Xanthine oxidase inhibition protects against Western diet-induced aortic stiffness and impaired vasorelaxation in female mice

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Running Title: Allopurinol prevents vascular stiffness

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Abstract

Consumption of a high-fat high-fructose diet (western diet, [WD]) promotes vascular stiffness, a critical factor in the development of cardiovascular disease (CVD). Obese and diabetic women exhibit greater arterial stiffness than men, which contributes to the increased incidence of CVD in these women. Further, high-fructose diets result in elevated plasma concentrations of uric acid via xanthine oxidase (XO) activation, and uric acid elevation is also associated with increased vascular stiffness. However, the mechanisms by which increased xanthine oxidase activity and uric acid contribute to vascular stiffness in obese females remain to be fully uncovered.

Accordingly, we examined the impact of XO inhibition on endothelial function and vascular stiffness in female C57BL/6J mice fed a WD or regular chow for 16 weeks. WD feeding resulted in increased arterial stiffness, measured by atomic force microscopy in aortic explants (16.19±1.72 vs 5.21±0.54 kPa, p<0.05), as well as abnormal aortic endothelium-dependent and independent vasorelaxation. XO inhibition with allopurinol (widely utilized in the clinical setting) substantially improved vascular relaxation and attenuated stiffness (16.9±0.50 vs 3.44±0.50 kPa, p<0.05), while simultaneously lowering serum uric acid levels (0.55±0.98 vs 0.21±0.04 mg/dL, p<0.05). In addition, allopurinol improved WD-induced markers of fibrosis and oxidative stress in aortic tissue, as analyzed by immunohistochemistry and transmission electronic microscopy. Collectively, these results demonstrate that XO inhibition protects against WD-induced vascular oxidative stress, fibrosis, impaired vasorelaxation and aortic stiffness in females. Further, excessive oxidative stress resulting from XO activation appears to play a key role in mediating vascular dysfunction induced by chronic exposure to WD consumption in females.

Key Words: Western diet, vascular stiffness, females, oxidative stress, allopurinol
Introduction

Widespread consumption of diets rich in saturated fat and fructose (western diet, [WD]) leads to insulin resistance and obesity, which are critical factors in the pathogenesis of type 2 diabetes mellitus (DM2) and cardiovascular disease (CVD) (31). In turn, CVD accounts for over 50% of deaths in subjects with DM2 (36, 52). Women affected by obesity, insulin resistance and DM2 are at an especially high risk of developing CVD, with a 50% increased risk of death from coronary artery disease (36) and 27% increased relative risk of stroke compared to men (57). Therefore, uncovering the mechanisms that link diet induced obesity to vascular dysfunction is of paramount importance. Notably, vascular stiffness is a parameter that can be used to evaluate vascular dysfunction and can be measured clinically using noninvasive techniques (60). Although it is a physiological phenomenon associated with aging, vascular stiffness is also a biomarker that correlates independently with increased risk of CVD-related morbidity and mortality (4, 12, 14, 34). Conditions such as obesity and DM2 are characterized by accelerated and enhanced vascular stiffness (69, 75). Furthermore, under these conditions augmented vascular stiffness occurs to a greater extent in women compared to men (56). Therefore, the increased incidence and greater severity of CVD in diabetic women can be partially related to increased vascular stiffness (14, 64).

Fructose in contemporary diet derives mostly from abundantly available sucrose in table sugar and high-fructose corn syrup, and appears to be a critical contributor to obesity and associated metabolic abnormalities arising from consumption of WD. Indeed, excess dietary ingestion of fructose strongly correlates with weight gain and impaired glucose tolerance in rodents (38) as well as in humans (68). High-fructose diets lead to increased levels of uric acid, via xanthine oxidase (XO) activation in the liver (10), and high uric acid levels are in turn
associated with increased vascular stiffness and CVD in women (16, 21, 47). Importantly, uric acid levels in the high-normal range have been associated with increased vascular stiffness in women (21) and beneficial CVD effects of XO inhibition have been documented even in the absence of frank hyperuricemia (39). In addition to uric acid production, XO activation in cardiovascular tissue results in enhanced production of reactive oxygen species (ROS) and increased expression of pro-inflammatory molecules in rodents and humans (28, 30, 48), which contribute to cardiovascular tissue damage. The association between hyperuricemia, obesity, and CVD is well recognized in the literature, and frequently coexists with other features of the metabolic syndrome, such as impaired glucose homeostasis (51). Conversely, pharmacologic XO inhibition results in improvements in systemic inflammatory markers in pre-clinical models of the metabolic syndrome (3, 46) as well as in humans (70).

