Uric Acid Promotes Left Ventricular Diastolic Dysfunction in Mice Fed a Western Diet

Guanghong Jia,* Javad Habibi,* Brian P. Bostick, Lixin Ma, Vincent G. DeMarco, Annya R. Aroor, Melvin R. Hayden, Adam T. Whaley-Connell,* and James R. Sowers*

Abstract—The rising obesity rates parallel increased consumption of a Western diet, high in fat and fructose, which is associated with increased uric acid. Population-based data support that elevated serum uric acids are associated with left ventricular hypertrophy and diastolic dysfunction. However, the mechanism by which excess uric acid promotes these maladaptive cardiac effects has not been explored. In assessing the role of Western diet–induced increases in uric acid, we hypothesized that reductions in uric acid would prevent Western diet–induced development of cardiomyocyte hypertrophy, cardiac stiffness, and impaired diastolic relaxation by reducing growth and profibrotic signaling pathways. Four-week-old C57BL/6J male mice were fed excess fat (46%) and fructose (17.5%) with or without allopurinol (125 mg/L), a xanthine oxidase inhibitor, for 16 weeks. The Western diet–induced increases in serum uric acid along with increases in cardiac tissue xanthine oxidase activity temporally related to increases in body weight, fat mass, and insulin resistance without changes in blood pressure. The Western diet induced cardiomyocyte hypertrophy, myocardial oxidative stress, interstitial fibrosis, and impaired diastolic relaxation. Further, the Western diet enhanced activation of the S6 kinase-1 growth pathway and the profibrotic transforming growth factor-β1/Smad2/3 signaling pathway and macrophage proinflammatory polarization. All results improved with allopurinol treatment, which lowered cardiac xanthine oxidase as well as serum uric acid levels. These findings support the notion that increased production of uric acid with intake of a Western diet promotes cardiomyocyte hypertrophy, inflammation, and oxidative stress that lead to myocardial fibrosis and associated impaired diastolic relaxation. (Hypertension. 2015;65:531-539. DOI: 10.1161/HYPERTENSIONAHA.114.04737.) ● Online Data Supplement

Key Words: cardiac remodeling ▪ inflammation ▪ obesity ▪ uric acid

It is increasingly recognized that consumption of a Western diet (WD) high in both fat and sugar promotes obesity and cardiovascular disease.1,2 The increase in sugar has largely been driven by increased intake of high-fructose corn syrup.3–5 An important component of sugar in WD is fructose, which makes up 50% of the content of sucrose and over half of the sugar content of high-fructose corn syrup. Fructose metabolism in the liver leads to increased synthesis of uric acid.6–9 This increase in serum levels of uric acid has been associated with instigating various components of the metabolic syndrome, including hypertension.4–6,8,9 However, telemetry studies in rodents10 and a systemic review and meta-analysis of clinical trials11 suggests that increased fructose intake and associated elevations in uric acid are not associated with elevations in blood pressure. Regardless, there is mounting evidence that elevated levels of uric acid are associated with increased cardiovascular disease risk.12–14 Emerging data suggests that fructose-induced rises in uric acid may promote hemodynamic abnormalities and heart failure via increases in inflammation, oxidative stress, endothelial dysfunction, and activation of the renin–angiotensin–aldosterone system.8,12–16

The cardiomyopathy that accompanies obesity and insulin resistance is characterized by both hypertrophy and impaired diastolic relaxation1 with left ventricular stiffness and impaired diastolic relaxation.17 The left ventricular stiffness and diastolic dysfunction associated with obesity-related cardiomyopathy is thought to be induced by interstitial fibrosis. However, little is known regarding the mechanisms linking interstitial fibrosis and diastolic dysfunction in obesity. In this context, there is evidence that the high fructose component of our diets promotes increased hepatic production of uric acid,
insulin resistance, inflammation, and an altered immune state that contribute to a proinflammatory state related to metabolic cardiomyopathy.18–20 Indeed, it has been reported that elevated serum levels of uric acid are associated with eccentric left ventricular hypertrophy and impaired left ventricular diastolic relaxation.17,21 However, the mechanisms by which elevated levels of uric acid contribute to the development of metabolic (obesity)-related cardiomyopathy is poorly understood.

We hypothesized that a WD would increase hepatic production of serum levels of uric acid that would in turn promote cardiomyocyte hypertrophy, inflammation, and oxidative stress and lead to myocardial tissue fibrosis and accompanying impairments in left ventricular diastolic relaxation. The corollary to this hypothesis was that targeting reductions in uric acid with xanthine oxidase inhibition would prevent development of cardiac fibrosis and diastolic dysfunction. Accordingly, in this investigation, we fed a WD high in fat and fructose to male C57BL/6J mice for 16 weeks with and without the xanthine oxidase inhibitor allopurinol to explore the role of elevated uric acid in the pathogenesis of WD-induced diastolic dysfunction.

