Mineralocorticoid Receptor Agonists Induce Mouse Aortic Aneurysm Formation and Rupture in the Presence of High Salt

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Objective—Elevated plasma aldosterone concentrations in patients have been linked to a spectrum of cardiovascular diseases. Mineralocorticoid receptor antagonists provide additional benefits in patients with heart failure. However, whether aldosterone and the mineralocorticoid receptor are involved in aortic aneurysm is unknown.

Approach and Results—We report that administration of deoxycorticosterone acetate (DOCA) and salt or aldosterone and salt, but not DOCA or salt alone, to C57BL/6 male mice induced abdominal and thoracic aortic aneurysm formation and rupture in an age-dependent manner. DOCA and salt- or aldosterone and salt-induced aortic aneurysm mimicked human aortic aneurysm with respect to elastin degradation, inflammatory cell infiltration, smooth muscle cell degeneration and apoptosis, and oxidative stress. Aortic aneurysm formation did not correlate with the increase in blood pressure induced by DOCA and salt. Systemic administration of the angiotensin-converting enzyme inhibitor, enalapril, or angiotensin type 1 receptor antagonist, losartan, did not affect DOCA and salt-induced aortic aneurysm. In contrast, the mineralocorticoid receptor antagonists, spironolactone or eplerenone, significantly attenuated DOCA and salt- or aldosterone and salt-induced aortic aneurysm.

Conclusions—The current study describes a novel aortic aneurysm animal model induced by mineralocorticoid receptor agonist and high salt, and reveals a previously unrecognized but potentially significant role of aldosterone in the pathogenesis of aortic aneurysm. These findings imply that mineralocorticoid receptor antagonists may be effective in the treatment of some aortic aneurysms. (Arterioscler Thromb Vasc Biol. 2013;33:1568-1579.)

Key Words: aldosterone ■ aneurysm ■ deoxycorticosterone ■ receptors ■ salt

Aortic aneurysms can be divided into thoracic aortic aneurysms (TAAs) and abdominal aortic aneurysms (AAAs) according to their location. TAAs occur in all age people without sexual dimorphism and are highly associated with hereditary conditions. In contrast, AAAs are typically associated with aging, male gender, atherosclerosis, and smoking but have weak genetic association. AAAs are the most common form of aortic aneurysms, affect 4% to 8% of men over the age of 60, and account for ≈2% of all deaths in Western countries. Currently, open surgery repair and endovascular repair are the only widely used therapies for treatment of aortic aneurysm, and no drug has been approved for treatment of this devastating disease.

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The mineralocorticoid receptor, also known as the aldosterone receptor or nuclear receptor subfamily 3, group C, member 2 (NR3C2), is a protein that in humans is encoded by the NR3C2 gene (Gene ID: 4306). Multiple steroid hormones, including aldosterone and its precursor deoxycorticosterone acetate (DOCA), can bind to and activate NR3C2, but aldosterone is considered the primary physiological ligand in humans. The mineralocorticoid receptor was initially identified in the epithelial cells of the kidney and has been well recognized for its pivotal role in regulation of salt excretion, plasma volume, and blood pressure (BP). Subsequent studies show that the mineralocorticoid receptor is also expressed in nonepithelial cells and tissues (eg, heart and aorta), which raises the possibility that the mineralocorticoid receptor may also exert functions beyond the kidney. Indeed, a growing body of evidence suggests that the mineralocorticoid receptor plays a critical role in cardiac fibrosis, heart failure, and myocardial infarction. In particular, evidence from 2 large clinical trials, the Randomized ALDactone Evaluation Study

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Aortic Aneurysms in DOCA and Salt Mouse Model