However, the impact of XO inhibition on vascular stiffness and function remains to be fully elucidated. A recent meta-analysis evaluating the effects of the XO inhibitor allopurinol on vascular stiffness in humans was inconclusive (18), thus underscoring the need for additional research in this area. In the present investigation, we test the hypothesis that XO inhibition results in reduced WD-induced vascular stiffness and impaired aortic vasodilatory responses, in part, via decreased vascular oxidative stress. Therefore, we utilized C57BL/6J female mice fed a WD for 16 weeks in the presence or absence of allopurinol, a widely used XO inhibitor, which reduces uric acid production and oxidative stress. We assessed in vivo and ex vivo vascular stiffness, as well as aortic vasomotor responses along with markers of fibrosis and oxidative stress both systemically and in aortic tissue.

Methods

Animal Models
Three week old C57BL/6J female mice (stock number 00664) were procured from Jackson Laboratories (Bar Harbor, ME). All procedures were approved in advance by the Institutional Animal Care and Use Committee of the University of Missouri and mice were cared for according to NIH guidelines. When mice were four weeks of age, they were randomly assigned to 1) control diet feeding (CD) (Test Diet 58Y2, Richmond, Indiana), 2) CD and allopurinol (CD-ALLO, 125 mg/L in drinking water), 3) WD containing high fat (46%) and high carbohydrate as sucrose (17.5%) and high fructose corn syrup (17.5%) (Test Diet 58Y1 with high refined carbohydrate and high fat, 5APC, Richmond, Indiana) (WD) or 4) WD and allopurinol (WD-ALLO, 125 mg/L in drinking water) for 16 weeks. The dose used was chosen based on previous studies from our laboratory that showed beneficial cardiac effects in males (30). The female mice were housed in pairs under a 12-hour light/dark regimen and water and food were provided ad libitum.

### Body composition and Biochemical Parameters

Mice were weighed before sacrifice. After 16 weeks of feeding, mice underwent body composition analysis for whole body fat mass, lean body mass, and total body water using an EchoMRI-500 for quantitative magnetic resonance analysis (Echo Medical Systems, Houston, TX), as previously described (41). Venous blood samples were collected from a subset of fasting mice in each treatment group, and plasma was stored at −80°C for glucose and uric acid measurements. Measurements of plasma uric acid and glucose were performed by automated clinical chemistry analyzer (AU680, Beckman-Coulter, Inc., Brea, CA) as previously described (30).

### Atomic Force Microscopy (AFM) Imaging and Force Measurement
AFM was used to evaluate stiffness of endothelial cells (ECs) in enface aortic preparations of the thoracic aorta as previously described (42). Briefly, a 2x2 mm segment of the thoracic aorta was obtained after sacrifice and opened longitudinally. The adventitial surface of each explant was fastened to a glass covered slip using cell tak opened longitudinally. Stiffness (elastic modulus) of the EC surface was measured by AFM using a nano-indentation protocol as previously described (17). A MFP-3D AFM 89 (Asylum Research Inc. Goleta, CA) mounted on an Olympus IX81 microscope (Olympus Inc.) was used for biomechanical measurements and estimate elastic modulus/stiffness. AFM measurements were conducted at room temperature (~25°C). For stiffness measurements, an AFM cantilever (MLCT, Bruker-nano, Goleta, CA) was used to perform repeated cycles of nano-indentation and retraction cycles on the cell surface. The parameters employed were 0.3 Hz sampling frequency, with an approach/retraction velocity of 960 nm•sec-1, 1600 nm traveling distance for one sampling cycle (indentation and retraction), and approximately 400-600pN loading force. Force curves were generated over a period of two minutes and analyzed using NForceR software (registration number TXu1-328-659) and MATLAB. The mean of these elastic modulus (i.e., stiffness) values was computed for each indentation site and then averaged together for each group. Estimations of Young’s modulus (E-modulus) were obtained using a length of 100-300 nm of the AFM indentation curve, after the initial point of contact that was fit with a Hertz model as shown in equation:

\[ F = \frac{2}{\pi (1 - \nu^2)} \tan \alpha \frac{E \delta^2}{\tan \alpha} \]

Where, E is the E-modulus, F is the force exerted by AFM probe on tissue surface, Δ is indentation depth into the sample, α is the half-opening angle of the AFM tip, and ν is the Poisson ratio. The tissues were considered as a gel and ν was assumed at 0.5. To obtain
topographical images of EC or VSMC, the AFM was operated in contact mode. The area of the tissue surface that was scanned in these experiments was 40 x 40 μm and the digital density of the scanned area was 512 x 512 pixels. Stylus type AFM probes (Model: MLCT-C, k = 15 pN/nm, Bruker, Santa Barbara, CA) were used to perform surface scanning at 0.4 Hz frequency with approximately 300-500 pN tracking force (17).

