Low-Dose Mineralocorticoid Receptor Blockade Prevents Western Diet–Induced Arterial Stiffening in Female Mice


Abstract—Women are especially predisposed to development of arterial stiffening secondary to obesity because of consumption of excessive calories. Enhanced activation of vascular mineralocorticoid receptors impairs insulin signaling, induces oxidative stress, inflammation, and maladaptive immune responses. We tested whether a subpressor dose of mineralocorticoid receptor antagonist, spironolactone (1 mg/kg per day) prevents aortic and femoral artery stiffening in female C57BL/6J mice fed a high-fat/high-sugar western diet (WD) for 4 months (ie, from 4–20 weeks of age). Aortic and femoral artery stiffness were assessed using ultrasound, pressurized vessel preparations, and atomic force microscopy. WD induced weight gain and insulin resistance compared with control diet–fed mice and these abnormalities were unaffected by spironolactone. Blood pressures and heart rates were normal and unaffected by diet or spironolactone. Spironolactone prevented WD-induced stiffening of aorta and femoral artery, as well as endothelial and vascular smooth muscle cells, within aortic explants. Spironolactone prevented WD-induced impaired aortic protein kinase B/endothelial nitric oxide synthase signaling, as well as impaired endothelium-dependent and endothelium-independent vasodilation. Spironolactone ameliorated WD-induced aortic medial thickening and fibrosis and the associated activation of the progrowth extracellular receptor kinase 1/2 pathway. Finally, preservation of normal arterial stiffness with spironolactone in WD-fed mice was associated with attenuated systemic and vascular inflammation and an anti-inflammatory shift in vascular immune cell marker genes. Low-dose spironolactone may represent a novel prevention strategy to attenuate vascular inflammation, oxidative stress, and growth pathway signaling and remodeling to prevent development of arterial stiffening secondary to consumption of a WD. (Hypertension. 2015;66:99-107. DOI: 10.1161/HYPERTENSIONAHA.115.05674.) ● Online Data Supplement

Key Words: aldosterone ■ obesity ■ spironolactone ■ vascular stiffness

In the setting of obesity and diabetes mellitus, women exhibit significant cardiovascular disease (CVD) more frequently and with higher severity than diabetic men.1,2 This is in contrast to lean nondiabetic premenopausal women that have lower incidences of CVD relative to similar men. Increased aortic stiffness independently predicts future CVD, especially in women.3,4 Aortic stiffening, which is a normal aging phenomenon, is inordinately deleterious in postmenopausal women compared with men.5,6 Moreover, women struggling from obesity have elevated arterial stiffness and are more vulnerable to CVD compared with men.7–9 Epidemiological studies reveal that measurement of aortic stiffness has a superior predictive value for determining CV risk compared with classical CV risk factors and, as such, has emerged as an important biomarker predictive of end organ damage and overall CVD risk.10,11 Given the ongoing epidemic of obesity, targeting arterial stiffness to reduce the risk for a first CV event9,11 with pharmacological or lifestyle interventions is urgently needed, especially for women. Importantly, few studies address the impact of important environmental factors, such as diet, in the genesis of CVD in females. This has led to a call by the National Institutes of Health and the American Heart Association to fill this void with more studies on CVD in females.12

Emerging evidence supports the notion that overactivation of vascular mineralocorticoid receptors (MRs) contribute to CVD.13–15 The sex-related differences in CVD are due, in part, to steroid hormones.16 Increasing evidence of a role for aldosterone...
and MR signaling in development of arterial stiffness has emanated from multiple studies.\textsuperscript{17–20} A recent clinical study reported an association between elevated serum aldosterone concentrations and increased aortic stiffening in normotensive overweight and obese adults aged 20 to 45 years.\textsuperscript{21} Moreover, accumulating evidence suggests improvement in arterial elasticity occurs with administration of MR antagonists (MRA).\textsuperscript{22–24}

Obesity-related vascular insulin resistance promotes endothelial cell (EC) dysfunction as a consequence of impaired insulin-mediated activation of endothelial nitric oxide synthase (eNOS) to reduce bioavailability of nitric oxide (NO).\textsuperscript{25} In obesity, impaired EC-mediated vascular relaxation could also result from MR signaling-mediated increased generation of reactive oxygen species with subsequent NO scavenging and reduction in bioavailable NO, which normally reduces extracellular matrix remodeling and stiffening.\textsuperscript{14,15} In addition, abnormal immune and inflammatory responses contribute to vascular dysfunction and stiffness.\textsuperscript{26} Extracellular matrix remodeling underlies age-related arterial stiffening.\textsuperscript{10,26}

We recently reported that low-dose spironolactone administered to female mice prevents Western diet (WD)-induced development of myocardial stiffness and diastolic dysfunction.\textsuperscript{27} That study demonstrated an important role for the MR in promoting myocardial oxidative stress, fibrosis, and impaired immunity associated with decreased left ventricular compliance resulting from consumption of a WD. The idea that an increase in vascular stiffness secondary to consumption of a WD can be mediated by excessive MR signaling is intriguing and raises the question whether using a low (subpressor) dose of an MRA could improve compliance in conduit and muscular arteries (eg, aorta and femoral artery). To test this hypothesis, we administered a low dose of the MRA, spironolactone to female C57BL/6J mice fed a WD high in fat and the refined sugars, sucrose, and high-fructose corn syrup (HFCS). Herein, we report that vascular MR signaling plays an important role in inflammatory and immune responses and development of aortic and femoral artery stiffening secondary to consumption of a WD. Importantly, the development of these CV abnormalities can be prevented with a low dose of spironolactone.

Methods
For detailed description of procedures, see Methods in the online-only Data Supplement.

