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

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Lastra G, Manrique C, Jia G, Aroor AR, Hayden MR, Barron BJ, Niles B, Padilla J, Sowers JR. Xanthine oxidase inhibition protects against Western diet-induced aortic stiffness and impaired vasorelaxation in female mice. Am J Physiol Regul Integr Comp Physiol 313: R67–R77, 2017. First published May 24, 2017; doi:10.1152/ajpregu.00483.2016.—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. Furthermore, high-fructose diets result in elevated plasma concentrations of uric acid via xanthine oxidase (XO) activation, and uric acid elevation is 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 wk. WD feeding resulted in increased arterial stiffness, measured by atomic force microscopy in aortic explants (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 arterial stiffness in females. Furthermore, 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.

Western diet; vascular stiffness; females; oxidative stress; allopurinol

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 more than 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 with 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 with 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 diets 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 correlates strongly 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 proinflammatory 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, pharmacological XO inhibition results in improvements in systemic inflammatory markers in preclinical 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 wk 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 no. 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 National Institutes of Health guidelines. When mice were 4 wk of age, they were randomly assigned to 1) a control diet feeding (CD; Test Diet 58Y2, Richmond, IN), 2) a CD and allopurinol (CD-ALLO; 125 mg/l in drinking water), 3) a WD containing high fat (46%) and high carbohydrate (17.5%) and high fructose corn syrup (17.5%) (Test Diet 58Y1 with high fructose corn syrup feeding (CD; Test Diet 58Y2, Richmond, IN), or 4) a WD and allopurinol (WD-ALLO, 125 mg/l in drinking water) for 16 wk. 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-h light-dark regimen, and water and food were provided ad libitum.

Body composition and biochemical parameters. Mice were weighed before being euthanized. After 16 wk 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 described previously (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, Brea, CA) as described previously (30).

Atomic force microscopy imaging and force measurement. Atomic force microscopy (AFM) was used to evaluate stiffness of endothelial cells (ECs) in enface aortic preparations of the thoracic aorta, as described previously (42). Briefly, a 2 × 2 mm segment of the thoracic aorta was obtained after euthanasia and opened longitudinally. The adventitial surface of each explant was fastened to a glass-covered slip using Cell-Tak, which was opened longitudinally. Stiffness (elastic modulus) of the EC surface was measured by AFM using a novel-indentation protocol, as described previously (17). An MFP-3D AFM 89 (Asylum Research, Goleta, CA) mounted on an Olympus IX81 microscope (Olympus) was used for biomechanical measurements and to 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 of sampling frequency with an approach/retraction velocity of 960 nm/s, 1,600 nm traveling distance for one sampling cycle (indentation and retraction), and ~400–600 pN loading force. Force curves were generated over a period of 2 min and analyzed using NForceR software (registration no. 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. Estimation of Young’s modulus (E-modulus) was 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 the equation

\[
E = \frac{2}{\pi} \frac{\delta^2}{F} \tan \alpha
\]

where \(E\) is the E-modulus, \(F\) is the force exerted by AFM probe on tissue surface, \(\delta\) is indentation depth into the sample, \(\alpha\) is the half-opening angle of the AFM tip, and \(v\) is the Poisson ratio. The tissues were considered as a gel, and \(v\) 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 × 40 µm, and the digital density of the scanned area was 512 × 512 pixels. Stylus-type AFM probes (model: MLCT-C, \(k = 15\) pN/µm; Bruker, Santa Barbara, CA) were used to perform surface scanning at 0.4-Hz frequency, with ~300–500 pN of tracking force (17).