**Ex vivo Vasomotor Responses of Aortic Rings**

Vasomotor responses were evaluated in the aorta via wire myography as previously described (17, 42). Briefly, a 2 mm segment of thoracic aorta was collected immediately after euthanasia and placed in the bathing physiological salt solution (PSS) containing (in mM): NaCl, 4.7 KCl, 1.2 NaH₂PO₄, 1.17 MgSO₄, 2 CaCl₂, 5 glucose, 2 pyruvate, 0.02 EDTA, 3 MOPS, and 1% bovine serum albumin, pH 7.4. Samples were maintained at 37°C and were continuously aerated with 95% O₂ - 5% CO₂. Before experimentation, the aortic contractile state was ascertained by KCl (80mM L⁻¹). Aortas were pre-constricted with U46619 (100nM). Vasorelaxation of arterial rings to acetylcholine (ACh, 10⁻⁹ to 10⁻⁴ M), and the nitric oxide (NO)-donor sodium nitroprusside (SNP, 10⁻⁹ to 10⁻⁴ M) were assessed by cumulative addition of agonist to the vessel bath. Aortic relaxation responses are presented as percent maximal relaxation, calculated as [(Fb – Fd)/(Fb – Fmin)] × 100, where Fd is force after a drug intervention, Fb is baseline force, and Fmin is force before the intervention. At the end of each experiment, the PSS bath solution was replaced with Ca²⁺-free PSS to determine minimal force during passive conditions.

**Vascular Fibrosis**

A 2 mm segment of thoracic aorta was fixed in 3% paraformaldehyde, dehydrated in ethanol, paraffin embedded, and transversely sectioned in 5μm slices. Four sections each for 4-5
mice per group were examined. Slides were stained with picrosirius red stain and Verhoeff-Von Gieson (VVG) stain to measure collagen accumulation. The areas and intensities of red color which were stained with picrosirius red and the intensities of pink color on the VVG stained sections indicative of collagen deposition were quantified as gray scale intensities using MetaVue software as previously described (29).

Measurement of vivo Aortic stiffness in vivo

We measured aortic stiffness in vivo by Pulse Wave Velocity (PWV). Doppler ultrasound (Indus Mouse Doppler System, Webster, TX) was used as previously described in our laboratory (17). Prior to sacrifice, isoflurane-anesthetized mice (1.75% in 100% oxygen stream) were placed supine on a heating board and legs secured to ECG electrodes. PWV was determined according to the transit time method, calculated as the difference in arrival times of a Doppler pulse wave at two locations along the aorta (aortic arch and descending aorta) which were set at a known distance apart (35 mm). Each of the pulse wave arrival times was measured as the time from the peak of the ECG R-wave to the leading foot of the pulse wave at which time velocity begins to rise at the start of systole. The distance between the two locations along the aorta was divided by transit time and data are expressed in mm/ms. Velocity waveforms were acquired at the aortic arch followed immediately by measurement at the descending aorta proximal to the iliac bifurcation (17).

Oxidative Stress

Aortic oxidative stress was assessed by immunostaining for 3-nitrotyrosine (3-NT) as previously described (77). 3-NT is a product of tyrosine nitration mediated by ROS such as
peroxynitrite, which promotes NO destruction as well as vascular inflammation (17, 29). Briefly, 5 µm paraffin embedded Aorta sections from different treatments were dewaxed, rehydrated, antigen retrieved and incubated overnight with 1:150 primary rabbit polyclonal anti-3-NT antibody (Millipore). Sections were washed and incubated 30 min with secondary antibodies, biotinylated anti rabbit, and streptavidin-HRP. After several rinses with distilled water, diaminobenzidine (DAB) was applied for 7 minutes, and sections were again rinsed and stained with hematoxylin for 90 seconds, dehydrated, and mounted with permount. For 3-NT quantification, all colors on the sections were deleted except the brown color which is indicative of 3-NT formation in the different component of the aorta. The slides were checked under a bright field (Nikon 50i) microscope (Nikon, Tokyo, Japan) and 40× images were captured with a Cool SNAP cf camera (Roper Scientific Germany, Trenton, NJ). The images were analyzed by MetaVue (Molecular Devices, Sunnyvale, CA) and the intensity of brown color was quantified as gray scale intensities. To quantify 3-NT in ECs, the endothelial layer was carefully traced and the brown color was quantified only in that region. For quantifying 3-NT generation in VSMCs, equal regions of interest were made in the media in all sections and the intensities of brown color were quantified and analyzed in these specific areas.

XO activity in aortic tissue was determined using a xanthine oxidase activity quantitative colorimetric/fluorimetric assay kit (BioAssay Systems, Hayward, CA). In addition we measured systemic oxidative stress by quantifying plasma Malondialdehyde (MDA) using a colorimetric/flurimetric assay kit (TBARS assay kit, Cayman Chemical, Ann Arbor, MI).