Results
WD-Induced Increases in Body and Fat Pad Weights and Systemic Insulin Resistance Were Not Prevented by Spironolactone

Body weights of 20-week-old WD control (WDC) and WD spironolactone (WDSp) mice were similarly heavier compared with their lean counterparts (Table S1 in the online-only Data Supplement). Percent body weight gain at the end of the study period was \(71\pm8\%\) and \(97\pm12\%\) for control diet control (CDC) and WDC (\(P=0.06\)), respectively, and \(74\pm7\%\) and \(98\pm7\%\) for CD spironolactone (CDSp) and WDSp (\(P<0.05\)), respectively, and weight gain was unaffected by spironolactone. Perireproductive and retroperitoneal fat pad masses were \(3\text{-}4\)-fold higher in WDC versus CDC (\(P<0.01\)), respectively, and these changes were not altered by spironolactone (data not shown). Systemic glucose homeostasis, evaluated by intraperitoneal glucose tolerance, was impaired after WD feeding. The area under the curve of the glycemic excursion after the intraperitoneal glucose challenge was increased in WD-fed mice versus CD-fed mice (\(P<0.05\); Figure S1). The area under the curve was unaffected by spironolactone in WD-fed mice.

Spironolactone Prevents WD-Induced Increases in Aortic Pulse Wave Velocity

In vivo pulse wave velocity (PWV), determined in mice after 2 and 3 months on CD or WD (Figure 1A; Table S2), was unaffected by diet or spironolactone; however, both WD and Sp affected PWV significantly at the 4-month time point. PWV was elevated in the WDC group compared with CDC (\(P=0.006\)) and Sp prevented the elevation in PWV (\(P<0.001\)). Mean arterial pressure and heart rate were not different among groups at the end of the study (Figure 1B).

Spironolactone Prevents WD-Induced Increases in Aortic EC and Vascular Smooth Muscle Cell Stiffness

To determine whether intact EC and vascular smooth muscle cell (VSMC) stiffened in response to WD, we measured surface mechanical stiffness of EC and VSMC in aortic explants using atomic force microscopy (Figure 1C–1H). Both EC and VSMC exhibited an \(5\)-fold increase in surface stiffness (\(P<0.05\), CDC versus WDC), and these effects were prevented by spironolactone administration (\(P<0.05\) for CDC versus WDSp and \(P>0.05\) for CDC versus WDSp).

Spironolactone Prevents WD-Induced Endothelial Dysfunction in the Aorta

Endothelium-dependent vasodilatory responses to acetylcholine were decreased in WDC compared with CDC (\(E_{\text{max}}=16.3\pm2.7\%\), \(n=4\) versus \(35.1\pm2.2\%\), \(n=6\) and \(4\), respectively), and these defects were prevented in the WDSp group (\(E_{\text{max}}=29.8\pm1.8\%\) \(n=4\); Figure 1J). Similarly, endothelium-dependent vasodilatory responses to insulin were also impaired in WDC mice (\(E_{\text{max}}=3.4\pm6.5\%\) versus \(86.1\pm1.7\%\); \(n=6\) and \(4\), respectively), and improved in the WDSp group (\(E_{\text{max}}=64.2\pm5.4\%\), \(n=4\); Figure 1J). Endothelium-independent vasodilatory responses to sodium nitroprusside were also decreased in WD compared with CDC (\(E_{\text{max}}=18.7\pm0.7\%\), \(n=4\) versus \(38.9\pm2.7\%\), \(n=6\) and \(4\), respectively), and these defects were abolished in the WDSp group (\(E_{\text{max}}=35.7\pm2.6\%\), \(n=4\); Figure 1K). Collectively, these data are consistent with improved aortic EC and VSMC function in WDSp animals.

Spironolactone Ameliorates WD-Induced Aortic Remodeling

The medial layer of the aorta was \(18\%\) thicker in WD-fed mice compared with CD-fed mice (\(P<0.01\)) and thickening was prevented by spironolactone treatment (Figure 2A; \(P<0.05\), WDC versus WDSp). Adventitial collagen accumulation was significantly enhanced in WD relative to CD-fed mice and this accumulation was prevented by spironolactone treatment (Figure 2B and 2C). Fibronectin accumulation predominately in the adventitia in all groups; however, compared with CD, WD induced an increase in adventitial fibronectin and spironolactone prevented this abnormality (Figure 2D).
WD-Induced Vascular Oxidative Stress Was Improved by Spironolactone

WD induced increases in oxidant stress, assessed by 3-nitrotyrosine staining, in each layer of the aorta compared with CDC (P<0.05 for each layer; Figure 2E). Staining was most intense in the endothelium, modest in the adventitia, and relatively minimal in the medial layer. Spironolactone largely prevented 3-nitrotyrosine accumulation in the endothelium and adventitia (P<0.05, WDC versus WDSp for both layers).

Spironolactone Prevented WD-Induced Activation of ERK1/2 and Impairment in Protein Kinase B (Akt)/eNOS in the Aorta

WD induced a 3-fold increase in ERK1/2 activation (P<0.05, CDC versus WDC) that was prevented by spironolactone (P<0.05, WDC versus WDSp; Figure 3A). WD induced decreases in the phosphorylation (p) of both p-AktSer473 and p-eNOSSer1177 in aorta protein extracts compared with those from CDC mice (P<0.05 for both proteins; Figure 3B and 3C). Spironolactone preserved the normal activation levels in both p-AktSer473 and p-eNOSSer1177 protein compared with WDC group (P<0.05 for each protein).