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Materials and Methods

Both DOCA and High Salt Are Required to Induce Aortic Aneurysm

To determine whether DOCA and high salt are able to induce aortic aneurysm, 10-month-old male mice were randomly divided into 3 groups that received (1) no treatment (control; n=12); (2) DOCA alone (n=10); or (3) DOCA and salt (n=45). The representative ultrasound images (Figure 1A) and quantitative data (Figure 1B) illustrate that mice administered DOCA and salt exhibited a significant increase in aortic dilation (1.09 mm [0 week; n=45] versus 1.50 mm [2 week; n=44] or 1.46 mm [3 week; n=38]; P<0.001). In contrast, no aortic dilation was found in control mice or in mice administered DOCA alone. In addition to lumen dilation, a significant increase in external diameters of abdominal aortas was observed in mice administered DOCA and salt compared with control or mice administered DOCA alone (1.48 mm [DOCA and salt; n=37] versus 0.67 mm [DOCA; n=10] or 0.74 mm [control; n=12]; P<0.001; Figure 1C). A similar increase in external diameter was also observed in thoracic aortas from DOCA and salt, but not from control or DOCA only mice (1.16 mm [DOCA and salt; n=37] versus 0.83 mm [DOCA; n=10] or 0.88 mm [control; n=12]; P<0.05; Figure 1C).

Human aortic aneurysm, including TAA and AAA, is defined as a permanent localized dilatation of aorta, having ≥50% increase in diameter compared with the normal diameter of aorta. Based on this definition, of the 45 mice administered DOCA and salt, 28 developed AAAs (Figure 1D and 1E). Of the 28 mice that developed AAAs, 20 mice also developed TAAAs, 8 mice died of aortic aneurysm rupture, and no TAAAs were seen in mice that did not also exhibit an AAA. The incidence of AAAs, TAAAs, and aortic aneurysm rupture is 62%, 44%, and 18%, respectively (Figure 1E). AAAs were only found in the suprarenal abdominal aorta, and TAAAs were only found in the descending thoracic aorta. On the contrary, no AAA, TAA, or aortic aneurysm rupture was observed in control or DOCA alone (Figure 1E).

Of the 8 aortic ruptures, 1 occurred at 1 week and 7 occurred at 3 weeks after DOCA and salt administration (Figure 1A in the online-only Data Supplement). We found a significant increase in maximal intraluminal diameters of abdominal aorta in ruptured aortas compared with that in control mice at 2 weeks after DOCA and salt administration (1.80 mm [DOCA and salt; n=7] versus 1.10 mm [Ctrl; n=12]; P<0.001; Figure 1B in the online-only Data Supplement). This result illustrates that most if not all mice that developed aortic ruptures were first seen with enlarged aortic diameters.

To investigate whether high salt alone is sufficient to induce aortic aneurysms, 10-month-old male mice received drinking water containing high salt for 3 weeks. Administration of high salt alone had no effect on aortic dilation as measured by ultrasound imaging and ex vivo aortic quantification (Figure 2A and 2B in the online-only Data Supplement). Importantly, neither AAA, TAA, nor aortic rupture was evident in mice administered high salt.

Aldosterone and Salt Induce Aortic Aneurysms

Because DOCA is a mineralocorticoid receptor ligand, we defined whether aldosterone, a physiological ligand of the mineralocorticoid receptor, can induce aortic aneurysms in a dose-dependent manner. Ten-month-old mice receiving salt were infused with 3 different doses of aldosterone (200, 500, and 700 μg/kg per day) for 4 weeks. Figure 2A shows that plasma aldosterone concentrations were significantly elevated in mice administered aldosterone and salt in a dose-dependent manner (0.27 nmol/L [ctrl; n=7] versus 10.58 nmol/L [200 μg/kg per day; n=8; P<0.05], 21.21 nmol/L [500 μg/kg per day; n=6; P<0.01] or 27.36 nmol/L [700 μg/kg per day; n=6; P<0.01]).

