Endothelial Mineralocorticoid Receptor Mediates Diet-Induced Aortic Stiffness in Females


Rationale: Enhanced activation of the mineralocorticoid receptors (MRs) in cardiovascular tissues increases oxidative stress, maladaptive immune responses, and inflammation with associated functional vascular abnormalities. We previously demonstrated that consumption of a Western diet (WD) for 16 weeks results in aortic stiffening, and that these abnormalities were prevented by systemic MR blockade in female mice. However, the cell-specific role of endothelial cell MR (ECMR) in these maladaptive vascular effects has not been explored.

Objective: We hypothesized that specific deletion of the ECMR would prevent WD-induced increases in endothelial sodium channel activation, reductions in bioavailable nitric oxide, increased vascular remodeling, and associated increases in vascular stiffness in females.

Methods and Results: Four-week-old female ECMR knockout and wild-type mice were fed either mouse chow or WD for 16 weeks. WD feeding resulted in aortic stiffness and endothelial dysfunction as determined in vivo by pulse wave velocity and ex vivo by atomic force microscopy, and wire and pressure myography. The WD-induced aortic stiffness was associated with enhanced endothelial sodium channel activation, attenuated endothelial nitric oxide synthase activation, increased oxidative stress, a proinflammatory immune response and fibrosis. Conversely, cell-specific ECMR deficiency prevented WD-induced aortic fibrosis and stiffness in conjunction with reductions in endothelial sodium channel activation, oxidative stress and macrophage proinflammatory polarization, restoration of endothelial nitric oxide synthase activation.

Conclusions: Increased ECMR signaling associated with consumption of a WD plays a key role in endothelial sodium channel activation, reduced nitric oxide production, oxidative stress, and inflammation that lead to aortic remodeling and stiffness in female mice. (Circ Res. 2016;118:935-943. DOI: 10.1161/CIRCRESAHA.115.308269.)

Key Words: inflammation ■ macrophages ■ nitric oxide ■ vascular stiffness ■ Western diet

It is well accepted that obesity is associated with increased arterial stiffness, which is a prognosticator for increased cardiovascular disease.1-3 In this context, data from the Framingham Heart Study including an analysis of 2232 participants support that arterial stiffness is an independent predictor of cardiovascular disease morbidity and mortality in the general population, hypertensive patients, the elderly, and patients with end-stage renal disease.4 Obesity is promoted by consumption of a Western diet (WD) high in fat and refined carbohydrates.2,3 There is accumulating evidence that plasma aldosterone levels are higher in overweight and hypertensive women and that the elevated plasma aldosterone is positively associated with cardiac and vascular dysfunction in females but not in males.5,6 Mineralocorticoid excess and enhanced mineralocorticoid receptor (MR) activation promote oxidative stress, inflammation, endothelial dysfunction, arterial remodeling as well as fibrosis.3,9

We recently reported that consumption of a WD contributed to both impairments in cardiac diastolic relaxation and aortic stiffening in young female mice, abnormalities that were prevented by MR antagonism.5,9 One fundamental understanding is that MR mediates the WD-mediated attenuation of
endothelial nitric oxide synthase (eNOS) activity and the increase in reactive oxygen species production that mediates the destruction of NO, leading to reduced bioavailable NO in the development cardiac and vascular stiffness.\(^{12,13}\) There is emerging evidence that enhanced MR activation increases activation of serum- and glucocorticoid-regulated kinase 1 (SGK1)\(^{10,11}\) and epithelial Na\(^+\) channel (ENaC) expression on the endothelial cell (EC) surface. This, in turn, leads to reduction of NO production and bioavailability which, in turn, increases cortical stiffness of the cytoskeleton.\(^{12,13}\) Thus, cell-specific endothelial MR signaling may play an important role in the pathogenesis of cardiovascular stiffness. We hypothesized that consumption of a WD would promote ECMR-mediated aortic stiffness via increases in endothelial ENaC expression, reductions in eNOS activity, enhanced oxidative stress, maladaptive inflammation, and subsequent vascular remodeling. The corollary to this hypothesis was that specific ECMR knockout (ECMR\(^{-/-}\)) mice, driven by a VE-cadherin promoter, would prevent WD-induced aortic pathophysiological changes and associated aortic stiffness.