**Statistical Analysis**
Results are reported as the mean ± SE. Statistical analysis was primarily done by two-way ANOVA followed by post hoc tests (Bonferroni) to examine effects of WD and allopurinol (Sigma Plot 13.0, Systat Software). The dose responses to ACh and SNP were analyzed using repeated measures ANOVA. Aortic dilator responses are presented as percent maximal relaxation, calculated as \[\frac{(F_b - F_d)}{(F_b - F_{\text{min}})} \times 100\], where \(F_d\) is force after a drug intervention, \(F_b\) is baseline force, and \(F_{\text{min}}\) is minimal force obtained during passive conditions.

Transmission electron microscopy (TEM)

Aorta samples were fixed in 2% paraformaldehyde, 2% glutaraldehyde in 100 mM sodium cacodylate buffer pH=7.35. Fixed tissues were rinsed with 100 mM sodium cacodylate buffer, containing 10 mM 2 mercaptoethanol and 130 mM sucrose (2-ME buffer). Secondary fixation was performed using 1% osmium tetroxide in 2-ME buffer. Specimens were next incubated at 4°C for 1 hour, then rinsed with 2-ME buffer. Block staining was performed using 1% aqueous uranyl acetate and incubated at 4°C overnight, then rinsed with distilled water. Sections were cut to a thickness of 85 nm using an ultramicrotome and stained using Sato’s triple lead solution stain and 5% aqueous uranyl acetate. Multiple images were acquired for study at various magnifications with a JEOL JEM 1400 transmission electron microscope at 80 kV. Specifically, twelve endothelial images per group were acquired X800 and/or X1000 magnification for measurement of EC length with existing scale bar on image. To measure EC length, 3 EC were measured randomly from each sample at their base where they joined the internal elastic lamina utilizing the scale bar of that image, for a total of 12 measurements for each sample.

Results
**WD results in increased body weight, adiposity and fasting glucose**

In agreement with previous data from our laboratory (41), 16 weeks of WD-feeding resulted in a significant increase in body weight as well as fat percentage in all cohorts. Administration of allopurinol did not affect these parameters (Fig 1A-B). In parallel, fasting blood glucose was elevated in the WD-fed cohorts relative to animals fed a control diet (281.0±10.30 vs 217.75±27.30 mg/dL, p<0.05). Administration of allopurinol did not induce significant changes in body composition or glucose metabolism in either the CD-fed or the WD-fed mice (Fig 1C). These data support the notion that the effects of XO inhibition are independent of changes on body weight, body composition or glucose metabolism. In male C57BL/6J mice, we have previously demonstrated and published that 16-weeks of WD resulted in a significant increase in uric acid levels (30). In the present investigation, WD resulted in a non-statistical significant elevation of uric acid in the WD-fed female mice relative to animals fed a CD (0.55±0.09 vs 0.40±0.05 mg/dL). Treatment with allopurinol did not affect uric acid levels in CD-fed animals, but it substantially lowered uric acid levels in WD-fed female mice compared to untreated WD-fed animals (0.21±0.04 vs 0.55±0.09 mg/dL, p<0.05) (Fig 1D). In addition, XO activity was increased by approximately 1.9 fold in WD female mice relative to animals fed a CD (6.86±2.97 vs 3.61±0.37 U/L, p=0.35), and was significantly decreased by allopurinol (1.51±0.27U/L, p<0.05). XO activity also trended down in CD-fed mice treated with allopurinol as well (2.07±0.08 U/L, p=0.17).

**XO inhibition protects against WD-induced aortic stiffness and impaired vasodilatory responses**
We have demonstrated and published previously that 16 weeks of WD feeding results in increased cardiovascular stiffness in female mice (17, 41). In the current investigation, we assessed vascular stiffness via AFM of thoracic aortic explants. We found that WD feeding resulted in a significant increase in aortic stiffness as compared to CD (16.9±0.50 vs 5.21±0.54 kPa) (Fig 2A). XO inhibition with allopurinol significantly reduced aortic stiffness to levels comparable to animals fed a CD (16.9±0.50 vs 3.44±0.50 kPa, p<0.05) (Fig 2A). In addition, we measured in vivo aortic stiffness by ultrasound-based PWV. We have previously demonstrated significant increases in PWV in female mice fed a WD for 16 weeks relative to mice fed a CD (17, 29, 42). In our experiments, treatment with allopurinol did not affect significantly PWV (3.82±0.25 mm/ms for untreated vs 3.67±0.16 mm/ms for allopurinol-treated, all fed a WD, p>0.05).