Spironolactone Ameliorates Immune and Inflammatory Changes in the Aorta

Inflammatory Markers
Levels of the proinflammatory M1 marker transcripts, monocyte chemoattractant protein-1 and CD 86, were elevated in aortic extracts of WDC and WDSp versus CDC mice (Figure 4A). The transcript level of interleukin (IL)-10, an anti-inflammatory M2 marker, was elevated only in WDSp. The ratios of M2 to M1 expression illustrates that there was a relative increase in macrophage polarization favoring M2 in the WDSp group compared with WDC (Figure 4B). In addition, the aorta of WDC mice exhibited significantly elevated levels of the macrophage marker, CD11b, which was not affected by spironolactone. Nonetheless, there was a relative increase in macrophage polarization favoring M2 in the aorta of WDSp mice compared with WDC mice (Figure 4C).
WDSp group compared with WDC as indicated by the significantly higher IL-10/CD11b ratio (Figure 4B).

**Acetylcholine-Induced Vasodilation and Arterial Structure in Femoral Arteries Are Significantly Improved by Spironolactone**

No significant effects of diet or in vivo spironolactone were observed on KCl- or phenylephrine-induced constrictions in femoral arteries (Figure 5A). Endothelium-dependent acetylcholine-induced vasodilation responses were not significantly affected by WD feeding. However, spironolactone significantly increased acetylcholine-induced vasodilatory responses in WD-fed mice, WDSp versus WDC (Emax=80.5±15.9%, n=4 versus 42.6±15.6%, n=5, respectively; P<0.05). This greater relaxation response associated with spironolactone was not observed in CD-fed mice (Figure 5B). No significant

---

**Figure 2.** Aortic remodeling in untreated Western diet–fed mice (WDC) mice is prevented by mineralocorticoid receptor antagonism (WDSp). **A,** Representative micrographs show medial wall thickening, periaortic fibrosis by **(B)** Verhoeff-Van Gieson, **(C)** picrosirius red staining, and **(D)** adventitial accumulation of fibronectin. **E,** Immunostaining analysis for 3-nitrotyrosine staining. The vessel lumen is indicated by the letter L. Values are mean±SE; n=5 per group. Post hoc comparisons within a time point; *P*<0.05 CDC vs WDC; †P<0.05 WDC vs WDSp. CDC indicates control diet control; CDSp, control diet spironolactone; WDC, Western diet control; and WDSp, Western diet spironolactone.

**Figure 3.** Immunoblot analysis of **(A)** activation of ERK1/2, **(B)** p-Akt, and **(C)** endothelial nitric oxide synthase (eNOS) expression relative to total Akt and eNOS expression, respectively. Values are mean±SE; n=3 per group. Post hoc comparisons within a time point; *P*<0.05 CDC vs WDC; †P<0.05 WDC vs WDSp. CDC indicates control diet control; CDSp, control diet spironolactone; WDC, Western diet control; and WDSp, Western diet spironolactone.
differences were found for the endothelium-independent vasodilatory responses to sodium nitroprusside between any of the groups (Figure 5C).

Effects of Spironolactone and Diet on Femoral Artery Structure
Femoral arteries from WD-fed mice were less distensible than those of CD-fed mice as indicated by a significant leftward shift in their circumferential wall stress–strain curves (Figure 5D). Femoral arteries of WDSp mice were significantly more distensible than those of WDC. The modulus of elasticity, used as an index of stiffness, was significantly elevated in femoral arteries of WDC mice compared with those of CDC and WDSp (Figure 5E). No significant differences were observed for the internal passive pressure–diameter relationships, an index of remodeling, or were there differences in wall/lumen ratios, mean wall thickness, and wall cross-sectional areas between any of the groups (Figure 5F–5I).

Discussion
Collectively, the results of this investigation support the hypothesis that MR plays an important role in the development of aortic stiffness in female mice in the setting of WD-induced insulin resistance. Herein, we demonstrate that aortic PWV is increased in female mice fed a diet high in fat and refined sugars (sucrose/HFCS). Although MR blockade had no effect on reducing excess body or fat pad weight, it completely prevented the increase in PWV observed in the untreated mice fed WD. Atomic force microscopy was used to further assess potential cellular contributions to aortic stiffness using ex vivo aortic explants to measure EC and VSMC stiffness. Intact explants enable measurement of stiffness of EC and VSMC that are still in contact with native extracellular matrix proteins. Our findings in both types of preparations identified abnormally high stiffness in WD-fed mice that was prevented with spironolactone.

Abnormalities in the insulin signaling cascade in the aorta, including decreased Akt activation, have been documented in models of obesity and insulin resistance.28,29 In this study, we observed decreased vascular Akt phosphorylation (activation) in untreated WD-fed mice compared with their CD-fed counterparts. We also examined phosphorylation of eNOS as a downstream target of insulin-Akt signaling. Compared with untreated mice fed CD, we observed a marked decrease in phosphorylated eNOS1177 and the ratio of p-eNOS1177/total eNOS in WD-fed female mice. Therefore, decreased eNOS phosphorylation may account, in part, for the impairment of aortic endothelial function and this may contribute to the observed aortic stiffening. A recent study indicated that aldosterone contributes importantly to a phenomenon known as stiff endothelial cell syndrome mediated by increased EC expression of the epithelial sodium channel and MR in concert with impaired generation of NO, and that spironolactone can prevent its manifestation and improve endothelial function.22 Therefore, therapies that increase EC NO bioavailability and improve EC function could potentially reduce aortic stiffening in overweight individuals.11

Inflammation because of maladaptive immune responses has recently been implicated in playing a role in development of vascular disease, including vascular stiffness.15,30 One of the immune mechanisms related to inflammation is polarization of macrophages.23 The activation of an M1 macrophage phenotype leads to a proinflammatory response and conversely, activation of M2 macrophages mount an opposing anti-inflammatory response. Nonetheless, no studies have linked consumption of a high fat/high sucrose/HFCS diet with aortic stiffening, dysfunction and remodeling, and an abnormal immune and inflammatory response and macrophage polarization. We observed increases in monocyte chemoattractant protein-1, and CD86 in the aorta of WD-fed mice suggestive of an inflammatory response. Even though spironolactone did not prevent the WD-induced increase in these proinflammatory markers, it significantly increased the M2 macrophage marker, IL-10, leading to improvement in the M1/M2 ratio suggesting suppression of the inflammatory response. Recent evidence indicates that IL-10 may contribute to improved cardiovascular insulin sensitivity.31