Methods

Animals and Treatments
ECMR\(^{-/-}\) mice were generated by crossing MR\(^{+/+}\) mice with VE-Cad-Cre\(^{+}\) mice as previously described.\(^{14,15}\) MR\(^{+/+}\) Cre-Cre\(^{-}\) littermates were used as controls. In these mice, exon 5 and exon 6 of the MR gene are flanked by loxP sites via homologous recombination (MR\(^{−/−}\)), as previously described.\(^{14,15}\) 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 National Institutes of Health guidelines. Groups of 4-week-old female mice were fed a WD consisting of high-fat (46%) and a high-carbohydrate component as constituted with sucrose (17.5%) and high-fructose corn syrup (17.5%) and water with or without amiloride (1 mg/kg per day) for 16 weeks.\(^{16}\) Parallel groups of age-matched female controls (ECMR\(^{-/-}\)) were fed regular mouse chow (CD) for the same period of time.

Aortic Stiffness by Pulse Wave Velocity In Vivo
Doppler ultrasound (Indus Mouse Doppler System, Webster, TX) was performed on mice according to a previously established protocol to evaluate pulse wave velocity.\(^{3}\)

Atomic Force Microscopy Imaging and Force Measurement
ECs were isolated using anti–platelet endothelial cell adhesion molecule (PECAM)-1 antibody-conjugated Dynabeads as previously described.\(^{17}\) To evaluate the stiffness of the endothelium in aortic preparations, a 2 x 2 mm segment of the thoracic aorta was obtained from mice after the 16-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 enface access by the atomic force microscopy to the EC surface. Stiffness of the EC surface was measured by atomic force microscopy. The stiffness of EC within intact aortic explants from mice and primary cultured ECs was measured using a nanoindentation protocol with atomic force microscopy according to previously described procedures.\(^{3,15}\)

Ex Vivo Aortic Activity and Flow-Induced Dilation
Aortic mechanical activity was measured by wire myography as previously described.\(^{3}\) Mesenteric resistance arteries were isolated and cannulated onto glass micropipettes, pressurized at 70 mm Hg without flow, and warmed to 37°C in commercial pressure myograph chambers (Living Systems Instrumentation, Burlington, VT) as previously described.\(^{3,19,20}\)

Chromatin Immunoprecipitation and Quantitative Reverse Transcription-Polymerase Chain Reaction
Chromatin immunoprecipitation analysis and qPCR were carried out as previously described.\(^{3,15}\)

Western Blot and Slot Blot
Protein expression was measured by Western blot as previously described.\(^{3}\) For slot blot, 5-µg proteins were added in the well of a slot blot apparatus (Hoefer Inc, Holliston, MA). Nitrocellulose was removed and blocked with 5% dry milk in tris-buffered saline and tweek 20 and subsequently was incubated overnight at 4°C with blocking buffer containing antibodies to 3-nitrotyrosine (Millipore, Billerica, MA).

Aortic Remodeling and Fibrosis
Aortic remodeling was evaluated by immunostaining and transmission electron microscopy as previously described.\(^{3,15}\)

Statistical Analysis
Histological data were collected by genotype- and treatment-blinded investigators. Data are reported as means±SEM. Differences in outcomes were determined using 1- or 2-way ANOVA and paired tests and were considered significant when \(P<0.05\). All statistical analyses were performed using Sigma Plot (version 12) software (Systat Software).

Results

ECMR\(^{-/-}\) Prevents Aortic and Endothelium Stiffness
As previously reported, the MR gene is specifically and completely recombined in ECs from Cre+ mice and MR mRNA is significantly reduced in primary ECs cultured from mouse lungs and hearts, but not in leukocytes from ECMR knockout mice.\(^{14}\) Consumption of a WD for 16 weeks by ECMR\(^{+/+}\) mice induced increases in whole body fat mass, \(>6\)-fold increase in perireproductive fat mass and insulin resistance compared with CD-fed mice.\(^{15}\) There were no significant difference in WD-induced changes in body composition or insulin sensitivity in ECMR\(^{+/+}\) versus ECMR\(^{+/+}\) mice.\(^{15}\) Also, there were also no significant differences in lean body weight or mean arterial pressures between any of the groups.\(^{15}\) WD-induced increases in in vivo pulse wave velocity and ex vivo endothelium stiffness in ECMR\(^{+/+}\) and these effects were prevented in WD ECMR\(^{-/-}\) (Figure 1A and 1B). Both measures were unaffected by CD feeding for both ECMR\(^{-/-}\) and ECMR\(^{+/+}\) mice (Figure 1A). To verify the effects observed on stiffness were aldosterone-MR dependent, we isolated and cultured ECs by anti–PECAM-1 antibody-conjugated Dynabeads and then exposed them to aldosterone (10⁻⁶ mol/L). Aldosterone induced EC stiffness in ECMR\(^{+/+}\)
and this was prevented in ECMR−/− (Figure 1C and 1D). These data are consistent with improved endothelium and aortic stiffness in WD ECMR−/− mice.