Structural and functional characteristics of the vasculature are closely linked (72), and vascular endothelial stiffness contributes to impaired vasomotor responses (74). Importantly, we have previously shown than 16 weeks of WD-feeding in female C57BL/6J results in impaired endothelial-dependent and independent vasodilatory responses (17). Therefore, we evaluated vasomotor responses only in the WD-fed cohorts. In isolated aortic rings, the cohort treated with XO inhibitor had greater vasodilatory response to ACh when compared with the WD-fed animals (E_max=16.38±1.94% vs E_max=27.46±2.80%, p<0.05) (Fig 2B). Similarly, the endothelial-independent vasodilatory response to SNP was significantly greater in the WD-ALLO when compared with WD (E_max=28.50±4.55% vs 44.29%, p<0.05) (Fig 2C). These responses were restored to a degree comparable to female mice fed a CD (E_max=35.1±2.20% and 38.9±2.70% for ACh and SNP respectively), as previously published by our laboratory (17). The present data
demonstrate that XO inhibition prevents WD-induced aortic stiffness in parallel with greater endothelial dependent and independent vasodilatory responses.

**XO inhibition ameliorates vascular stiffness in association with a reduction in vascular oxidative stress and fibrosis**

We analyzed systemic markers of oxidative stress by measuring Malondialdehyde (MDA) concentrations in plasma. WD fed mice demonstrated a significant elevation of MDA concentrations (5.48±0.59 vs 2.15±0.34 μM, p<0.05), which was prevented by treatment with allopurinol (3.90±0.82 μM) (Fig 3A). Further, XO inhibition reduced MDA to levels similar to those found in allopurinol treated control mice (2.05±0.26 μM, p>0.05).

Previously, we have determined that in females WD-induced aortic stiffness is related to increased oxidative stress and vascular remodeling(17). Similarly, in the present investigation we found increased 3-NT staining in the WD-fed cohort group when compared with CD (Fig 3B). In addition, XO inhibition significantly ameliorated oxidative stress (WD vs. WD-ALLO, Fig 3B). These data, in addition to our results showing WD-induced increments in XO activity in aortic tissue which is ameliorated by allopurinol, collectively suggest that XO inhibition is protective against WD-induced vascular oxidative stress in female mice.

We also studied the impact of WD-induced enhancement of XO activity on fibrosis in aortic tissue by immunohistochemistry. Relative to mice fed a CD, WD feeding for 16 weeks resulted in increased collagen deposition, as measured by picrosirius red immunostaining (Fig 4). Allopurinol administration resulted in significant reductions in this marker of fibrosis in WD-fed animals relative to untreated animals also fed a WD (WD-ALLO vs. WD). These data suggest a beneficial influence of XO inhibition on vascular fibrosis in conditions of WD feeding (Fig 4).
Finally, we analyzed ultrastructural changes via TEM in aortic samples. Our findings demonstrate increased deposition of highly organized fibrillary collagen in WD-fed mice, which correlates with our immunohistochemistry findings reported above (Fig 5). Furthermore, we also demonstrated an abnormal phenotype characterized by shortening and lifting of ECs, which is suggestive of increased EC contractility (Fig 6). Similar to our AFM and vasomotor responses, these abnormalities were corrected by allopurinol.

Discussion

The central aim of the current investigation was to evaluate the impact of XO inhibition on vascular stiffness in a female rodent model of chronic over-nutrition with a diet high in fat and fructose (WD). Overall, our data demonstrate that XO inhibition improves WD-induced vascular stiffness, as well as aortic vasodilatory responses. In addition, we show that these actions of XO inhibition occur in concert with decreased oxidative stress and aortic fibrosis in females.

Fructose is ubiquitously consumed in industrialized countries, mostly derived from sucrose present in table sugar and high-fructose corn syrup, and typically leads to increased levels of uric acid (71). As opposed to glucose, which is readily utilized by most cells to produce energy, fructose must be further metabolized prior to joining the glycolytic pathway. In the liver, fructokinase phosphorylates fructose to fructose-1-phosphate in an unregulated manner, leading to depletion of adenosine triphosphate (ATP), which in turn results in stimulation of key enzymes involved in purine nucleotide synthesis. Ultimately, this pathway leads to increased activity of XO and subsequent production of uric acid (32, 33, 43).
Although there is controversy in regards to the relative contribution of dietary fructose in the development of CVD (62), high-fructose diets are currently under scrutiny due to their potential impact on obesity, DM2 and CVD (6, 9, 40, 50, 63, 67). It is accepted that increased levels of uric acid as occurs with WD consumption are linked to increased risk for CVD (61), as well as chronic kidney disease in humans (49). Nonetheless, other mechanisms have been postulated to contribute to the development of vascular dysfunction and CVD, including fructose-induced cardiovascular oxidative stress (30, 62).