Figure 4. Effect of spironolactone on macrophage polarization. A, mRNA expression of M1 markers, MCP-1 and CD86, as well as the total macrophage cell marker, CD11b, in aorta. B, mRNA expression of the M2 marker, interleukin (IL)-10, and the ratios of M2/M1 and M2/total macrophage expression in aorta. Values are mean±SE; n=3 per group; *P<0.05 CDC vs WDC; †P<0.05 WDC vs WDSp. CDC indicates control diet control; CDSp, control diet spironolactone; MCP-1, monocyte chemoattractant protein-1; WDC, Western diet control; and WDSp, Western diet spironolactone.
In addition, IL-10 released from regulatory T cells has also been shown to improve endothelial function by suppressing NADPH oxidase–mediated oxidative stress. Therefore, the apparent improvement in M2 macrophage polarization observed in this study may contribute to prevention of vascular injury by MR blockade. Although insulin metabolic signaling through Akt/eNOS pathway is impaired, growth factor signaling through activation of ERK1/2 pathway is either not affected or stimulated. In this regard, ERK activation modulates vascular remodeling and inflammatory response. Thus, ERK activation may contribute to vascular inflammation, remodeling, and arterial stiffness, as observed in this study.

In addition to the aorta, we also examined the function and structure of the femoral artery. As established by the recent Hoorn study, local arterial stiffness measures, specifically in the femoral and carotid arteries, but not brachial artery, independently predict CVD risk. This is the first study to show that WD feeding results in impairments in femoral artery distensibility and stiffness and that low-dose MRA prevents these impairments. In this study, endothelium-dependent and endothelium-independent vasodilatory responses to acetylcholine and sodium nitroprusside in the femoral artery were not affected by WD feeding, but spironolactone treatment significantly enhanced endothelial-dependent vasodilation in femoral arteries (Figure 5).

Figure 5. Effect of spironolactone on Western diet (WD)-induced femoral artery endothelial dysfunction and vascular stiffness. Femoral artery constriction to phenylephrine (A), vasodilation to acetylcholine (B), and sodium nitroprusside (SNP; C). Femoral artery strain–stress relationship and femoral artery modulus of elasticity–pressure curve. Femoral artery remodeling is indicated by pressure–diameter relationships (D), wall/lumen–pressure relationships (E), mean wall thickness–pressure relationship (F), and cross-sectional area–pressure relationship (G). Values are mean±SE; n=3 to 7 per group; †P<0.05 WDSp vs WDC. CDC indicates control diet control; CDSp, control diet spironolactone; CSA, cross sectional area; WDC, Western diet control; and WDSp, Western diet spironolactone.
Mineralocorticoid Blockade and Aortic Stiffness

In this study, we have used a low dose of spironolactone, and the rationale for this is 3-fold. First, we previously established that this dose does not affect blood pressure. Second, in addition to the MR, spironolactone binds to androgen, progesterone, and glucocorticoid receptors; however, binding to non-MR is likely to require much higher doses of spironolactone to induce antiandrogenic or progesterone actions. Finally, addition of low dose of a MRA to standard therapy has been shown to reduce morbidity and mortality among patients with heart failure.

Thus, it could be reasoned that addition of low-dose MRAs could prevent further progression of vascular stiffening and the associated risk of heart failure in obese females regardless of diabetic status and with minimal risk of side effects. Moreover, it is likely that the antistiffening effects of MRAs relate largely to preventing aldosterone because opposed to glucocorticoid-mediated MR signaling in the vasculature. This is because both vascular endothelial and smooth muscle cells express 11β hydroxysteroid dehydrogenase, an enzyme that limits glucocorticoid signaling through the MR receptor.

In contrast to our study, others have reported resistance to the metabolic and CV complications associated with high-fat diets in female mice, suggesting estrogen-mediated CV protection. Such studies have mostly used high-fat diets rather than diets high in both fat and refined sugars like the diet used in this investigation. The abrogation of CV protection manifested in our model as an increase in aortic stiffness is consistent with clinical studies reporting increases in aortic PWV in overweight women. Data from the Framingham Heart Study indicate that women have higher serum aldosterone concentrations that correlate with a pattern of left ventricular concentric remodeling. We previously reported that young female C57BL6 mice exhibit higher serum levels of aldosterone compared with males (≈50% higher in females), but WD had no effect on aldosterone levels in males or females. Therefore, we speculate that the interaction of higher aldosterone levels in females and consumption of a WD high in fat, sucrose, and HFCS may act synergistically to promote the observed increases in oxidative stress, inflammation, and vascular stiffness. However, a recent in vitro study showed that estrogen can suppress aldosterone-mediated expression of genes in EC that contribute to CV dysfunction and disease. Thus, it is possible that this antagonistic effect of estrogen on deleterious MR signaling in the vasculature may be abrogated with high fat/sucose/HFCS diet.

The results of this study indicate that the effects of spironolactone on prevention of WD-induced vascular stiffness occurred in the absence of an increase in mean arterial pressure and HR. Moreover, aortic stiffness was not increased in control mice at the end of the feeding trial, therefore, the effects seen in WD are not because of normal aging processes. The potential influence of the female estrous cycle on PWV is a potential limitation of this study. Nonetheless, a previous report demonstrated that PWV does not vary over the course of the menstrual cycle in women.