**ECMR Modulates ENaC Expression**

Recent data suggest endothelial ENaC is tightly regulated by MR signaling and also promoted by increased serum sodium, thereby promoting cell swelling, stiffness, and impaired relaxation. In this context, WD feeding in ECMR+/+ mice was associated with a significant increase in expression of MR (Figure 2A) and its binding to the ENaC promoter, which contains at least 6 of 8 conserved nucleotides of the consensus sequence (NGNACAnnnTGTNCN; Figure 2B and 2C).22 WD indirectly increased an increase in ENaC promoter activity (Figure 2C) and ENaC expression with upregulation of SGK1 in ECs of ECMR+/+ mice (Figure 2; Online Figure IA and IB). However, these effects were attenuated in ECMR−/− female mice (Figure 2). In primary culture EC, aldosterone (10−8 mol/L) increased the expression of ENaC in the cultured ECMR+/+ ECs that was prevented in ECMR−/− cells (Figure 2D).

**ENaC Antagonist Prevents Endothelial Dysfunction**

To further investigate ENaC in the development of EC stiffness, amiloride (1 mg/kg per day), an antagonist for ENaC, was administered in the WD ECMR+/+ female mice for 16 weeks. Amiloride treatment increased endothelium vasodilatory responses to acetylcholine (Figure 3A) in aorta, but not to sodium nitroprusside (Figure 3B), suggesting that amiloride could improve endothelium-dependent relaxation in aorta but not endothelium-independent responses. Furthermore, ECMR+/+ (Figure 3C) and amiloride (Figure 3D), respectively, improved flow-induced mesenteric artery dilation, suggesting that both ECMR and ENaC participate in promoting endothelial dysfunction in ECMR+/+ mice.

**ECMR Signaling Promotes Aortic Relaxation Dysfunction Through Attenuation of eNOS Activation**

Endothelium-dependent vasodilatory responses to both acetylcholine (Figure 4A) and insulin (Figure 4B) were attenuated and this decrease occurred in concert with reduced p–protein kinase B/eNOS signaling in WD ECMR+/+ compared with CD ECMR+/+ (Figure 4A and 4D). However, these adverse effects were not present in the WD ECMR−/− vasculature (Figure 4). Meanwhile, acetylcholine-induced bioavailable NO was greater in aortic explants of ECMR−/− ex vivo compared with ECMR+/+ (Online Fig. II). Thus, ECMR-mediated impairment of aortic relaxation with WD consumption is driven by a reduction in activation of eNOS and bioavailable NO.

**ECMR Signaling Mediates Oxidative Stress, Maladaptive Proinflammatory Cytokine Generation, and Macrophage M1/M2 Polarization**

Vascular nicotinamide adenine dinucleotide phosphate oxidase generated superoxide anions react with NO to generate peroxynitrite, thereby reducing bioavailable NO. In wild-type mice, WD consumption promoted aortic oxidant stress as indicated by increases in nicotinamide adenine dinucleotide phosphate oxidase subunits Nox2, Nox4, and p22phox as well as 3-nitrotyrosine, a marker for peroxynitrite formation (Figure 5). The attendant increase in oxidant stress was associated with increases in aortic tissue expression of the M1 macrophage markers, CD86 and CD11c (Figure 5). ECMR−/− deletion prevented WD-induced increases in M1 macrophage polarization. Furthermore, vasculature from ECMR−/− mice also displayed increased M2 marker expression (CD206 and interleukin [IL]10) and an increased M2/M1 marker gene expression ratio suggesting a shift in polarization to a M2 phenotype in the ECMR−/− (Figure 5).