Materials and Methods
Additional materials and methods are provided in the online-only Data Supplement.

Animals and Treatments
All animal procedures were performed in accordance with the Animal Use and Care Committee at the University of Missouri-Columbia, the Subcommittee for Animal Safety at the Truman VA, and National Institutes of Health Guide for the Care and Use of Laboratory Animals. C57BL/6J male mice were obtained from The Jackson Laboratory. Groups of 4-week-old male mice were fed a WD consisting of high fat (46%) and a high carbohydrate component as described previously.22,23 Venous blood samples were collected from a subset of fasted mice in each treatment group, and plasma was stored at −80°C for glucose and insulin assay and homeostatic model assessment of insulin resistance as previously described.17,21 Cardiac xanthine oxidase activity was determined in tissue protein supernatant using a xanthine oxidase assay kit (Abcam, Cambridge, MA).

Blood Pressure Measurements
Average systolic, diastolic, and mean arterial pressures were determined by catheterization of the right carotid artery under isoflurane anesthesia as previously described.21

In Vivo High-Resolution Cine-MRI
Noninvasive cine-MRI scans were performed on mice using a horizontal-bore 7 Tesla Bruker AVANCE III BioSpec MRI system (Bruker Corp, Billerica, MA) equipped with a 35 mm quadrature detection radiofrequency coil. Left ventricular morphology and functions were measured and analyzed using a similar method as established previously.22,23

Quantification of Myocardial Interstitial Fibrosis
Five micron sections of heart from all of the treatments were stained with picro-Sirius-red for evaluation of interstitial fibrosis. Slides were checked with a Nikon50i microscope, and 5 images were randomly captured from the left ventricle and right ventricle with a cool Snap-cap camera and auto leveled with Photoshop. Morphometric analysis was performed using MetaVue software. In each image, the areas of hot pink color and their intensities, which are representative of interstitial fibrosis, were quantified. Collagen 1 and 3 were evaluated using immunostaining with specific antibodies for each isoform.23

Quantification of Cardiomyocyte Hypertrophy
Five micrometer of paraffin-embedded heart sections were dewaxed, rehydrated with ethanol series and 4-(hydroxyethyl)-1-piperazinmethanesulfonic acid wash buffer, and stained with wheat Germ Agglutinin. Two images from different treatments were randomly captured from the left ventricle by using biphoton confocal microscope. On each image, the area of 10 cardiomyocytes were quantified by MetaVue and averaged. Average cardiomyocyte areas from different treatments were compared.

3-Nitrotyrosine
3-Nitrotyrosine was quantified as previously described.22,23

Ultrastructure Analysis With Transmission Electron Microscopy
Briefly, left ventricular tissues were cut into 2 mm squares and placed immediately in primary transmission electron microscopy fixative as previously described.22,23 Specimens were then placed in resin and polymerized at 60°C for 24 hours. Ultrathin sections (85 nm) were stained with 5% uranyl acetate and Sato’s Triple lead stain. A JOEL 1400-EX TEM (Joel, Tokyo, Japan) was used to view all samples.

Western Blot
Protein concentrations of cardiac tissue homogenates were measured as previously described.

RNA Isolation and Quantitative PCR
Total RNA was isolated using the TRizol reagent (Sigma) method as previously described.21 Real-time PCR was done using 8 μL cDNA, 10 μL SYBR green PCR master mix (Bio-Rad Laboratories), and forward and reverse primers (10 pmol/L/μL; Integrated DNA Technologies, San Diego, CA) using a real-time PCR system (CFX96; Bio-Rad Laboratories).

Gelatin Zymography for Matrix Metalloproteinase Activity
Tissue samples were extracted in 50 mmol/L Tris, 0.2% Triton X-100, 10 mmol/L CaCl2, pH 7.5, 100 μM phenylmethylsulfonyl fluoride, and a protein inhibitor cocktail (Sigma, St Louis, MO) by a method established in our laboratory.22,23

Statistical Analysis
Results are reported as the mean±SE. Differences in outcomes were determined using 2-way ANOVA or paired t tests and were considered significant when P<0.05. All statistical analyses were performed using Sigma Plot (version 12) software (Systat Software).

Results
Experimental Parameters
Compared with the control group, WD significantly promoted increases in fat mass (9.34±1.13 g), visceral fat weight (2.67±0.18 g), body weight normalized to tibia length (20.0±0.7 g/mm), and homeostatic model assessment of insulin resistance (5.49±0.58); however, there were no significant
improvements with allopurinol treatment (Table). Allopurinol treatment did reduce plasma uric acid, urine uric acid levels, and cardiac xanthine oxidase activity (Table). Of note, there were no significant changes in between groups for lean body weight or blood pressure (Table).