Ex vivo vasomotor responses of aortic rings. Vasomotor responses were evaluated in the aorta via wire myography, as described previously (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) 145 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 (80 mM). Aortas were preconstricted with U-46191 (100 nM). Vasorelaxation of arterial rings to acetylcholine (ACH, 10⁻⁹ to 10⁻⁴ M) and the nitric oxide (NO)-donor sodium nitroprusside (SNP, 10⁻⁷ to 10⁻³ M) were assessed by cumulative addition of agonist to the vessel bath. Aortic relaxation responses are presented as percent maximal relaxation, calculated as \([F_{a} - F_{b}] / F_{a}\) × 100, where \(F_{a}\) is force before a drug intervention, \(F_{b}\) is baseline force, and \(F_{a}\) 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 5-µm slices. Four sections each for four to five 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 that were stained with picrosirius red and the intensities of pink color on the VVG-stained sections indicative of collagen deposition were quantified as grayscale intensities, using MetaVue software as described previously (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 described previously in our laboratory (17). Before euthanasia, 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 and 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

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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 millimeters per millisecond. Velocity waveforms were acquired at the aortic arch, followed immediately by measurement at the descending aorta proximal to the iliac bifurcation.  

Oxidative stress. Aortic oxidative stress was assessed by immunostaining for 3-nitrotyrosine (3-NT), as described previously (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 and rehydrated, and antigen was retrieved and incubated overnight with 1:150 primary rabbit polyclonal anti-3-NT antibody (Millipore). Sections were washed and incubated for 30 min with secondary antibodies, biotinylated anti-rabbit, and streptavidin-horseradish peroxidase. After several rinses with distilled water, diaminobenzidine was applied for 7 min, and sections were again rinsed and stained with hematoxylin for 90 s, dehydrated, and mounted with permount. For quantifying 3-NT generation in VSMCs, equal endothelial layer was carefully traced and the brown color quantified as grayscale intensities. To quantify 3-NT in ECs, the antigen was retrieved and incubated overnight with 1:150 primary rabbit polyclonal anti-3-NT antibody (Millipore). Sections were dewaxed and rehydrated, and sections were again rinsed and stained with hematoxylin for 90 s, dehydrated, and mounted with permount. For 3-NT quantification, all colors on the sections were deleted except for the brown color, which is indicative of 3-NT formation in the different component of the aorta. The slides were checked under a brightfield (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 grayscale intensities. To quantify 3-NT in ECs, the endothelial layer was carefully traced and the brown color quantified only in that region. For quantifying 3-NT generation in VSMCs, equal regions of interest were measured 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/fluorimetric assay kit (TBARS assay kit; Cayman Chemical, Ann Arbor, MI).

Statistical analysis. Results are reported as means ± SE. Statistical analysis was done primarily 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 \[
\left(\frac{F_{max} - F_0}{F_{max} - F_{min}}\right) \times 100.
\]

Transmission electron microscopy. Aorta samples were fixed in 2% paraformaldehyde and 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 then incubated at 4°C for 1 h and then rinsed with 2-ME buffer. Block staining was performed using 1% aqueous uranyl acetate and incubated at 4°C overnight and 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, 12 endothelial images per group were acquired at ×800 and/or ×1,000 magnification for measurement of EC length with existing a scale bar on image. To measure EC length, 3 ECs 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 wk 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. 1, A and 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 demonstrated previously and published that 16 wk of WD feeding results in aortic stiffness compared to animals fed a CD (2.07 ± 0.54 vs. 0.55 ± 0.09 mg/dl, P < 0.05; Fig. ID). In addition, XO activity was increased ~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 ± 0.27 U/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 wk 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 compared with CD (16.9 ± 0.50 vs. 5.21 ± 0.54 kPa; Fig. 2A). XO inhibition with allopurinol significantly reduced aortic stiffness to levels comparable with 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 wk relative to mice fed a CD (17, 29, 42). In our experiments, treatment with allopurinol did not significantly affect 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 shown previously that 16 wk of WD feeding in female C57BL/6J results in impaired endothelium-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 endothelium-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 with female mice fed a CD (E_max = 35.1 ± 2.20 and 38.9 ± 2.70% for ACh and SNP, respectively), as published previously by our laboratory (17). The present data demonstrate that XO inhibition prevents WD-induced aortic stiffness in parallel with greater endothelium-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 MDA concentrations in plasma. WD-fed mice demonstrated a significant elevation in 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). Furthermore, 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 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