We performed our studies in cycling female mice because of the translational relevance related to observations in premenopausal women. To this point, the impact of dietary fructose on vascular stiffness appears to have more severe implications in females than males. Indeed, in a cohort of over 88,000 women, the consumption of high-fructose diet in the form of >2 sugar-sweetened beverages resulted in a 35% increased risk of coronary artery disease relative to men (24). In a Japanese cohort of over 38,000 subjects, sugar-sweetened beverages were associated with ischemic stroke in only women (20). Similarly, it has been shown that high plasma levels of uric acid are linked to increased vascular stiffness in apparently healthy women but not in men (21). Furthermore, women exhibit increased vascular stiffness with aging, and insulin resistance worsens this abnormality to a greater degree compared to men (56). Therefore, the augmented incidence and greater severity of CV disease in obese and diabetic women can be partially related to the presence of increased vascular stiffness (14, 64). Indeed, women with DM2 are at an especially higher risk of developing coronary artery disease (36) and stroke compared to men (57).

Our data support the notion that a key mediator of WD-induced vascular stiffness is increased oxidative stress. The key role played by excess production of ROS in the pathogenesis
of CVD is well established and described (13, 45). In addition, data from our laboratory have demonstrated that ROS contributes to vascular remodeling, as well as stiffness in rodent models of chronic over-nutrition (17, 29, 42). In this study, we evaluated systemically oxidative stress by measuring plasma levels of MDA, as well as at the tissue level via measurements of XO and 3-NT in aortic tissue. Our results demonstrate significantly increased plasma levels of MDA and 3-NT in vascular smooth muscle and endothelial cells, which were substantially reduced by administration of allopurinol, to levels comparable to mice fed a CD and treated with allopurinol. Moreover, similar results were demonstrated in our measurements of XO oxidase activity, which is independently a major source of ROS and is known to participate in the pathogenesis of vascular dysfunction leading to CVD (23). Therefore, our data do support a critical role for oxidative stress as a mediator of WD-induced vascular stiffness in female mice. Mitochondrial enzymes, activation of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase enzymatic complex, and endothelial nitric oxide synthase (eNOS) uncoupling are major alternative sources of ROS as well (37). Conversely, there is extensive work about the role of multiple antioxidants in protecting the vasculature against oxidative stress-induced damage. Generic antioxidants, as well as different inhibitors of the NADPH oxidase enzymatic complex and mitochondrial oxidative stress have been associated with improved markers of vascular stiffness, fibrosis remodeling and endothelial dysfunction (15, 22, 73). However, our findings that allopurinol decreased WD-induced vascular stiffness and fibrosis, strongly support an important role for tissue XO activation as a key mediator of vascular dysfunction as well. Furthermore, our findings are in agreement with previous data in cardiovascular tissue in rodents treated chronically with XO inhibition (19, 44).
High-fructose diets can indeed trigger activation of alternative sources of ROS such as NADPH oxidase (1, 5, 11). However, in our experiments treatment with allopurinol decreased markers of oxidative stress and fibrosis, thus supporting the role of vascular XO activation as a critical contributor to elevated ROS production in the setting of WD feeding. Indeed, we have previously reported that WD feeding increases cardiovascular XO activity in males, and that allopurinol treatment for 16 weeks mitigates this WD-mediated increase (30).

Interestingly, administration of allopurinol resulted in improvements in endothelial stiffness in the absence of significant changes aortic stiffness in vivo (measured by PWV). These findings suggest that improvement in endothelial cell stiffness is an earlier event that precedes further changes in arterial stiffness in whole aorta. In agreement, data from our laboratory obtained in female mice also fed a WD for 16 weeks have also reported changes in endothelial stiffness in response to other interventions such as exercise, which do not result in in vivo changes in PWV (54).

We also found improved vasomotor responses associated with XO inhibition ex vivo. Utilizing wire myography, we detected increased both endothelium-dependent and independent responses in aorta in our allopurinol-treated mice in conditions of WD feeding. Interestingly we have previously reported aortic maximal relaxation reaching roughly 35-40% for ACh and approximately 70% for SNP in female mice fed a CD (17, 29, 42). Available literature has reported comparable results to our published data (58, 59), whereas others studies report higher vasomotor responses compared to our results in control animals under similar conditions (55, 76). In addition, we have demonstrated under the same conditions as in our experiments, that WD feeding for 16 weeks results in reduction of maximal vasorelaxation in aorta of approximately 30% and 35% for ACh and SNP, relative to CD (17, 29, 42). In our current
investigation, we found even more substantial reductions in vasomotor responses by WD, approximately 54% for ACh and 61% for SNP, thus confirming a very significant impact of WD on vasomotor response, both endothelium dependent and independent. Although reports are variable, reflecting that vascular function is influenced by multiple variables including genetic background and experimental conditions, our data not only confirm the profound impact of WD on vasomotor responses, but demonstrates a critical contribution of enhanced XO activity to vascular abnormalities induced by WD.

We utilized allopurinol for XO inhibition since it is known to be safe and effective in the clinical setting for the treatment of hyperuricemia. Furthermore, available literature has reported potential additional benefits of this medication (18), such as improvements in endothelial function (25), oxidative stress (26) and modulation of inflammation (2), which are contributors to vascular stiffness. Nonetheless, the efficacy of allopurinol to improve vascular stiffness has not been established (18).