**Cell-Specific ECMR Signaling Mediates WD-Induced Aortic Fibrosis/Remodeling**

Oxidant stress and inflammation contribute to maladaptive tissue remodeling, and in this study we observed that WD ECMR+/+ exhibited increased aortic thickness and fibrosis, findings prevented in WD ECMR−/− (Figure 6A and 6B). Transmission electron emission analysis revealed Western diet–induced increases in thickened electron dense plasma-lemma and free ribosomes, which was largely corrected in WD ECMR−/− (Figure 6C). Consistent with the aortic remodeling, WD also enhanced expression of p-extracellular signal–regulated kinase 1/2 signaling (Figure 6D), fibroblast growth factor 23 (Online Figure IIIB), osteopontin (Online Figure IIIC), and promotes elastin fiber breakdown (Online Figure IV) in aorta. However, ECMR−/− prevented these abnormalities (Figure 6).
Discussion

This investigation demonstrates that consumption of a WD for 16 weeks in female mice results in aortic stiffness, indicated by increases in pulse wave velocity, impairment of aortic relaxation, and endothelial stiffness. Aortic stiffness was associated with enhanced membrane ENaC, oxidative stress, attenuated eNOS activity, classical macrophage polarization and inflammation. We previously observed that WD was associated with impaired diastolic relaxation at an earlier age in female mice than in male mice and systemic MR receptor blockade with spironolactone prevented the development of cardiac and vascular stiffness in females. Here, we further demonstrate that EC-specific deletion of the MR prevents the development of WD-induced aortic stiffness in female mice. In this regard, WD promotion of endothelial membrane ENaC expression, eNOS inactivity, oxidative stress, inflammation, M1/2 macrophage polarization, and associated aortic fibrosis was also prevented in ECMR−/− female mice. To the best of our knowledge, this is the first study to examine the impact of EC-specific deletion of the MR on aortic stiffness and endothelial function in females. Moreover, this is the first endeavor to investigate the impact of EC-specific MR deletion on vascular stiffness associated with consumption of a highly translational WD.

Mechanical stiffening of EC cortex in concert with impairment of eNOS activation characterizes the stiff endothelial cell syndrome. One study indicated that aldosterone and MR activation contribute to stiff endothelial cell syndrome by increasing membrane ENaC and mechanical stiffening of the EC cortex in concert with impaired generation of NO. Furthermore, spironolactone prevents stiff endothelial cell syndrome manifestation and improves endothelial function. In addition, studies conducted in mice with Liddle syndrome, a disease characterized by impaired ubiquinization of endothelial membrane ENaC have also demonstrated the critical role of this sodium channel in regulation of endothelial function and blood pressure. Our study further highlights the role of ECMR signaling in increasing membrane ENaC localization to promote aortic stiffness and decrease endothelium-dependent relaxation in a dietary model of vascular disease. The current data support the notion that both ECMR and...
ENaC activation integratively promote stiffness and reduce eNOS activity, NO bioavailability, and endothelium-mediated relaxation.26

It is noteworthy that the magnitude of dilatory responses to acetylcholine that we observed in the thoracic aorta of 20-week-old female ECMR+/+ and ECMR−/− are similar to those observed in some previous studies,27–29 yet lower than those reported in other studies, despite the fact that most previous investigations have examined aortic vasoreactivity only in male mice.30,31 In this regard, murine vascular function measurements are known to be influenced by a variety of genetic, physiological, and experimental factors, including genetic background, age, vascular collection time relative to the time of mouse euthanization, circadian cycle, and the...
segment of the vessel tested. For example, 1 study reported that endothelium-dependent and endothelium-independent vasodilation in response to carbachol and nitroprusside differ in magnitude between thoracic and abdominal aorta, as well as in arteries from other vascular beds. Other studies also found the similar results in C57BL16J and C57BI/6N background mice. Furthermore, current data are consistent with our previous study in 20-week-old female C57Bl/6J mice using the same technique.