**Xanthine Oxidase Inhibition Improves Diastolic Relaxation**

Previous work from our laboratory and others support the notion that WD-induced obesity alters myocardial structure and function.17,23 In this regard, the earliest manifestation is that of an impairment in diastolic relaxation generally referred to as diastolic dysfunction. To determine the effect of xanthine oxidase inhibition on diastolic dysfunction, we performed cine-MRI in vivo. WD increased cardiac left ventricular diastolic relaxation time (34.81±1.95 ms versus 28.56±0.21 ms; P<0.05) and decreased left ventricular initial filling rate (0.28±0.06 μL/ms versus 0.37±0.03 μL/ms; P<0.05; Figure 1). Treatment with allopurinol normalized the initial filling rate (0.42±0.07 μL/ms; P<0.05) and diastolic

### Table. Effects of Allopurinol on Characteristics of Mice Fed a Western Diet

<table>
<thead>
<tr>
<th>Measures</th>
<th>CD</th>
<th>CD-Allo</th>
<th>WD</th>
<th>WD-Allo</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fat mass (g)</td>
<td>3.09±0.24 (7)</td>
<td>2.66±0.35 (7)</td>
<td>9.34±1.13 (8)*</td>
<td>10.37±1.32 (7)*</td>
</tr>
<tr>
<td>Lean mass (g)</td>
<td>23.04±0.25 (7)</td>
<td>23.11±0.31 (7)</td>
<td>23.07±0.69 (8)</td>
<td>24.21±0.30 (7)</td>
</tr>
<tr>
<td>Visceral fat weight (g)</td>
<td>0.82±0.06 (18)</td>
<td>0.76±0.06 (17)</td>
<td>2.67±0.18 (19)*</td>
<td>2.45±0.15 (17)*</td>
</tr>
<tr>
<td>Body weight/tibia length (g/mm)</td>
<td>15.1±0.3 (18)</td>
<td>14.3±0.2 (17)</td>
<td>20.0±0.7 (22)*</td>
<td>18.9±1.2 (17)*</td>
</tr>
<tr>
<td>Heart weight/tibia length (mg/mm)</td>
<td>62.5±1.5 (18)</td>
<td>57.9±1.0 (17)</td>
<td>69.9±2.1 (22)*</td>
<td>57.7±3.6 (17)*</td>
</tr>
<tr>
<td>HOME-IR (arbitrary units)</td>
<td>2.79±0.18 (9)</td>
<td>2.25±0.11 (6)</td>
<td>5.49±0.58 (9)*</td>
<td>5.09±1.44 (5)*</td>
</tr>
<tr>
<td>Plasma uric acid (mg/dL)</td>
<td>0.50±0.06 (15)</td>
<td>0.20±0.03 (8)*</td>
<td>0.68±0.07 (17)*</td>
<td>0.29±0.05 (8)*</td>
</tr>
<tr>
<td>Urine uric acid (mg/dL)</td>
<td>8.42±0.48 (3)</td>
<td>7.84±0.99 (4)</td>
<td>13.72±1.74 (5)*</td>
<td>10.60±0.70 (4)*</td>
</tr>
<tr>
<td>Cardiac xanthine oxidase activity (mU/mL)</td>
<td>0.061±0.008 (3)</td>
<td>0.040±0.013 (3)</td>
<td>0.312±0.016 (4)*</td>
<td>0.107±0.013 (3)*</td>
</tr>
<tr>
<td>Systolic BP (mm Hg)</td>
<td>99±5 (3)</td>
<td>101±4 (8)</td>
<td>110±13 (3)</td>
<td>101±4 (9)</td>
</tr>
<tr>
<td>Diastolic BP (mm Hg)</td>
<td>63±3 (3)</td>
<td>70±3 (8)</td>
<td>69±5 (3)</td>
<td>68±4 (9)</td>
</tr>
<tr>
<td>MAP (mm Hg)</td>
<td>75±4 (3)</td>
<td>80±3 (8)</td>
<td>83±7 (3)</td>
<td>79±4 (9)</td>
</tr>
<tr>
<td>Pulse pressure (mm Hg)</td>
<td>37±1 (3)</td>
<td>31±2 (8)</td>
<td>41±8 (3)</td>
<td>33±3 (9)</td>
</tr>
</tbody>
</table>

Values are mean±SE. (n) represents the number of group. BP indicates blood pressure; CD, control diet control; CD-Allo, control diet allopurinol; HOMA-IR, homeostatic model assessment of insulin resistance; WD, Western diet; and WD-Allo, Western diet allopurinol.

*P<0.05 compared with CD.
†P<0.05 compared with WD.