In conclusion, we have elucidated a role for the MR in development of aortic stiffness and the associated abnormalities in immune responses, oxidative stress, endothelial/smooth muscle function, and structural remodeling in a clinically relevant female model of weight gain because of consumption of a WD high in fat, HFCS, and sucrose. Importantly, aortic stiffness was detected not only in vivo, but also on the aortic EC and VSMC surfaces of ex vivo preparations. These findings may be particularly relevant to overweight and obese women who may be at increased risk for development of aortic stiffness and CVD.

Perspectives

Results of this investigation suggest not only that MR play a critical role in development of WD-induced aortic stiffness, but also that a low dose of spironolactone exerts profound prophylaxis to maintain vascular health in individuals consuming a high fat/sucose/HFCS diet. Targeting the MR with adjunctive low-dose spironolactone may be an effective strategy to limit vascular disease progression in individuals struggling from overweight/obesity that are at risk for a first CV event. Our results suggest that this strategy could minimize the likelihood of side-effects associated with spironolactone (hypokalemia or hypotension) and maximize the efficacy of MRA. Thus, the rationale for this strategy parallels the rationale for use of a low dose of spironolactone to treat severe heart failure reported in Randomized Aldactone Evaluation Study (RALES).

Acknowledgments

We gratefully acknowledge Brenda Hunter for editorial assistance, Nathan Rehmer, Alex Meuth, Terry L. Carmack, and Lisa D. Watkinson for technical support. This work was supported with resources and facilities at the Harry S. Truman Memorial Veterans Hospital in Columbia, MO, including the Biomolecular Imaging Center (MRI) and the Small Animal Ultrasound Imaging Center (Echocardiography), as well as the Atomic Force Microscopy Center in the Dalton Cardiovascular Research Center.

Sources of Funding

This work was supported by the Department of Medicine Research Council (V.G. DeMarco), National Institutes of Health (R01-HL073101 R01-HL107910 to J.R. Sowers, R01 HL088105 to L.A. Martinez-Lemus, and P01 HL095486 to G.A. Meininger), Department of Veterans Affairs Biomedical Laboratory Research and Development (CDA-2 IK2 BX002030 to S.B. Bender, 0018 to J.R. Sowers).

Disclosures

None.

References


10.3317/jraas.2006.004.

10.3317/jraas.2006.004.
Novelty and Significance

What Is New?
- These data indicate that consumption of a Western diet, high in fat, and refined sugars promotes vascular insulin resistance and stiffness in female mice.
- Development of this vascular phenotype can be prevented by a low dose of the mineralocorticoid receptor blocker, spironolactone.

What Is Relevant?
- Vascular stiffness is a strong predictor for cardiovascular events and under insulin resistant conditions women have greater stiffness compared with men.

Summary
Results of this investigation suggest that enhanced mineralocorticoid receptor activation plays an integral role in development of vascular resistance that is promoted by consumption of a Western diet in females.
Low-Dose Mineralocorticoid Receptor Blockade Prevents Western Diet–Induced Arterial Stiffening in Female Mice


Hypertension. 2015;66:99-107; originally published online May 26, 2015; doi: 10.1161/HYPERTENSIONAHA.115.05674

Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2015 American Heart Association, Inc. All rights reserved.
Print ISSN: 0194-911X. Online ISSN: 1524-4563

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://hyper.ahajournals.org/content/66/1/99

Data Supplement (unedited) at:
http://hyper.ahajournals.org/content/suppl/2015/05/26/HYPERTENSIONAHA.115.05674.DC1

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

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

Subscriptions: Information about subscribing to Hypertension is online at:
http://hyper.ahajournals.org//subscriptions/
Low Dose Mineralocorticoid Receptor Blockade Prevents Western Diet-induced Arterial Stiffening in Female Mice

*Vincent G. DeMarco1,3,5, *Javad Habibi1,5, *Guanghong Jia1,5, Annayya R. Aroor1,5, Francisco Ramirez3,6, Luis Martinez-Lemus3,6, Shawn B. Bender3,4,6, Mona Garro1,5, Melvin R. Hayden1,5, Zhe Sun3,6, Gerald A Meininger3,6 Camila Manrique1,5, Adam Whaley-Connell2,5, and James R. Sowers1,3,5

Department of Medicine, Division of Endocrinology, Diabetes and Metabolism1 and Division of Nephrology2, Department of Medical Pharmacology and Physiology3, Department of Biomedical Sciences4, University of Missouri Columbia School of Medicine, Research Service, Harry S. Truman Memorial Veterans Hospital5, and the Dalton Cardiovascular Research Center6, Columbia, MO, USA

Running Title: Mineralocorticoid blockade ameliorates aortic stiffness

*contributed equally to this work

Co-corresponding Authors:
Dr Vincent G. DeMarco, PhD and Dr James Sowers, MD
Department of Medicine
Division of Endocrinology, Diabetes and Metabolism
University of Missouri-Columbia School of Medicine
One Hospital Drive
Columbia, MO 65212
Phone: (573) 884-5530
E-mail: sowersj@missouri.edu
E-mail: demarcov@missouri.edu

Key Words: Aldosterone, spironolactone, pulse wave velocity, atomic force microscopy

Sources of Funding: This work was supported by the Department of Medicine Research Council (VGD), National Institutes of Health (R01-HL073101 R01-HL107910 to JRS, R01 HL088105 LAM-L and P01 HL095486 to GAM), Department of Veterans Affairs Biomedical Laboratory Research & Development (CDA-2 IK2 BX002030 to SBB, 0018 to JRS).