Normally, the EC is protected by a well-developed glyocalyx and membrane expression of ENaC is maintained in an attenuated state of activity. Thus, the access of Na\(^+\) into the EC is limited, eNOS activation is optimized, and vasodilation is maintained. In states of obesity and activated renin–angiotensin aldosterone system, there is evidence that increased ENaC membrane abundance, together with a damaged glyocalyx, facilitate Na\(^+\) entry into EC and triggers the polymerization of G–actin to F–actin. Under these conditions, normal caveolar function and eNOS activation are compromised and the endothelial plasma membrane and immediate submembrane cytoskeletal compartment stiffens. In this study, SGK1 was increased with consumption of a WD, and this was prevented with ECMR deletion. This is important as SGK1 inhibits ubiquinization by upregulation of E3 ubiquitin ligase (Nedd4-2), and thus increases membrane insertion of this sodium channel. Therefore, these data suggest that enhancement of ECMR, SGK1, and ENaC coordinately contribute to aortic stiffness in female mice fed a WD.

One of the important new observations is that cell-specific ECMR activation also mediates WD-induced nicotinamide adenine dinucleotide phosphate oxidase activity as evidenced by increases in p22phox, Nox2, and Nox4. The resultant oxidative stress and inflammation responses are well known to play key roles in the development of aortic stiffness. To this point, increases in free radicals and inflammation directly suppress eNOS activation and reduce NO bioavailability. In addition, increases in vascular reactive oxygen species result in destruction of generated NO, which normally exerts a negative feedback to suppress ENaC activity by unclear signaling pathways. One study has shown that H\(_2\)O\(_2\) increased ENaC activity by both activation of phosphatidylinositol 3-kinases and inactivation of phosphatase and tensin homolog in renal tubular epithelium. Both IL6 and tumor necrosis factor activate EnaC and stimulate sodium uptake, in part, by activation of the extracellular signal–regulated kinase 1/2 signal pathway.

MR activation may also promote EC injury by increasing inflammatory cytokines such as vascular cell adhesion molecule (VCAM-1) and intercellular adhesion molecule (ICAM-1). VCAM-1 expression is upregulated by oxidized low-density lipoprotein (oxLDL) and Mrp2 deletion, and this was prevented with ECMR deletion. This is important as Mrp2 deletion increases cellular uptake of oxLDL, which is a prodifferentiation stimulus for EC. Thus, these data suggest that ECMR deletion prevents oxLDL–Mrp2–VCAM-1 activation without normalizing Mrp2 expression. Therefore, these data suggest that ECMR deletion prevents oxLDL–Mrp2–VCAM-1 activation without normalizing Mrp2 expression.
molecule 1 and intercellular adhesion molecule 1, which attract and promote immune cell adhesion to and transmigration through the endothelial barrier and to the artery wall from the bloodstream. Our previous study showed that WD induces an increase in CD11b in aorta, which represents a total macrophage cell marker. Interestingly, this data indicate that ECMR signaling also mediates WD-induced macrophage M1/M2 polarization because this was prevented in the ECMR−/− mice. M1/M2 polarization as indicated by both increases in M1 markers CD86 and CD11c and decreases in M2 markers CD206 and IL10 have been posited to be involved in the pathogenesis of tissue fibrosis and stiffness. The increase in M1/M2 polarization with consumption of a WD is consistent with previous results in mice showing that deletion of MRs in macrophages resulted in reduction in M1 phenotype and mRNA levels for markers of vascular inflammation and fibrosis. These observed pathophysiological changes leading to aortic fibrosis and remodeling were associated with increases in p-extracellular signal–regulated kinase 1/2, fibroblast growth factor 23, osteopontin, broken elastin fibers, and ultrastructural abnormalities were also induced by consumption of a WD. To this point, Erk1/2 is a family of serine/threonine protein kinases activated as an early response to a variety of cytokines, growth factors, and regulate transcription factor activation, ultimately contributing to vascular cell differentiation, proliferation, and vascular remodeling. In this study, phospho-Erk1/2 protein levels were significantly increased in mice fed a WD, and this abnormality was prevented in ECMR knockout mice, suggesting a role for extracellular signal–regulated kinase 1/2 in ECMR-mediated aortic remodeling. Thus, activation of ECMR in the setting of obesity resulting in increased reactive oxygen species, inflammation response, and macrophage M1/M2 polarization could contribute to aortic remodeling and fibrosis that is characteristic of EC dysfunction and vascular stiffness in females with obesity and cardiovascular disease.