**Figure 1.** Western diet (WD)–induced cardiac diastolic dysfunction is prevented with xanthine oxidase inhibition. **A**. Representative midventricle short-axis cine-MRI images that correspond to end-diastole, end-systole, and early diastole phases of cardiac cycle from WD-fed mouse (WD, middle row) and WD-fed mouse treated with allopurinol (WD+Allo, lower row) compared with the control diet (CD)–fed mouse (upper row). Left ventricular diastolic relaxation time (B) and Left ventricular initial filling rate (C) derived from in vivo cine-MRI. *P<0.01 compared with CD; †P<0.05 compared with WD. CD-allo indicates control diet allopurinol; and WD-allo, Western diet allopurinol.
relaxation time (27.7±0.87 ms; \(P<0.05\)) that correlated with reduced circulating plasma uric acid (\(R^2=0.26\); \(P=0.01\); Table). These beneficial effects occurred in the absence of improvements in blood pressure, body weight, or insulin sensitivity (eg, homeostatic model assessment of insulin resistance). Of note, systolic function was unaffected by WD consumption or allopurinol treatment in WD-fed mice (see Table I in the online-only Data Supplement).

**Xanthine Oxidase Inhibition Attenuates Left Ventricular Hypertrophy Through S6 Kinase-1**

Diastolic dysfunction in obesity has been associated with left ventricular remodeling and hypertrophy initiated by growth kinases that occur independent of pressure-dependent responses on left ventricular concentric remodeling. Recent data highlight the importance of the nutrient sensing S6 kinase-1 (S6K1) in the development of cardiac hypertrophy. Intake of a WD had no effect on blood pressure, yet induced increases in heart weight and cardiomyocyte size compared with controls (Table; Figure 2). These pathological changes were related to increases in threonine phosphorylation of S6K1. Treatment with allopurinol had little effect on blood pressure, body weight, or homeostatic model assessment of insulin resistance, yet had a significant effect on reducing heart weight, cardiomyocyte size, and phosphorylation of S6K1.

**Xanthine Oxidase Inhibition Reduced Left Ventricular Interstitial Fibrosis and Tissue Remodeling in Concert With Reduced Transforming Growth Factor \(\beta_1/\text{Smad}2/3\) Signaling Cascade and Reductions in Matrix Metalloproteinase-9**

The structural abnormalities associated with impaired diastolic relaxation have been attributed to tissue remodeling and increased fibrosis that may, in part, be mediated by signaling through TGF-\(\beta_1\). To determine the improvements in cardiac diastolic relaxation related to allopurinol treatment, we evaluated myocardial fibrosis by Verhoeff-van Gieson staining for total collagen and immunostaining collagen 1 in mice fed a WD with or without allopurinol treatment. WD induced an increase in cardiac fibrosis that was temporally related to increases in TGF-\(\beta_1\), phosphorylation of SMAD2/3, and matrix metalloproteinase (MMP)-9 activity. Importantly, allopurinol treatment led to reductions in both interstitial fibrosis and collagen 1 that correlated with the alterations in initial filling rate on cine-MRI (\(R^2=0.20\), \(P=0.03\) and \(R^2=0.19\), \(P=0.03\); respectively). These findings also occurred in relation to the reductions in the TGF-\(\beta_1\), Smad2/3, and MMP-9 activity, suggesting that increased TGF-\(\beta_1/\text{Smad2/3}\) signaling cascade and MMP-9 activity were involved in the cardiac interstitial fibrosis and cardiac left ventricular remodeling induced by a WD (Figure 3).

**Xanthine Oxidase Inhibition Attenuated Myocardial Oxidative Stress and Macrophage M1/M2 Polarization**

The left ventricular tissue remodeling seen with obesity-related diastolic dysfunction has been associated with excess myocardial oxidative stress and inflammatory cytokines. The nitration of protein tyrosine residues can be evaluated by 3-nitrotyrosine immunostaining, which is an indirect marker of increased peroxynitrite formation and oxidative stress. Indeed, WD-induced reductions in initial filling rate was associated with an increase in myocardial 3-nitrotyrosine staining that was improved with allopurinol treatment (\(R^2=0.23\) and \(P=0.01\); Figure 4). In this context, the oxidant stress observed with WD occurred in concert with increases in M1 macrophage CD11b expression (Figure 4), whereas allopurinol treatment was associated with increases in M2 marker CD206, interleukin 10 expression, and the ratio of M2/M1 markers gene expression (Figure 4).
Xanthine Oxidase Inhibition Improved WD-Induced Myocardial Ultrastructural Abnormalities

Transmission electron emission analysis revealed WD-induced myocardial cellular remodeling, which consisted of excessive mitochondria accumulation with abnormally enlarged mitochondria with loss of mitochondrial matrix electron density, fragmentation, and loss of cristae in the intermyofibrillar, perinuclear, and subsarcolemma regions, which was largely corrected with allopurinol treatment. These abnormalities occurred in conjunction with sarcomere disorganization (Figure 5). WD-fed mice also displayed a decrease in endothelial transcytotic vesicle and lipid droplets and an increase in accumulation of lysosomes in subsarcolemma and perinuclear regions (not shown), which were corrected with allopurinol treatment.