Remarkably, consumption of a WD induced only a non-significant increase in plasma uric acid levels in our female mice. This modest response has been previously reported in several studies dealing with high fructose diets used in humans (27) as well as in rodents (65), and several reasons have been argued for this particular finding. Unlike humans in which uricase (the enzyme that catalyzes conversion of uric acid into allantoin and allows its excretion) is absent, this enzyme is present in rodents. Therefore, the impact of high-fructose diets on uric acid levels in mice differs significantly, and elevations in serum levels of uric acid in response to high fructose diets (66) are expected to be less significant compared to humans. Furthermore, similar to glucose, fructose can be reabsorbed from the filtrate in the renal proximal tubule via transporters such as GLUT 5 and GLUT 9, and hyperglycemia –present in our WD-fed mice- is
associated with impaired function of proximal tubules leading to decreased urate reabsorption (7, 8). In addition, also in the setting of hyperglycemia, high concentrations of fructose reaching the renal proximal tubules competitively inhibit urate reabsorption via GLUT 9, which can transport both glucose and fructose (35). Collectively these phenomena result in reduced reabsorption of urate and therefore to decreased plasma uric acid levels, as found in our experiments. Furthermore, there is a dimorphic response regarding uric acid handling in mice and it has been shown that elevations in uric acid are more substantial in males relative to females. Certainly, we have previously published data demonstrating significant increases in plasma uric acid levels in male mice fed a WD for 16 weeks (30), which in addition confirms the adequacy of the feeding paradigm chosen for our study. Also, it has been shown that males have greater metabolism of fructose in the proximal tubule, while females exhibit more substantial distal tubule abnormalities regarding electrolyte balance (65).

In humans, available clinical studies support the therapeutic role of XO inhibition even in the absence of frank hyperuricemia. In heart failure subjects, a high dose of allopurinol improved forearm endothelial function as assessed by venous occlusion plethysmography (25). Remarkably, these findings were not dependent of reduction of uric acids levels and might have been related to decreased oxidative stress (25). Another recent analysis of an elderly hypertensive population dataset in the United Kingdom showed that in hypertensive individuals, treatment with allopurinol was associated with 50% decreased risk of stroke and 30% risk of cardiac events (39). Importantly, a multicenter, prospective, randomized, open-label, blinded-endpoint clinical study, the PRIZE study, is currently underway and will assess the effect of a newer XO inhibitor – febuxostat – on carotid intima thickness in a population of asymptomatic hyperuricemia subjects (53). This trial, designed to include 500 participants with uric acid >7.0
mg/dL and carotid intima-media thickness ≥1.1 mm, will likely add valuable information regarding the role of XO inhibition in the treatment of CVD.

Our findings of a reduction in WD-mediated vascular stiffness with allopurinol contributes to a better understanding of the impact of XO activation on vascular stiffness in females. However, additional studies comparing males to females are warranted and will contribute to determining a possible differential CVD benefit from XO inhibition in females compared to males. In addition, additional studies in cardiac tissue in females are needed to better understand the sexual dimorphic impact of a WD, elevated uric acid, and XO activity on cardiovascular stiffness and cardiac impaired relaxation. In this regard, data from our laboratory in male mice fed a WD have already shown benefits of allopurinol on markers of cardiac stiffness and diastolic dysfunction (30).

Our results do not show a significant impact of XO inhibition on body weight, fat percentage or fasting glycemia in mice exposed to WD. These data are suggestive that the actions of allopurinol were not mediated by an indirect effect of XO inhibition on these metabolic parameters. Instead, the beneficial actions of XO inhibition are likely resulting from direct vascular effects such as decreased oxidative stress. We did not specifically measure markers of insulin resistance, however the finding that the above-mentioned parameters remained unchanged also supports the possibility of mechanisms not affecting insulin sensitivity or glucose homeostasis.

In summary, this investigation demonstrates that XO inhibition and subsequent reductions in systemic and vascular tissue oxidative stress play a protective role against WD-induced vascular stiffness in female mice. Because population studies suggest that hyperuricemia has a more severe impact on vascular stiffness in females compared to males, our findings
stimulate the possibility that females consuming a WD may be specially benefited from pharmacologic XO inhibition. Additional studies are required to determine the extent to which reduced vascular stiffness with XO inhibition is mediated by a reduction in uric acid, and whether this pharmacological treatment results in a significant reduction in the excessive CVD morbidity and mortality found in insulin resistant women relative to men.

Acknowledgements: We acknowledge the work by Dr. Javad Habibi, PhD, who captured and analyzed immunohistochemistry data. We also acknowledge Ms. Brenda Hunter for her valuable editorial assistance and formatting of this manuscript. We acknowledge the National Institutes of Health for funding this investigation, as well as the Department of Veterans Affairs.