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 the Harry S Truman VA Research Center and mice were cared for according to NIH guidelines. When mice were four weeks of age mice they were randomly assigned to one of four groups that included 1) mice fed a control diet (CD) (Test Diet 58Y2, Richmond, Indiana) and implanted with a control placebo pellet (CDC), 2) mice fed a CD and implanted with a
pellet designed to deliver 1 mg•kg\(^{-1}\)•day\(^{-1}\) Sp subcutaneously (CDSp) (Innovative Research of America, Sarasota, FL), 3) mice fed a WD containing high fat (46%) and high carbohydrate as sucrose (17.5%) and high fructose corn syrup (HFCS) (17.5%) (Test Diet 58Y1) and implanted with a control pellet (WDC) and 4) mice fed a WD and implanted with a Sp pellet (WDSp). Pellets were implanted on the back between the scapulae during brief isoflurane anesthesia. Mice were housed in pairs under a 12-hour light/dark regimen and water and food were provided ad libitum. The rationale for choosing a dose of 1 mg•kg\(^{-1}\)•day\(^{-1}\) Sp was based on established evidence that this dose neither reduces blood pressure nor induces anti-androgenic and progestogenic effects.\(^2\) In fact, it has been shown that 1 mg•kg\(^{-1}\)•day\(^{-1}\) Sp inhibits just 35% of \textit{in vivo} aldosterone binding to the MR.\(^2\)

### Assessment of Whole-Body Insulin Sensitivity

Intraperitoneal glucose tolerance test (IPGTT) was performed following a 5-hour fast as previously described.\(^3\) Briefly, dextrose (1 g • kg\(^{-1}\)) was injected intraperitoneally and the glucose excursion was monitored over time and compared between treatment groups. Blood samples were analyzed for glucose (AlphaTRACK, Abbott, Ill., USA) at time 0 and 15, 30, 45, 60, and 120 min following dextrose injection. The cumulative glycemic excursion was evaluated as the area under the curve (AUC).

### Aortic Stiffness by \textit{in vivo} Pulse Wave Velocity

Doppler ultrasound (Indus Mouse Doppler System, Webster, TX) was performed on female mice according to a previously established protocol\(^4\) to evaluate pulse wave velocity (PWV), the gold standard technique for \textit{in vivo} determination of arterial stiffness. Determination of PWV is based on the transit time method utilized to determine the difference in arrival times of a Doppler pulse wave at two locations along the aorta a known distance apart. Each of the pulse wave arrival times is 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 is divided by the difference in arrival times and is expressed in m/s. Velocity waveforms were acquired at the aortic arch followed immediately by measurement at the descending aorta 35 mm distal to the aortic arch. Ultrasound procedures were performed on isoflurane-anesthetized mice (1.75% in 100% oxygen stream).

### Blood Pressure

While under isoflurane (1.75% isoflurane in 100% O\(_2\)) anesthesia, the right carotid artery was catheterized with a 1.2 French mouse pressure catheter (Transonic) so the tip was proximate to the aortic arch. After blood pressure was stable following a brief acclimation period, average mean arterial pressure (MAP) was determined with an Advantage Data Acquisition System (Seisense, Ontario Canada) as previously reported.\(^5\)

### Preparation of \textit{en face} \textit{ex vivo} Aortic Explants for AFM

To evaluate the stiffness of EC and VSMC in aortic preparations a 2x2 mm segment of the thoracic aorta was obtained from mice following the sixteen-week experimental period. The aorta was opened longitudinally and the adventitial surface of each explant was fastened to a glass cover slip using cell tak allowing \textit{en face} access by the AFM to the EC surface. Stiffness of the EC surface was measured by...
AFM. VSMC stiffness was evaluated in separate but similar aortic samples with the exception that the EC surface layer was gently removed by rubbing prior to attachment to the cover slip.

AFM Imaging and Force Measurement

The stiffness of EC or VSMC within intact aortic explants from mice was measured using a nano-indentation protocol with AFM according to previously described procedures. A MFP-3D AFM (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: \[ F = \frac{2 E \delta^2}{\pi (1 - \nu^2) \tan \alpha} \]

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 \( \nu \) is the Poisson ratio. The tissues were considered as a gel and \( \nu \) 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.

Ex vivo Vasomotor and Mechanical Responses of Conduit Arteries

Vascular functional parameters were evaluated in the aorta and femoral arteries from the same mice.

Aorta: A 2 mm segment of thoracic aorta was collected immediately after euthanasia and placed in ice-cold 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. Arterial rings were preconstricted with KCl (80 mM•L⁻¹). Dilation of arterial rings to acetylcholine (1 nm to 10 mM), the NO-donor sodium nitroprusside (1 mm to 10 mM), and to insulin ((Novolin R, Novo Nordisk; 0.1 to 300 ng•ml⁻¹) was assessed by cumulative addition of agonist to the vessel bath. The doses of insulin utilized represent physiological (fasting ~0.2 ng•ml⁻¹; post-prandial ~2 ng•ml⁻¹), pathophysiological (>5 ng•ml⁻¹) and pharmacological (>10 ng•ml⁻¹) levels. At the end of each
experiment, the PSS bath solution was replaced with Ca\textsuperscript{2+}-free PSS to determine maximal passive diameter.

**Femoral Arteries:** The proximal femoral artery was isolated and cannulated onto glass micropipettes, pressurized at 70 mmHg without flow, and warmed to 37°C in commercial myograph chambers (Living Systems Instrumentation, Burlington, VT, USA) as previously described.\textsuperscript{9, 10} To test for viability, the cannulated arteries were allowed to stabilize for 40 min and then exposed to physiological saline solution (PSS) in which NaCl was substituted equi-molar substituted with 80 mM KCl. Only arteries that constricted more than 20% to this 80 mM K\textsuperscript{+} solution were used in the analyses.