Discussion

The main findings of this investigation were that consumption of a WD for 16 weeks resulted in elevated serum and urine uric acid, increases in myocardial xanthine oxidase activity and oxidative stress, increases in MMP-9, M1 macrophage polarization, fibrosis, and cardiomyocyte hypertrophy. These
abnormalities occurred in concert with impaired diastolic relaxation as determined by high resolution MRI. Importantly, pharmacological inhibition of xanthine oxidase activity with allopurinol prevented the development of WD-associated diastolic relaxation in conjunction with reduced plasma and urine uric acid and myocardial xanthine oxidase activity, oxidative stress, cardiac remodeling, and M1/2 macrophage polarization. Further, these improvements with xanthine oxidase inhibition occurred in the absence of any systemic effect on body weight, systemic insulin sensitivity, and limited effects on

Figure 4. Western diet (WD)-induced myocardial oxidative stress and M1 macrophage expression are ameliorated by xanthine oxidase inhibition. A, Representative images of left ventricular sections stained for 3-nitrotyrosine (NT), a marker of oxidant stress from accumulation of oxidant peroxynitrite (ONOO−). B, PCR expression of M1 macrophage CD11b expression. Allopurinol induced M2 macrophage marker IL10 and CD206 mRNA expression in WD-fed mice by using real-time PCR. C, Ratio of macrophage M2/M1 marker mRNA. *P<0.05 compared with control diet (CD); †P<0.05 compared with WD. CD-allo indicates control diet allopurinol; and WD-allo, Western diet allopurinol.

Figure 5. Ultrastructural observations of the myocardium using transmission electron microscopy (TEM). Note the excessive mitochondrial accumulation in the intermyofibrillar regions and the disorganization and thinning of sarcomeres in Western diet (WD) as compared with the control diet (CD) and allopurinol-treated controls (CD-Allo). Also, note the loss of lipid droplets (encircled) in the WD as compared with the CD and CD-Allo, which were restored with allopurinol treatment. Additionally, note that allopurinol treatment did not completely restore intermyofibrillar mitochondria to that of CD; however, allopurinol did partially restore sarcomeric disorganization and thinning. Magnification, ×600; scale bar, 2 μm. WD-allo indicates Western diet allopurinol.
blood pressure. The WD-associated activation of both growth (S6K) and profibrotic (TGF-β/Smad2/3) signaling pathways were prevented by allopurinol inhibition of WD-induced uric acid production. Taken together, these data suggest that increased production of uric acid associated with a WD plays an integral role in the development of obesity/metabolic cardiomyopathy. To our knowledge, this is the only preclinical data to support a link between hyperuricemia and cardiac diastolic dysfunction in rodents fed a WD.27,28 The WD used in this investigation reflects the increased carbohydrate and fat that is often consumed in Western cultures and thus is translationally relevant.

It has been proposed that the link between hyperuricemia and chronic heart failure might be mediated by an increase in oxidative stress and inflammation, in part, caused by elevated xanthine oxidase activity and consequently increased production of oxygen free radicals.29 Cardiac xanthine oxidase activation generates superoxide anions, which may contribute to the damage of cellular proteins and membranes and thereby induces cellular dysfunction or death through apoptosis and necrosis.30 Meanwhile, reactive oxygen species induced by xanthine oxidase reduce nitric oxide bioavailability, MMP activation, fibroblast proliferation and collagen synthesis.30 Furthermore, uric acid and xanthine oxidase activity have been associated with a proinflammatory state in human subjects and particularly with an increase in inflammatory markers during the development of cardiomyocyte hypertrophy.31 In epidemiological studies, elevated levels of serum uric acid have been linked to obesity, hypertension, insulin resistance, and left ventricular hypertrophy.21,32 For example, in an analysis of 3305 individuals leading to cardiac hypertrophy.17,35 In this regard, investigators have shown that there is increased activity of the progrowth serine kinase S6K1, increased inflammation and fibrosis associated with a WD and this increased inflammation and fibrosis may be driven by hyperuricemic-mediated activation of growth, as well as profibrotic signaling pathways. Indeed, the increases in uric acid observed in WD-fed mice were associated with increased activity of the progrowth serine kinase S6K1, which is a critical convergence point for the various signals leading to cardiac hypertrophy.17,35 In this regard, endogenous cardiac S6K1 activation has been reported to be significantly elevated in conjunction with pathological hypertrophy and left ventricular diastolic dysfunction.17,36