Collectively, results of this investigation suggest a pivotal role of ECMR activation in development of aortic stiffness.
and endothelial dysfunction. These vascular changes are associated with increases in endothelial membrane ENaC, reduced eNOS activity, oxidative stress, and maladaptive immune responses in the aorta of female mice fed a WD. These preclinical highly translational data fill a gap in our knowledge of the role of cell-specific MR signaling in promotion of vascular stiffness. Further investigation of the precise role of MR signaling in other cells (including vascular smooth muscle cells and macrophages) in promotion of vascular stiffness in conjunction with dietary factors is an important area of future investigation.

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Disclosures

None.

References


Novelty and Significance

**What Is Known?**

- Arterial stiffness is increased to a greater extent in women during obe-
sity and diabetes than in men.
- Consumption of a Western diet (WD) high in saturated fat and refined sugar increases plasma aldosterone and enhances vascular mineralocorticoid receptor (MR) activation.
- Vascular MR contributes to vascular stiffness by enhancing endo-
thelial dysfunction and upregulating oxidase stress, inflammation, and fibrosis.

**What New Information Does This Article Contribute?**

- Activation of endothelial MR signaling is associated with consumption of a WD.
- Enhanced endothelial cell MR (ECMR) activation and resultant increases in endothelial sodium channel (ENaC) activation lead to a reduction in nitric oxide production and bioavailability and associated vascular stiffness.

- ECMR-mediated promotion of oxidative stress, M1 macrophage polarization, leads to further decreases in nitric oxide bioavailability and associated aortic fibrosis and stiffness in females consuming a WD.

In comparison with male mice fed a WD, pulse wave velocity is elevated earlier in female mice. This abnormality is prevented by MR antagonist. Here, we show that ECMR-mediated activation of ENaC on EC leads to reduction of nitric oxide production and bioavailability. We found that WD consumption prompts ECMR to bind the hormone-response element on the site of ENaC promoter and increases ENaC expression in ECs. This ECMR-mediated response was associated with increased aortic vascular and EC stiffness. WD increased aortic remodeling with an increase in macrophage M1 markers CD86 and CD11c, and this inflammatory immune response was prevented by ECMR deletion in WD-fed female mice. These findings suggest that increased ECMR signaling plays a key role in ENaC activation, reduced nitric oxide bioavailability, and macrophage recruitment that lead to aortic stiffness in females.

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SUPPLEMENTAL MATERIAL

Endothelial mineralocorticoid receptor mediates diet induced aortic stiffness in females

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Expanded Methods:

**Aortic stiffness by pulse wave velocity in vivo**

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 streams).

**AFM Imaging and force measurement**

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, 92 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 TXu1-328-97 659) 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. E-99 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:

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

Where, \( E \) is the E-modulus, \( F \) is the force exerted by AFM probe on tissue surface, \( \delta \) is indentation depth into the sample, \( a \) is the half-opening angle of the AFM tip, and \( \nu \) is the Poisson ratio. The tissues were considered as a gel and the Poisson ratio \( \nu \) was assumed at 0.5. To obtain topographical images of EC, 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 108 scanning at 0.4 Hz frequency with approximately 300-500 pN tracking force.

**Ex vivo aortic activity and flow-induced dilation**

A 2 mm segment of thoracic aorta was collected immediately after euthanasia and placed in ice-cold physiological salt solution (PSS) containing (in mM): 119 NaCl, 4.7
KCl, 2.5 CaCl, 1.18 KH₂PO₄, 1.17 MgSO₄, 0.027 EDTA, 5.5 glucose, and 25 NaHCO₃, pH 7.4. Aortic contractile state was ascertained by KCl (80 mM•L⁻¹). Aortas were preconstricted with U46619 (100 nM). Dilation of arterial rings to acetylcholine (1 nm to 100 µM), the NO-donor sodium nitroprusside (1 nM to 100 µM), 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 Ca²⁺-free PSS to determine maximal passive force. Aortic dilator responses are presented as percent maximal relaxation, calculated as \[ \frac{(F_b - F_d)}{(F_b - F_{min})} \times 100 \], where \( F_d \) is force after a drug intervention, \( F_b \) is baseline force, and \( F_{min} \) is maximal passive force.