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Fig. 2. XO inhibition improves WD-induced aortic stiffness and increases endothelium-dependent and -independent vasodilatory responses in the setting of WD feeding. A: aortic endothelial stiffness was assessed via atomic force microscopy (AFM) of aortic explants from C57BL/6J female mice fed a WD for 16 wk in the presence or absence of ALLO in drinking water. *P < 0.05, control diet (CD) vs. WD; #P < 0.05, WD vs. WD-ALLO; n = 4–7 for all groups. In addition, vasomotor responses were evaluated in C57BL/6J female mice fed a WD for 16 wk in the presence or absence of ALLO in drinking water. B and C: responses of isolated aortic rings to the endothelium-dependent dilator acetylcholine (B) and the endothelium-independent vasodilator sodium nitroprusside (C). §P < 0.05, WD vs. WD + ALLO; n = 4–7.
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 wk 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 transmission electron microscopy 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). Similarly 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 overnutrition 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, derived mostly 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 before joining the glycolytic pathway. In the liver, fructokinase phosphorylates fructose to fructose 1-phosphate in an unregulated manner, leading to depletion of adenosine triphosphate, 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 with regard 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

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**Fig. 4.** XO inhibition decreases aortic fibrosis. *Left:* analysis of fibrosis by picrosirius red staining in aortas from C57BL/6J female mice fed WD for 16 wk in the presence or absence of ALLO in drinking water. *Right:* collagen deposition quantified as average grayscale intensities. *P* < 0.05, WD vs. CD; #*P* < 0.05 WD vs. WD + ALLO; *n* = 4 all groups.

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**Fig. 5.** ALLO 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 wk in the presence or absence of ALLO in drinking water (representative images). CD (*A*) and CD + ALLO (*B*) 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. *C:* layering of highly organized, compact, dense, fibrillar collagen (*C*; demarked by dashed line). *D:* ALLO treatment protects against additional layering of mature, highly organized, compact, dense, fibrillar collagen relative to WD-fed animals in *C.* Magnification, ×400. Scale bar, 5 μm; *n* = 12 for all groups.
vascular stiffness appears to have more severe implications in females than in males. Indeed, in a cohort of more than 88,000 women, the consumption of high-fructose diet in the form of two sugar-sweetened beverages resulted in a 35% increased risk of coronary artery disease relative to men (24). In a Japanese cohort of more than 38,000 subjects, sugar-sweetened beverages were associated with ischemic stroke only in 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 with men (56). Therefore, the augmented incidence and greater severity of CV disease in obese and diabetic women can be related partially 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 with 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 overnutrition (17, 29, 42). In this study, we evaluated systemically oxidative stress by measuring plasma levels of MDA and 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 with mice fed a CD and treated with allopurinol.

Moreover, similar results were demonstrated in our measurements of XO 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 reported previously that WD feeding increases cardiovascular XO activity in males and that allopurinol treatment for 16 wk mitigates this WD-mediated increase (30).
Interestingly, administration of allopurinol resulted in improvements in endothelial stiffness in the absence of significant changes in 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 male mice also fed a WD for 16 wk 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 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 ~70% for SNP in female mice fed a CD (17, 29, 42). Available literature has reported comparable results with our published data (58, 59), whereas others studies report higher vasomotor responses compared with 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 wk results in reduction of maximal vasorelaxation in aorta of ~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, ~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 demonstrate 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 nonsignificant increase in plasma uric acid levels in our female mice. This modest response has been reported previously 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 with humans. Furthermore, similarly to glucose, fructose can be reabsorbed from the filtrate in the renal proximal tubule via transporters such as glucose transporter (GLUT)S and GLUT9, and hyperglycemia, which 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 GLUT9, 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 wk (30), which additionally 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, whereas 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 on reduction of uric acid levels and might have been related to decreased oxidative stress (25). Another recent analysis of an elderly hypertensive population data set in the UK 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 PRISE 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 contribute to a better understanding of the impact of XO activation on vascular stiffness in females. However, additional studies comparing males with females are warranted and will contribute to determining a possible differential CVD benefit from XO inhibition in females compared with 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 with males, our findings stimulate the possibility that females consuming a WD may benefit specially from pharmacological 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.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS


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