Cardiac remodeling associated with obesity is often characterized by increased cardiac fibrosis and stiffness. To this point, consumption of a WD resulted in the activation of the TGF-β1/Smad2/3 signaling pathway, which was prevented by inhibiting the production of uric acid via allopurinol administration. In this regard, TGF-β1 is a powerful initiator for the synthesis of collagens and other major extracellular matrix components in many organ systems.37 TGF-β1 regulates fibroblast proliferation and extracellular matrix production, particularly of collagen and fibronectin, although reducing degradation of these components through Smad2/3,38 MMP-9 activity, as determined by gelatin zymography, was increased in left ventricular tissue from mice fed a WD for 16 weeks, and this increase was mitigated by xanthine oxidase inhibition. MMP-9 is secreted by several cell types, including macrophages and fibroblasts.39 In this regard, investigators have shown that there is increased MMP-9 in cardiac fibroblasts under conditions of increased oxidative stress.40 Activation of the renin–angiotensin–aldosterone system,41 as well as increases in inflammatory cytokines,42 increases MMP-9 activity and associated fibroblast migration. Polarized macrophages are also a substantive source of MMP-9. MMP-9 has a role in immune cell function, and its upregulation is associated with increases in myocardial infarction.39 Increased MMP-9 activity has been shown to promote collagen synthesis and the transition of cardiac fibroblasts to myofibroblasts which, in turn, produce extracellular matrix.43 Indeed, targeted deletion of MMP-9 attenuates cardiac hypertrophy and collagen accumulation after myocardial injury.44 The attenuation of MMP-9 activity with xanthine oxidase inhibition likely played a role in the reduction of inflammation, macrophage polarization, and fibrosis induced by consumption of a WD. Interestingly, the activity of gelatinase MMP-9 but not MMP-2 was increased in WD-fed mice, and this effect was prevented by xanthine oxidase inhibition. Indeed, a previous study reported an increase in the abundance and activity of MMP-3 and MMP-9 accompanied by either increased or decreased MMP-2 expression level in the left ventricular tissue of failing hearts.45 Collectively, results reported herein and by others suggest that MMP-9 may be involved in the regulation of the structural integrity of the endothelial cell membrane and cardiac fibrosis.

Our results suggest that the enhanced cardiac xanthine oxidase activity with the consumption of a WD plays a role in the generation of oxygen-free radicals because inhibition of xanthine oxidase with allopurinol treatment prevented the rise in myocardial 3-nitrotyrosine levels and related fibrosis. Our finding that allopurinol reduced myocardial indices of oxidative stress as well as cardiac tissue xanthine oxidase highlights the potential direct antioxidant effects of this compound as well as its uric acid and tissue xanthine oxidase lowering properties. However, results of the current study also implicate inflammatory macrophage polarization as a potential instigator of the increased inflammation and fibrosis associated with a WD and accompanies elevations in cardiac xanthine oxidase and hyperuricemia. Indeed consumption of a WD promoted a proinflammatory macrophage M1/M2 polarization, which was prevented by inhibition of the increased uric acid production accompanying consumption of the WD. Macrophage polarization favoring an enhanced M1 proinflammatory response and suppressing an M2 anti-inflammatory response occurs in obesity and the cardiorenal metabolic syndrome.41 The proinflammatory M1...
macrophages secrete inflammatory cytokines to cause insulin resistance and cardiac dysfunction. In contrast, M2 macrophages secrete interleukin 10, which lessens the development of cardiomyocyte hypertrophy and cardiac fibrosis. Further investigation of the precise role of uric acid in promoting inflammatory macrophage polarization is an important area of future investigation.

**Perspective**

The National Institutes of Health have recently instituted clinical trials to evaluate the clinical utility of lowering serum uric acid levels with allopurinol, including the PERL study which is a multicenter clinical trial of allopurinol’s ability to reduce kidney injury and proteinuria in patients with type 1 diabetes mellitus. In a translational model of consumption of the contemporary WD high in fat and fructose, increased production of uric acid is an instigator of cardiomyocyte hypertrophy, interstitial fibrosis, and an obesity-related cardiomyopathy characterized by cardiac stiffness and impaired diastolic relaxation. Results suggest that enhanced myocardial xanthine oxidase activity and macrophage polarization drive growth and profibrotic signaling pathways that precipitate these cardiac abnormalities. These preclinical data may provide potential mechanisms and markers to be followed in the National Institute of Health clinical trials.

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Uric Acid in Cardiac Diastolic Dysfunction

Novelty and Significance

**What Is New?**

- Western diet increases serum uric acid along with an increase in cardiac tissue xanthine oxidase activity temporally related to increases in body weight, fat mass, and insulin resistance without changes in blood pressure.
- Uric acid and xanthine oxidase activity are involved in left ventricular hypertrophy and diastolic dysfunction by upregulation of S6 kinase-1, transforming growth factor β1/Smad signaling cascade, and matrix metalloproteinase (MMP)-9.
- Uric acid and xanthine oxidase activity regulate myocardial oxidative stress and macrophage M1/M2 polarization.

**What Is Relevant?**

- Pharmacological inhibition of xanthine oxidase activity prevents the development of obesity-associated diastolic relaxation in conjunction with reduced uric acid and myocardial xanthine oxidase activity, oxidative stress, cardiac remodeling, and M1/2 macrophage polarization.
- Blocking xanthine oxidase activity may be a novel therapeutic strategy in prevention of cardiovascular diseases, such as hypertension.