Mesenteric resistance arteries were bathed in albumin-free physiological saline solution (PSS) containing (in mM) 145.0 NaCl, 4.7 KCl, 2.0 CaCl₂, 1.0 MgSO₄, 1.2 NaH₂PO₄, 0.02 EDTA, 2.0 Pyruvic Acid, 5.0 Glucose and 3.0 MOPS buffer, and perfused with PSS containing 0.15 Albumin, pH of 7.4. 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). To test for viability, the cannulated arteries were allowed to stabilize for 40 min and then exposed to PSS in which NaCl was substituted equimolarly with 80 mM KCl. Only arteries that constricted more than 20% to this 80 mM K⁺ solution were used in the analyses. After the exposure to high K⁺, the arteries washed three times with fresh PSS and allowed to stabilize for 10 min. Vessels were subsequently pre-constricted with 10⁻⁵ M phenylephrine for 5 min and then exposed to eight increasing steps in flow ratios (0 to 26 µl/min) to determine flow (shear stress)-induced endothelium-mediated vasodilation. The changes in flow were induced with a peristaltic pump placed in the inflow pipette, while a pump in the outflow pipette was used to maintain intravascular pressure constant at 70 mmHg. Vessels remained at each flow ratio for 5 min until internal diameter became stable. Subsequently, flow was interrupted and the vessels washed three times as before. Amiloride (1 µM) was then added both intra- and extraluminally to the vessel. After a 20-min incubation in amiloride, the arteries where pre-constricted and exposed to increasing flow ratios as before, in the presence of amiloride. At the end of each experiment flow was stopped and the arteries exposed to Ca²⁺-free PSS with 2 mM ethylene glycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid (EGTA) and 10⁻⁴ M adenosine for 10 min to obtain maximal passive diameter.

Measurement of ex vivo production of NO
Ex vivo production of NO in aortic explant was measured by using 4-amino-5-methylamino-2,7-difluorofluorescein (DAF-FM) diacetate (Molecular Probes, Eugene). Aortic explants were incubated with 5 µM DAF-FM diacetate for 30 min in DMEM medium at 37°C. After washing the aortic explants in DMEM medium for removing the excess dye, aortic explants were exposed to 10 µM acetylcholine for 10
min. Intracellular NO levels were determined from the fluorescence intensity of DAF-FM via confocal microscopy.

**Chromatin immunoprecipitation (ChIP) and quantitative RT-PCR**

ChIP analysis was carried out with the Imprint ChIP kit (Sigma, St. Louis, MO, USA). To release the chromatin, 20 mg of aortic tissue was homogenized and cross-linked with 1% formaldehyde. This was then digested with micrococcal nuclease (2 U/ml; Sigma) to provide ~500 bp of fragmented genomic DNA. The extract was either aliquoted as genomic input DNA or immunoprecipitated using MR antibody (Cell Signaling Technology, Danvers, MA). Following hydrolyzation of the crosslinks, the DNA was collected and quantitative PCR were performed on both genomic input and ChIP DNA. PCR primers were designed to flank the two MR consensus sequences in the ENaCα promoter. Quantitative PCR of the ChIP products and genomic input DNA was performed by real-time PCR using SYBR green (Bio-Rad, Hercules, and CA). The amount of ChIP DNA present in each sample was reported as percentage of genomic input DNA.