All 3 doses of aldosterone and salt markedly increased maximal intraluminal diameters of suprarenal abdominal aortas 2 weeks after administration of mice with aldosterone and salt (Figure 2B; Table II in the online-only Data Supplement). Similar significant increases were also seen in maximal external diameters of abdominal and thoracic aortas 4 weeks after administration of mice with aldosterone and salt (Figure 2C; Table III in the online-only Data Supplement). Accordingly, all 3 doses of aldosterone and salt also markedly induced AAAs (65%, 58%, and 67%, respectively), TAAAs (45%, 42%, and 55%, respectively), and aortic aneurysm rupture (35%, 25%, and 44%, respectively; Figure 2D).
Interestingly, 200 μg/kg per day aldosterone infusion plus high salt seemed to be sufficient for induction of aortic aneurysm formation because effects were not dose dependent above this aldosterone dose (Figure 2B through 2D). To rule out the possibility that the vehicle (50% dimethyl sulfoxide [DMSO]) may have had an effect on aldosterone and salt-induced aortic aneurysm formation, mice were infused with 50% DMSO plus high salt for 4 weeks. Figure IIIA and IIIB in the online-only Data Supplement illustrate that DMSO and salt did not cause aortic dilation as measured by ultrasound imaging and ex vivo aortic diameter quantification. In addition, neither AAAs, TAAs, nor aortic ruptures were seen in mice administered DMSO and salt.

Aortic aneurysms induced by DOCA and salt versus those induced by aldosterone and salt had similar features, but there were some differences. First, in contrast to the massive and diffusive aortic aneurysms induced by DOCA and salt (Figure 1D), aortic aneurysms induced by aldosterone and salt were more discretely localized to the suprarenal abdominal aorta (Figure 2E). Second, aortic aneurysm ruptures occurred more frequently in mice administered aldosterone and salt compared with those administered DOCA and salt (35%, 25%, and 44%, respectively, with the 3 doses of aldosterone [Figure 2D] versus 18% in DOCA and salt [Figure 1E]).

### Vascular Pathology of DOCA and Salt- or Aldosterone and Salt-Induced Aortic Aneurysms

Human aortic aneurysms are characterized by elastin and collagen degradation, matrix metalloproteinase (MMP) upregulation, inflammatory cell infiltration, vascular smooth muscle cell degeneration, and oxidative stress. To investigate whether DOCA and salt- or aldosterone and salt-induced aortic aneurysms have these pathological features, sequential 5-μm cross-sections were collected throughout the entire aorta (Figure IV in the online-only Data Supplement). Of the 12 aortas sectioned, elastin staining in aortas from mice administered DOCA and salt or aldosterone and salt demonstrated pronounced elastin breaks and extensive vascular remodeling (Figure 3A through 3F). Quantitative analysis showed that elastin breaks were significantly increased in aortas administered DOCA and salt.
salt or aldosterone and salt compared with controls (Figure VIIB in the online-only Data Supplement). In addition to elastin, collagen content was also markedly suppressed in the media and adventitia of aortas from mice administered DOCA and salt or aldosterone and salt compared with that of control mice (Figure 3G through 3I).

MMP2 and MMP9 are enzymatic proteins that play a critical role in extracellular matrix degradation and vascular remodeling.13 MMP2 immunostaining was evident in the endothelial/medial layer of aortas from mice administered DOCA and salt or aldosterone and salt, and was apparent at elastin degradation sites of aortas compared with the control mouse aorta in which MMP2 protein was barely detectable (Figure 3J through 3L). The similar immunostaining pattern was also seen for MMP9, although it was less evident than MMP2 immunostaining (Figure 3M through 3O). Moreover, in situ zymography illustrated that total MMP activity was markedly elevated in the medial and adventitial layers of aortas from mice administered DOCA and salt or aldosterone and salt compared with that in control mice (Figure VIII in the online-only Data Supplement).