After the exposure to high K\textsuperscript{+}, the arteries were washed three times with fresh PSS containing (in mM) 145.0 NaCl, 4.7 KCl, 2.0 CaCl\textsubscript{2}, 1.0 MgSO\textsubscript{4}, 1.2 NaH\textsubscript{2}PO\textsubscript{4}, 0.02 EDTA, 2.0 Pyruvic Acid, 5.0 Glucose and 3.0 MOPS at a pH of 7. Vessels were subsequently exposed to increasing concentrations of phenylephrine (10\textsuperscript{-8} to 10\textsuperscript{-4} M) to examine adrenergic-dependent vasoconstriction. After removing phenylephrine with fresh PSS, arteries were pre-constricted with 10\textsuperscript{-5} M phenylephrine and exposed to increasing concentrations of acetylcholine (ACh, 10\textsuperscript{-9} to 10\textsuperscript{-5} M) to determine endothelium-dependent vasodilation. The washing and pre-constriction procedures were repeated to test endothelium-independent vasodilatory responses as vessels were exposed to increasing concentrations of sodium nitroprusside (SNP) (10\textsuperscript{-8} to 10\textsuperscript{-4} M). All vasoactive agents were added in a cumulative fashion to the bath solution at increments of 10\textsuperscript{-4.5} M. Each concentration was maintained in the bath for 2 min. At the end of each experiment, arteries were exposed to Ca\textsuperscript{2+}-free PSS with 2 mM ethylene glycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid (EGTA) and 10\textsuperscript{-4} M adenosine to obtain maximal passive diameter. Vessels were then exposed to consecutive 2 min changes in intraluminal pressure from 5 to 120 mmHg while under passive conditions to determine the elastic properties of the arteries as previously described.\textsuperscript{11, 12} Throughout the experiment, chambers were mounted on inverted microscopes with CCD cameras. Luminal diameter and wall thicknesses were recorded using a video caliper (Living Systems Instrumentation, Burlington, VT, USA) and a Powerlab data acquisition system (ADInstruments Inc, Colorado Springs, CO, USA).

**mRNA isolation and quantitative RT-PCR**

Total RNA was isolated from thoracic aorta extracts using TRIzol reagent (Sigma). RNA yield was determined using a Nanodrop spectrophotometer (Thermo Scientific, Wilmington, DE). First-strand cDNA synthesis and real-time PCR was performed as previously described.\textsuperscript{13} The primer sequences for M1 macrophage markers included: MCP-1, Forward: 5’-GTCTCAGCCGATGCAGTTAAT-3’; Reverse: 5’-CTGCTGGTATTCTCTTGATGT-3’; CD 86, Forward: 5’-GACCGTTGTGTTGTTCTGG-3’, Reverse:5’-GATGAGCAGCATCAAGAGA-3’. The primer sequences for a marker of total macrophage number, CD11b, are Forward: 5’-CCAAGACGATCTCATCATA-3’, Reverse:5’-TTCTGGCTTGCTGAATCTCTT-3’. The primer sequences for the M2 macrophage marker, IL-10, Forward:5’-CCAAGCCTTATCGGAAATGA-3’,Reverse:5’-TTTTCACAGGGGAGAATCG-3’; and GAPDH, Forward: 5’- GGAGAAACCTGCAAGTGATGA-3’, Reverse: 5’-TCCTCAGTGAGCAGAACATGA-3’. Primer set specificity was analyzed by performing a melting curve. Cycling conditions 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, each in triplicate. Results were normalized to the housekeeping gene, GAPDH.

**Protein isolation and quantitation**
Aortic tissues were collected and lysed in lysis buffer and the protein concentration of the lysate was determined by Bio-Rad protein assay. Proteins were resolved by SDS-PAGE and transferred to nitrocellulose membranes. Non-specific proteins were blocked by incubation in blocking buffer and the membranes were incubated overnight at 4°C with blocking buffer containing antibodies to p-ERK1/2\textsuperscript{Thr202/Tyr204}, ERK1/2, p-Akt\textsuperscript{Serine473}, Akt (Cell Signaling Technology, Danvers, MA) and p-eNOS\textsuperscript{Serine1177} and eNOS (BD Biosciences, San Jose, CA).

Vascular Remodeling

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. To evaluate aortic fibrosis sections were stained with picrosirius red (PR) and Verhoeff-von Gieson (VVG) stain for the determination of collagen accumulation. The areas and the intensities of red color on the images which were stained with picro-Sirius red and the areas and intensities of pink color on the VVG stained sections which are the indicative of collagen deposition were quantified as gray scale intensities by using MetaVue software. Fluorescent immunohistochemistry was used to quantify fibronectin deposition. Aorta samples of were prepared as described above. Five µm sections were dewaxed, rehydrated, and placed in 95°C citrate buffer for 25 minutes for antigen retrieval. Non-specific binding cites were blocked with 5% BSA and 5% donkey serum. Next, sections were incubated with 1:50 rabbit polyclonal anti-fibronectin (Epitomic) and 1:200 mouse monoclonal smooth muscle actin (Dako) antibodies separately overnight at room temperature. After several washes the sections were incubated with appropriate secondary antibodies, mounted with Mowiol and the section were checked under a bi-photon confocal microscope (Ziess). The areas and the intensities of red color were quantified by using MetaVue.

Oxidative Stress

Aortic oxidative stress was assessed by immunostaining for 3-nitrotyrosine as previously described.\textsuperscript{14}

Statistical Analysis

Results are reported as the mean ± SE. Statistical analysis was by two-way ANOVA and post hoc t-tests (Bonferroni) to examine differences in outcomes between mice fed CD or WD and treated with placebo or spironolactone (Sigma Plot 12.0, Systat Software). Aortic dilator responses are presented as percent maximal dilation, calculated as \([(Dd – Db)/(Dmax – Db)]\cdot100\), where Dd is diameter after a drug intervention, Db is baseline diameter, and Dmax is maximal passive diameter. All differences were considered significant when p<0.05. For femoral arteries, changes in luminal diameters of arteries to phenylephrine-induced constriction were normalized to the maximal constriction induced by 80 mM KCl, whereas relaxation responses to ACh and SNP were normalized to the maximal constriction produced by Phenylephrine-induced pre-constriction. From the concentration response curves two parameters were obtained, the maximal effect produced by the agonist (\(E_{max}\)), and the half maximal effective concentration (EC\textsubscript{50}). The statistical analyses were performed using R (The R ProjectStatistical computing and graphics software, R 3.1.1 GUI 1.65). Two-way ANOVA) with post hoc Tukey-Kramer HSD (Honestly Significant Difference) tests were applied to adjust for multiple comparisons. Statistical significance was considered at P ≤ 0.05. All data are presented as means ± SEM, and n represents the number of animal used in each group.


