dP/dt$_{min}$). Volume calibration was achieved by bolus injection of 15 % saline at the end of the experiment.

**Measurement of XOR Activity**

XOR activity was measured using the horseradish peroxidase-linked Amplex Red fluorescence assay (Molecular Probes, Invitrogen Detection Technologies), as previously described$^8$. LV tissue was homogenized with a Polytron (3X15 s) in cell lysis buffer (Cell Signaling Tech., Beverly, MA). The homogenization buffer also contained 1 mM phenylmethyl sulfonyl fluoride (PMSF) and Protease Inhibitor Cocktail (Roche Applied Science, Indianapolis, IN). The homogenate was centrifuged at 4°C at 14,000 g for 30 min. The supernatants were recovered and protein concentrations were determined using BCA reagent (Pierce Biotechnology, Rockford, IL) and bovine serum as a standard. The LV homogenates was added to a working solution containing Amplex Red reagent (50 µM), xanthine (0.1 mM) and horseradish peroxidase type II (0.1 U/ml), and the H$_2$O$_2$ production was measured in the absence or presence of oxypurinol (1 mM) in order to subtract the background. The reactions were incubated at 37 °C for 30 minutes. Fluorescence readings were made in duplicate in a 96-well
plate at Ex/Em = 544/590 nm using 100-µl total volume per each well. XOR activity was calculated by using a standard curve and was normalized to the loaded amount of protein concentration.

**Measurement of mRNA Expression by Quantitative Polymerase Chain Reaction (qPCR)**

Fluorescence based real time qPCR was used to determine the mRNA expression of the following genes: XOR, atrial natriuretic peptide (ANP), B-type natriuretic peptide (BNP), α-myosin heavy chain (α−MHC), β-myosin heavy chain (β−MHC), and α-skeletal actin (αSA). The primer sets for these genes were chosen from the published cDNA sequences. Total LV RNA was isolated, cDNA was synthesized and each sample was run in duplicate on a GeneAmp 7900 Sequence Detection System (PE Applied Biosystems, Foster City, CA) and was analyzed using SDS 2.0 software (Applied Biosystems) as we have described.

**GSH/GSSG Ratio:**

Determination of the GSH/GSSG ratio was performed by using the glutathione assay kit (Cayman chemical, Ann Arbor, MI). Briefly, animals were sacrificed, the hearts harvested and perfused with cold PBS and snap frozen. The tissue was homogenized and the supernatant obtained after centrifugation (10,000 g, 15 min) was deproteinated following the instructions of the manufacturer of the kit. The deproteinated sample was divided in two aliquots: one for measurement of total GSH and the other for measurement of GSSG. The samples were assayed spectrophotometrically at 405 nm.
Measurement of NADPH Oxidase Activity

NADPH-dependent superoxide (O$_2^-$) production was measured in LV homogenates (mentioned in XO activity) using lucigenin-enhanced chemiluminescence (βNADPH 300 μM; at room temperature) on a microplate luminometer (Veritas, Turner Biosystems, Sunnyvale, CA).

A low lucigenin concentration (5 µM) was employed to minimize artifactual O$_2^-$ production due to redox cycling. Briefly, proteins were diluted in modified Krebs-HEPES buffer and βNADPH and lucigenin were added to wells just before reading. Chemiluminescence readings were expressed as integrated light units/mg protein/minute. Experiments were also performed in the presence of the flavoprotein inhibitor diphenyleneiodonium (DPI, 10 µM), or allopurinol (100µM) or the nitric oxide synthase inhibitor Nω-Nitro-L-arginine methyl ester hydrochloride (L-NAME, 100 µM).

Western-Blotting:

Whole heart proteins were prepared and western blots analysis was performed as described$^{10}$. The blots were incubated with primary anti-p47$^{phox}$ antibody (1:500, Upstate, Lake Placid, NY), anti-p22$^{phox}$ antibody (1:500, Santa Cruz Biotechnology, Santa Cruz, CA), anti-p67$^{phox}$ antibody, anti-gp91$^{phox}$ antibody (1:500, BD Transduction Laboratories, San Diego, CA), anti-sarcoplasmatic reticulum Ca$^{+2}$ ATPase (SERCA2) antibody (1:500, Affinity BioReagents, Golden, CO), anti-Na$^+/Ca^{+2}$ exchanger (NCX) antibody (1:500, Alpha Diagnostic Inc, San Antonio, TX), anti-phospholambam (PLB) antibody (1:1000, Affinity BioReagents), anti-extracellular signal-regulated kinase (ERK) antibody (1:1000, Cell Signaling Tech.), or anti-pERK antibody (1:1000, Cell Signaling Tech.). A monoclonal anti-GAPDH (glyceraldehyde-3-phosphate dehydrogenase) antibody (1:100000, Research Diagnostic Inc., Flanders, NJ) was used separately as a normalizer. Membranes were incubated for 1 hour with peroxidase-
conjugated chicken anti-rabbit or anti-mouse IgG antibodies (Santa Cruz Biotechnology) in 1:5000 dilutions. Bands were visualized by chemiluminescence (SuperSignal Substrate kit, Pierce, Rockford, IL) and quantified using the NIH Image software.

Reference List


calcium cycling: independent impact of neuronal and endothelial nitric oxide synthases. 