To investigate inflammatory cell infiltration, aorta cross-sections were immunostained with F4/80, Ly-6B.2, and CD90.2 antibodies, which recognizes monocytes/macrophages, neutrophils, and T cells, respectively. Monocyte/macrophage immunostaining was minimal in control aortic sections, but it was readily seen in the endothelial/medial layer of aortic sections from mice administered DOCA and salt or aldosterone and salt (Figure 3P through 3R). The similar immunostaining pattern was also seen for neutrophils, although it was less evident than monocyte/macrophage immunostaining (Figure 3S through 3U). Interestingly, no obvious T Cells were observed in the endothelial/medial layer of aortic sections (Figure 3V through 3X), although they were
Deoxycorticosterone acetate (DOCA) and salt or aldosterone and salt increase elastin and collagen degradation, matrix metallo-proteinase 2 (MMP2) and MMP9 protein expression, vascular macrophage and neutrophil accumulation, smooth muscle degeneration, and apoptosis in abdominal aortas. Abdominal aortas were isolated from control mice (A, D, G, J, M, P, S, V, Y, and BB) and mice administered DOCA and salt for 3 weeks (B, E, H, K, N, Q, T, W, Z, and CC) or aldosterone and salt for 4 weeks (C, F, I, L, O, R, U, X, AA, and DD). Paraffin-embedded cross-sections of abdominal aortas were stained with Elastic-Van Gieson staining for elastin (A through F), Masson trichrome for collagen (G through I), MMP2 Ab (J through L), MMP9 Ab (M through O), F4/80 Ab for macrophages (P through R), Ly6B.2 Ab for neutrophils (S through U), CD90.2 Ab for T cells (V through X), caldesmon (smooth) Ab for vascular smooth muscle cells (VSMCs; Y through AA), and terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) for apoptosis (BB through DD). Among representative images, A through C images are of low magnification, whereas the rest are of higher magnification. Arrows indicate elastin degradation sites.

To further investigate the role of vascular inflammation in DOCA and salt-induced aortic aneurysm, we determined mRNA expression of several inflammatory genes, including vascular cell adhesion molecule 1 (Vcam-1), chemokine (C-C motif) ligand 2 (Ccl2, also known as monocyte chemoattractant protein-1), and tumor necrosis factor (Tnf, also known as TNFα) in both abdominal and thoracic aortas from mice administered DOCA and salt or control mice. We found that Vcam-1, Ccl2, and Tnf were all markedly upregulated thoracic aortas from mice administered DOCA and salt compared with control mice (Figure XA–XC in the online-only Data Supplement). Interestingly, Vcam-1 and Ccl2, but not Tnf, were also significantly upregulated in abdominal aorta from mice administered DOCA and salt compared with control mice (Figure XD–XF in the online-only Data Supplement).

To investigate possible vascular smooth muscle apoptosis, aortic sections were stained with an antibody that specifically recognizes smooth muscle–specific caldesmon. In control aortas, caldesmon protein was highly expressed in the media of the vessel. In contrast, in aortas from mice administered DOCA and salt or aldosterone and salt, caldesmon protein expression was markedly suppressed (Figure 3Y through 3AA). Aortic sections were also stained with a terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) kit that recognizes apoptotic cells. In contrast to caldesmon staining, TUNEL-positive cells were absent in control aortas but could be found in aortas from mice administered DOCA and salt or aldosterone and salt (Figure 3BB through 3DD). Importantly, TUNEL staining was present in the same areas as elastin degradation, and the loss of immunostaining of caldesmon in sections was examined. Quantitative analysis also showed that TUNEL-positive cells in the tunica media of aortic section were significantly increased in mice administered DOCA and salt or aldosterone and salt compared with that in control mice (Figure XI in the online-only Data Supplement).

To investigate a potential role for oxidative stress, cross-sections of abdominal aortas from mice administered DOCA and salt were stained with dihydroethidium. In contrast to control aortas, in which only auto-florescence of elastin was seen, additional nuclear staining derived from dihydroethidium was observed in abdominal aortas from mice administered DOCA and salt (Figure XIA in the online-only Data Supplement). To investigate the mechanism that may
be responsible for the DOCA and salt-induced increase in oxidative stress, we determined the time course of DOCA and salt-induced Ncf1 (also known as p47phox; Gene: ID: 179669) mRNA expression in thoracic and abdominal aortas. We selected Ncf1 because NCF1 has been shown to be upregulated in human aortic aneurysms14 and has been demonstrated to be critical in Ang II–induced aortic aneurysms.15 Figure XIIIB and XIIIC in the online-only Data Supplement illustrate that Ncf1 mRNA was upregulated in a time-dependent manner in both thoracic and abdominal aortas in response to DOCA and salt. Importantly, Ncf1 mRNA upregulation by DOCA and salt preceded aortic aneurysm formation (9 days versus 3 weeks). In addition, a similar increase in Cyba (also known as p22phox; Gene ID: 13057) mRNA was also observed (data not shown).