**Summary**

Increased production of uric acid with intake of a Western diet promotes cardiomyocyte hypertrophy, inflammation, and oxidative stress that lead to myocardial fibrosis and associated impaired diastolic relaxation.
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Expanded Methods:

Structural and biochemical parameters
Briefly, mice were placed into a thin-walled plastic container while awake and under no anesthesia or distress. All measurements were performed during the same time of day. Mice were then weighed and euthanized via exsanguination under isoflurane anesthesia (above). Heart weights and visceral fat weights were obtained after harvesting along with tibial lengths measured to normalize weights and eliminate confounding effects of differences in size.

Blood pressure measurements
At the end of the 16 weeks feeding trial and immediately prior to being euthanized for tissue collections, mice were anesthetized with isoflurane (1.75% isoflurane in 100% O₂). The right carotid artery was isolated and a high fidelity 1.2 French mouse pressure catheter (Trasonic) was inserted and advanced to a position proximate to the aortic arch. After a brief acclimation period and when blood pressures were stable, average systolic (SBP), diastolic (DBP) and mean arterial pressures (MAP) were determined utilizing the Avantage Data Acquisition System (Scisense, Ontario Canada).

In Vivo High Resolution Cine-MRI
Animals were weighed and anesthetized using 1.8–2.7% isoflurane on a nose cone nonrebreathing system supplying continuous oxygen. ECG and respiratory monitoring and gating were performed with a small animal monitoring system (SA Instruments, Stony Brook, NY). Warm air was circulated through the MRI bore to maintain body temperature. ECG/respiratory gated gradient echo sequences were acquired with 1-mm slice thickness and 65 × 45- and 45 × 45-mm² field of views for the LV in long- and short-axis images, respectively. LV functional parameters were determined using a series of cine images of the LV in long-axis view acquired at 20 equally spaced time points throughout the entire cardiac cycle with a frame rate of 8–12 ms/frame. At each time point, the endocardial borders were traced to measure the LV chamber area using VnmrJ software (Agilent) by two experienced MRI readers. LV volumes (LVVs) at each phase were calculated with the following modified ellipsoid equation: \[ LVV = \frac{8A^2}{3\pi L}, \] where \( A \) is the endocardial area and \( L \) is the length of the LV long-axis chamber. The LVV curve was plotted as LVV versus time throughout a cardiac cycle. For the LV diastolic function measurements, the first derivatives of LVV against time were calculated to extract the diastolic filling rates and relaxation time. Diastolic IFR was defined as the slope of the first four time points on the early diastolic curve. Diastolic peak filling rate (PFR) was defined as the maximum derivative of the LVV curve. Diastolic relaxation time (DRT) was defined as the time duration from the end of systolic phase to the peak filling phase. Normalized DRT, which is the ratio of DRT to the R-R interval, was used to compare LV diastolic relaxation among groups, where normalized DRT = \[ \frac{DRT \times (HR / 6,000)}{.} \]

Myocardial interstitial fibrosis and immunohistochemistry
4-μm longitudinal and transvers sections of the LV were stained with VVG. Slides were blindly analyzed by one or two observers with a Nikon50i (Nikon, Tokyo, Japan) microscope. To keep uniformity and avoid error, each section was thoroughly checked.
Five representative areas were captured with ×40 images from each section with a CoolSNAP cf camera (Roper Scientific Germany, Trenton, NJ). The areas and intensities of pink regions, which are indicative of interstitial fibrosis, were quantified on both transverse and longitudinal sections of the LV using MetaVue software (Molecular Devices, Sunnyvale, CA). The average grayscale intensity due to collagen was recorded. An average value of these intensities was determined for each animal. Immunohistochemistry was performed according to previously published protocols in our group using antibodies previously described for collagen I and III (Abcam, Cambridge, MA).

**3-Nitrotyrosine**

Sections were then washed and incubated with secondary antibodies (biotinylated linked and streptavidin-HRP conjugated) for 30 min each. After several rinses with Tris-buffered saline-Tween 20, diaminobenzidine was applied for 8 min, and sections were then rinsed several times with distilled water, stained with hematoxylin for 80 s, dehydrated, and mounted with a permanent media. Slides were inspected under a bright-field (50i, Nikon) microscope, and ×40 images from each section were captured with a CoolSNAP cf camera. Signal intensities of brownish color, which is indicative of the 3-NT level, were quantified by MetaVue software.

**Western-blot**

Samples (40 μg/lane) were separated by SDS-PAGE and transferred to nitrocellulose membranes. Blots were incubated overnight at 4°C with primary antibodies against TGF-β (Abcam, Cambridge, MA), p-Smad 2/3 (Santa Cruz Biotec, Dallas, Texas), Smad 2/3 (Santa Cruz Biotec, Dallas, Texas), T389 p-S6K (Cell Signaling Technology, Danvers, MA), S6K (Cell Signaling Technology, Danvers, MA), and Pan-actin (Cell Signaling Technology, Danvers, MA). After a rinse, blots were incubated with secondary antibodies (1:5,000 dilution of each antibody) for 1 hour at room temperature. Bands were visualized by chemiluminescence, and images were recorded using a Bio-Rad ChemiDoc XRS image-analysis system. Quantitation of phosphorylated protein band density, normalized to the density of total protein for each sample, was performed using Image Lab (Bio-Rad).