Table S1. Body weight of C57BL/6J mice fed a western diet (WD) high in fat and high fructose corn syrup for four months compared to mice fed a control diet (CD). Although mice on the CDs gained weight with age, WD caused an additional increment of body weight gain. Administration of spironolactone (Sp) had no effect on body weight under either dietary regime. Sample sizes are noted in parentheses. Performed 2 way ANOVAs 7/06/14.

<table>
<thead>
<tr>
<th>Body Wt</th>
<th>Main Effect</th>
<th>P value</th>
<th>CDC (6)</th>
<th>CDSp (8)</th>
<th>WDC (6)</th>
<th>WDSp (10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre-Treatment (g)</td>
<td>Diet</td>
<td>0.798</td>
<td>12.3</td>
<td>11.9</td>
<td>12.4</td>
<td>12.1</td>
</tr>
<tr>
<td></td>
<td>Treatment</td>
<td>0.491</td>
<td>±06</td>
<td>±0.3</td>
<td>±0.3</td>
<td>±0.5</td>
</tr>
<tr>
<td></td>
<td>Interaction</td>
<td>0.887</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Diet</td>
<td>0.001</td>
<td>20.9</td>
<td>20.6</td>
<td>24.2*</td>
<td>23.8‡</td>
</tr>
<tr>
<td>Post-Treatment (g)</td>
<td>Treatment</td>
<td>0.657</td>
<td>±0.5</td>
<td>±0.3</td>
<td>±1.1</td>
<td>±0.9</td>
</tr>
<tr>
<td></td>
<td>Interaction</td>
<td>0.954</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Delta (g)</td>
<td>Diet</td>
<td>0.001</td>
<td>8.6</td>
<td>8.7</td>
<td>11.9*</td>
<td>11.7‡</td>
</tr>
<tr>
<td></td>
<td>Treatment</td>
<td>0.965</td>
<td>±0.6</td>
<td>±0.6</td>
<td>±1.3</td>
<td>±0.6</td>
</tr>
<tr>
<td></td>
<td>Interaction</td>
<td>0.885</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>% Increase</td>
<td>Diet</td>
<td>0.007</td>
<td>71</td>
<td>74</td>
<td>97a</td>
<td>98‡</td>
</tr>
<tr>
<td></td>
<td>Treatment</td>
<td>0.813</td>
<td>±8</td>
<td>±7</td>
<td>±12</td>
<td>±7</td>
</tr>
</tbody>
</table>

Values are mean ± SE. Control Diet Control (CDC), Control Diet Sp (CDSp), Western Diet Control (WDC), and Western Diet Sp (WDSp). Post-hoc comparisons; *P<0.05 CDC vs WDC; † P<0.05 WDC vs WDSp; ‡ CDSp vs WDSp. a P=0.06.
**Table S2.** Time course of pulse wave velocity changes of C57BL/6J mice fed a western diet (WD) high in fat and sugar compared to mice fed a control diet (CD). Administration of spironolactone (Sp) prevented the increase in PWV in WD fed mice. Sample sizes are noted in parentheses.

<table>
<thead>
<tr>
<th>Pulse Wave Velocity</th>
<th>Main Effect</th>
<th>P value</th>
<th>CDC (6)</th>
<th>CDSp (8)</th>
<th>WDC (6)</th>
<th>WDSp (10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 months PWV (m•s(^{-1}))</td>
<td>Diet</td>
<td>0.451</td>
<td>3.46 ±0.21</td>
<td>3.48 ±0.12</td>
<td>3.23 ±0.11</td>
<td>3.36 ±0.07</td>
</tr>
<tr>
<td></td>
<td>Treatment</td>
<td>0.418</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Interaction</td>
<td>0.479</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3 months PWV (m•s(^{-1}))</td>
<td>Diet</td>
<td>0.772</td>
<td>3.34 ±0.10</td>
<td>3.47 ±0.11</td>
<td>3.68† ±0.30</td>
<td>3.22 ±0.09</td>
</tr>
<tr>
<td></td>
<td>Treatment</td>
<td>0.325</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Interaction</td>
<td>0.078</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4 months PWV (m•s(^{-1}))</td>
<td>Diet</td>
<td>0.026</td>
<td>3.36 ±0.07</td>
<td>3.37 ±0.08</td>
<td>3.83*† ±0.07</td>
<td>3.38 ±0.08</td>
</tr>
<tr>
<td></td>
<td>Treatment</td>
<td>0.005</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Interaction</td>
<td>0.034</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Values are mean ± SE. Control Diet Control (CDC), Control Diet Sp (CDSp), Western Diet Control (WDC), and Western Diet LSp (WDSp). Post-hoc comparisons; *P<0.05 CDC vs WDC; † P<0.05 WDC vs WDSp.

**Figure S1.** Four months of Western diet (WD) feeding results in systemic insulin resistance without an effect of MR blockade. Insulin sensitivity was measured during an intra-peritoneal glucose tolerance test performed after 5 hour-fast. Graphs show (A) time course of glucose levels and (B) area under the curve calculations. AUC data for CDSp mice are not shown for clarity. Control Diet Control (CDC), Western Diet Control (WDC), and Western Diet Sp (WDSp). Values presented as means ± SE. *p < 0.05 compared to control diet (CDC). n = 5-8 per group.