Sources of Funding: This work was supported by National Institutes of Health (1K08HL132012-01A1 to GL, 1K08-HL129074-01 to CM; K01-HL125503 and R21-DK105368 to JP; R01-HL073101 and R01-HL107910 to JRS), Department of Veterans Affairs Merit Award 1BX001981 to JRS.


Figure 1. XO inhibition does not impact changes in body weight, adiposity or fasting glucose induced by WD. Body weight (A), body fat composition (percent of total body weight) by quantitative magnetic resonance (echoMRI-500) (B), fasting plasma glucose concentration (C) and plasma uric acid levels (D) (samples obtained under anesthesia). * p<0.05 CD versus WD, # p<0.05 WD versus WD-ALLO. CD, control diet; ALLO, allopurinol; n = 4-8 for all groups.

Figure 2. XO inhibition improves WD-induced aortic stiffness, and increases endothelial dependent and independent vasodilatory responses in the setting of WD-feeding. (A) Aortic endothelial stiffness was assessed via AFM of aortic explants from C57BL/6J female mice fed a WD for 16 weeks in the presence or absence of allopurinol in drinking water. * p<0.05 CD versus WD, # p<0.05 WD versus WD-ALLO. CD, control diet; ALLO, allopurinol; n = 4-5 for all groups. In addition, vasomotor responses were evaluated in C57BL/6J female mice fed a WD for 16 weeks in the presence or absence of allopurinol in drinking water. (B) Responses of isolated aortic rings to the endothelium-dependent dilator acetylcholine, and (C) to the endothelium-independent vasodilator sodium nitroprusside § p<0.05 WD vs WD + Allopurinol. n=4-7

Figure 3. Allopurinol decreases WD-induced oxidative stress systemically and in aortic tissue. (A) Levels of Malondialdehyde (MDA) were measured in plasma to determine systemic markers of oxidative stress, in C57BL/6J female mice fed a WD for 16 weeks, without and without treatment with allopurinol. (B) Analysis of oxidative stress by 3-nitrotyrosine (3-NT) staining in sections from aortas. CD, control diet; WD, western diet; ALLO, allopurinol. VSMC,
vascular smooth muscle cells *p<0.05 WD vs CD; # p<0.05 WD vs WD + ALLO. n=3-6 all groups

**Figure 4.** XO inhibition decreases aortic fibrosis. Analysis of fibrosis by picrosirius red staining in aortas from C57BL/6J female mice fed WD for 16 weeks in the presence or absence of allopurinol in drinking water. CD, control diet; WD, Western diet, ALLO, allopurinol. *p<0.05 WD vs CD; # p<0.05 WD vs WD + ALLO. n=4 all groups

**Figure 5.** Allopurinol decreases collagen deposition in WD-fed female mice. Analysis of fibrosis by Transmission Electron Microscopy (TEM) in aortas from C57BL/6J female mice fed WD for 16 weeks in the presence or absence of allopurinol in drinking water (representative images). Panel A, control diet (CD) and B, control plus allopurinol (CD+ALLO) demonstrate the normal appearance and extension of the fibrillar collagen adjacent to the homogeneous electron dense external elastic lamina (EEL) on the abluminal side of the media. Panel C demonstrates layering of highly organized, compact, dense, fibrillar collagen (C) demarked by dashed line). In panel D, Allopurinol treatment protects against additional layering of mature, highly organized, compact, dense, fibrillar collagen relative to WD-fed animals (panel C). Magnification X400; bar = 5 µm. CD, control diet; WD, Western diet, ALLO, allopurinol. n=12 for all groups

**Figure 6.** Allopurinol improves endothelial cell (EC) phenotype in WD-fed female mice. Analysis of EC morphology by transmission electron microscopy (TEM) in aortas from C57BL/6J female mice fed WD for 16 weeks in the presence or absence of allopurinol in drinking water (representative images). Panel C demonstrates a characteristic abnormal
phenotype of descending thoracic aorta EC (luminal side - closed arrows) showing lifting and separation (open arrows) from the internal elastic lamina (IEL) as compared to the control diet (CD) and CD treated with allopurinol (CD-Allo) models in panels A and B respectively. Importantly, notable contraction-retraction of ECs as depicted in insert (c) where there is loss of EC elongation of cytoplasm and nucleus, which illustrates their contracted phenotype (magnification X600; bar = 2µm). Panel D illustrates that WD models are protected from lifting; separation from the IEL and contraction of the ECs. Magnification X800; bar = 2µm in panels A-D. EL = medial elastic lamina; N = endothelial nucleus; VSMC = media vascular smooth muscle cell. n=12 for all groups.
~ 15-18 µm

~ 15 µm

~ 30 µm

~ 15 µm

EEL

EEL

EEL

EEL

Adv

CD

CD+ALLO

3 µm

5 µm

Media

Loose Areolar Collagen