**RNA isolation and quantitative PCR**

The yield of RNA was quantified using using a Nanodrop spectrophotometer (Thermo Scientific, Wilmington, DE). First-strand cDNA synthesis was done using 1 μg total RNA with oligo dT (1 μg), 5× reaction buffer, MgCl2, dNTP mix, RNAse inhibitor, and Improm II reverse transcriptase as per Improm II reverse transcription kit (Promega, Madison, WI). After the first strand synthesis, real-time PCR was done using 8 μl cDNA, 10 μl SYBR green PCR master mix (Bio-Rad Laboratories) and forward and reverse primers (10 pM/μl) (Integrated DNA Technologies, San Diego, CA) using a real-time PCR system (CFX96; Bio-Rad Laboratories). The primer sequences used were: CD11b, Forward: 5’- CCAAGACGATCTCAGCATCA-3’, Reverse:5’-TTCTGGGCTTGTGGAATCTGTT-3’; IL-10, Forward:5’- CCAAGCCCTTATCGAAATGA-3’, Reverse:5’- TTTTACAGGGGAAGAAATCG-3’; CD206, Forward: 5’- CAAGGAAGGGGGCTTGGATT-3’, Reverse:5’- CTTTCAGTCTTGGCAAGC-3’;
GAPDH, Forward: 5’-GGAGAAACCTGCCAAGTATGA-3’, Reverse: 5’-TCCTCAGTGTAGCCCAAGA-3’. The specificity of the primers was analyzed by running a melting curve. The PCR cycling conditions used were 5 min at 95°C for initial denaturation, 40 cycles of 30 s at 95°C, 30 s at 58°C and 30 s at 72°C. Each real-time PCR was carried out using three individual samples in triplicates, and the threshold cycle values were averaged. Calculations of relative normalized gene expression were done using the Bio-Rad CFX manager software based on the ∆Ct method. The results were normalized against housekeeping gene GAPDH.

Gelatin zymography for MMP activity
Samples were run on 8% SDS-PAGE containing gelatin (1.0 mg/mL). After electrophoresis, the gels were washed in Triton X-100 and incubated for 18 h in 50 mmol/L Tris-HCl buffer (pH 7.5) containing 0.2 mol/L NaCl and 10 mmol/L CaCl₂. Gels were stained with Brilliant Blue R250 and detained. Gelatinolytic activity of MMPs was evident as a clear band against the blue background of the stained gel. Quantitation of band density was performed using Image Lab (Bio-Rad).

Data Supplement:

**Supplementary Table S1: Allopurinol on the cardiac function in mice fed a Western diet**

<table>
<thead>
<tr>
<th>Measures</th>
<th>CD (8)</th>
<th>CD-Allo (3)</th>
<th>WD (8)</th>
<th>WD-Allo (6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heart Rate (bpm)</td>
<td>528 ± 37</td>
<td>465 ± 13</td>
<td>480 ± 23</td>
<td>428 ± 14</td>
</tr>
<tr>
<td>End-Diastolic Volume (μL)</td>
<td>46.2 ± 2.6</td>
<td>44.6 ± 3.3</td>
<td>47.8 ± 3.2</td>
<td>47.9 ± 4.1</td>
</tr>
<tr>
<td>End-Systolic Volume (μL)</td>
<td>16.1 ± 0.9</td>
<td>17.4 ± 1.4</td>
<td>16.7 ± 1.3</td>
<td>16.8 ± 1.1</td>
</tr>
<tr>
<td>Stroke Volume (μL)</td>
<td>30.1 ± 1.9</td>
<td>27.2 ± 2.0</td>
<td>31.1 ± 2.2</td>
<td>31.1 ± 3.0</td>
</tr>
<tr>
<td>Ejection Fraction (%)</td>
<td>65.1 ± 0.8</td>
<td>61.1 ± 0.7 *†</td>
<td>65.0 ± 1.5</td>
<td>64.6 ± 0.7</td>
</tr>
<tr>
<td>Cardiac Output (mL/min)</td>
<td>16.8 ± 0.7</td>
<td>12.6 ± 0.6 *</td>
<td>14.7 ± 0.9</td>
<td>13.3 ± 1.3</td>
</tr>
</tbody>
</table>

Values are mean ± SE. (n) represents the number in group. Control Diet Control (CD), Control Diet Allopurinol (CD-Allo), Western Diet (WD), and Western Diet Allopurinol (WD-Allo). *P<0.05 compared with CD; †P<0.05 compared with WD.