For qPCR, total RNA was isolated from thoracic aorta extracts using TRIzol reagent (Sigma, St Louis). RNA yield was determined using a Nanodrop spectrophotometer (Thermo Scientific, Wilmington, DE). First-strand cDNA synthesis was done using 1 μg total RNA with oligo dT (1 μg), 5× reaction buffer, MgCl₂, dNTP mix, RNase inhibitor, and Improm II reverse transcriptase as per Improm II reverse transcription kit (Promega, Madison, WI). After the first strand synthesis, real-time PCR was done using 8 μl cDNA, 10 μl SYBR green PCR master mix (Bio-Rad Laboratories) and forward and reverse primers (10 pM/μl) (Integrated DNA Technologies, San Diego, CA) using a real-time PCR system (CFX96; Bio-Rad Laboratories). The primer sequences used were: MR, Forward: 5'-CAGCTAGCTTTGGCAGTTC-3', Reverse: 5'-GTGTGACCTTGGAGCTTATT-3'; ENaCα, Forward: 5'-AGACAAACCGCATGAAGA-3', Reverse: 5'-CCTCGAAGCAGAAGCAAAC-3'; MCP-1, Forward: 5'-GATGCAGTTAATGCCCCACT-3'; Reverse: 5'-TTCTCTTTGAGGTCAGCAC-3'; IL17, Forward: 5'-CTTCACCTTGGACTCTGAGC-3'; Reverse: 5'-TGGCGGACAATAGAGGAAAAC-3'; CD86, Forward: 5'-GACCGTTGTTGTTGTTCTGG-3', Reverse: 5'-GATGAGGACACATCACAAGGA-3'; CD11c, Forward: 5'-ATGAAGAACCTCGGGAAAT -3', Reverse: 5'-GCTTAGATCATGCGGGTTTTG-3'; CD11c, Forward: 5'-ACACAGTGTGCTCCAGTATG-3', Reverse: 5'-GCCCAGGATATGTCACAGC-3'; IL10, Forward: 5'-CCAGCTTTATCGGAAATGA-3', Reverse: 5'-TTTTACACAGGGGAAATCG-3'; CD206, Forward: 5'-CAAGGAAGTTTCCCTTTG-3', Reverse: 5'-CCTTTCAGTCTCCTTTGGAAG-3'; GAPDH, Forward: 5'-GGAGAAACCTCGCCAAGTG-3', Reverse: 5'-TCCTCAGTGTACGCGGAA-3'. The specificity of the primers was analyzed by running a melting curve. The PCR cycling conditions used were 5 min at 95°C for initial denaturation, 40 cycles of 30 s at 95°C, 30 s at 58°C and 30 s at 72°C. Each real-time PCR was carried out using three individual samples in triplicates, and the threshold cycle values were averaged. Calculations of relative normalized gene expression were done using the Bio-Rad CFX manager software based on the ΔCt method. The results were normalized against housekeeping gene GAPDH.
Western blot
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 Thr202/Tyr204, Erk 1/2, p-Akt Serine473, Akt (Cell Signaling Technology, Danvers, MA) and p-eNOS 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) 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 which are the indicative of collagen deposition were quantified as gray scale intensities by using MetaVue software. Fluorescent immunohistochemistry was used to quantify ENaC, SGK1, osteopontin, and Nox4 expression. Aorta samples of were prepared as described above. Five μm sections were dewaxed, rehydrated, and placed in 95ºC 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 antibody to ENaCα, SGK1, osteopontin, and Nox4 expression (Cell Signaling Technology, Danvers, MA) 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.
Online Figure I: The higher magnification images in the expression of ENaC (A) and SGK1 (B) in aortic endothelium with corresponding measures of average gray scale intensities. Scale bar = 50 μm. n=4 to 5 per group. *P<0.01 compared with CD ECMR+/+; # P<0.05 compared with WD ECMR+/+.
Online Figure II: (A) Acetylcholine-induced bioavailable NO is greater in aortic explants of ECMR<sup>++</sup> ex vivo. n=2 per group. (B) Vasodilator responses of isolated aortic rings to the endothelium-independent vasodilator, sodium nitroprusside. n=4 to 5 per group. *P<0.05 compared with CD ECMR<sup>++</sup>; # P<0.05 compared with WD ECMR<sup>++</sup>. 
Online Figure III: Western diet induced Nox4, FGF23, and osteopontin is prevented in ECMR−/− mice. Representative images immunostaining for Nox4 (A), FGF23 (B), and osteopontin (C) with corresponding measures of average gray scale intensities below. n=5-6 per group. Scale bar = 50 μm. *P<0.05 compared with CD ECMR+/+; # P<0.05 compared with WD ECMR+/+. 
Online Figure IV: Western diet induced the broken elastin fibers are prevented in ECMR+/− mice. Quantified the broken elastin fibers were quantified in in each 40X bright-field images. n=5-6 per group. *P<0.05 compared with CD ECMR+/++; # P<0.05 compared with WD ECMR+/−.