**DOCA and Salt-Induced Aortic Aneurysm Is Age Dependent**

In humans, the prevalence of AAAs increases with age.2 Therefore, we compared aortic aneurysm formation in response to DOCA and salt administration in 10-week-old mice versus 10-month-old mice. DOCA and salt administration induced a significant increase in abdominal aortic dilation in mice of both ages (Figure 4A). However, luminal dilation was more pronounced in 10-month-old mice compared with 10-week-old mice (eg, 2 weeks after DOCA and salt, 1.50 mm [10 months old; n=44] versus 1.18 mm [10 weeks old; n=12]; P<0.001). Similarly, maximal external diameters of the thoracic aorta were also greater in 10-month-old mice administered DOCA and salt than that in 10-week-old mice (1.16 mm [10 months old; n=37] versus 0.80 mm [10 weeks old; n=10]; P<0.01; Figure 4B). A trend toward increased maximal external diameters of abdominal aortas was also seen in 10-month-old mice compared with 10-week-old mice (1.48 mm [10 months old; n=37] versus 1.06 mm [10 weeks old; n=10]; P=0.06; Figure 4B). A trend toward increased incidences of AAAs (42% [5 of 12; 10 weeks old] versus 64% [29 of 42; 10 months old]; P=0.10) and TAAs (17% [2 of 12; 10 weeks old] versus 44% [20 of 45; 10 months old]; P=0.20) was observed in 10-month-old mice compared with 10-week-old mice. In contrast, there was no difference of aortic aneurysm ruptures between 2 groups of mice (17% [2 of 12; 10 weeks old] versus 18% [8 of 45; 10 months old]; P=0.33). Moreover, DOCA and salt-induced aortic aneurysms in 10-month-old mice were more severe and diffuse, and often extended from the suprarenal abdominal aorta to the descending thoracic aorta (Figures 4C and 1D). In contrast, 10-week-old mice administered DOCA and salt had aortic aneurysms that primarily localized to the suprarenal abdominal aorta (Figure 4C).

**Mineralocorticoid Receptor Antagonism, But Not Ang II Type 1 Receptor Antagonism, Attenuates DOCA and Salt-Induced Aneurysms**

Although plasma renin and Ang II concentrations are suppressed in animals administered DOCA and salt,11 results suggest that a local vascular Ang II may be increased.16 Moreover, infusion of Ang II is an established model of abdominal and ascending aortic aneurysms,17,18 and Ang II is a primary stimulus for aldosterone release.4 Therefore, we administered either an angiotensin-converting enzyme inhibitor (enalapril) or an Ang II receptor blocker (losartan) to define the role of Ang II in DOCA and salt-induced aneurysms. Administration of losartan or enalapril resulted in a significant reduction in basal BP (Figure 5A), demonstrating effective blockage of the renin–angiotensin system. However, neither enalapril nor losartan significantly reduced maximal intraluminal diameters of suprarenal abdominal aortas (Figure 5B), maximal external diameters of thoracic and abdominal aorta (Figure 5C), or the incidence of AAAs, TAAs, and aortic aneurysm ruptures induced by DOCA and salt administration (Figure 5D).

To define the role of mineralocorticoid receptor in DOCA and salt-induced aortic aneurysm, we determined whether a mineralocorticoid receptor antagonist spironolactone affects DOCA and salt-induced aortic aneurysm. Administration of spironolactone resulted in a significant reduction in maximal intraluminal diameter of suprarenal abdominal aorta.
aortas (eg, 2 weeks after treatment, 1.35 mm [DOCA and salt plus spironolactone; n=16] versus 1.50 mm [DOCA and salt; n=44]; P<0.01; Figure 6A), maximal external diameters of abdominal aortas (1.04 mm [DOCA and salt plus spironolactone; n=14] versus 1.48 mm [DOCA and salt; n=37]; P<0.05; Figure 6B), and the incidence of AAAs (25% [4/16, DOCA and salt plus spironolactone] versus 62% [28 of 45; DOCA and salt]; P<0.05; Figure 6C). In addition, a trend toward reduced incidence of TAAs (25% [4/16, DOCA and salt plus spironolactone] versus 44% [20 of 45; DOCA and salt]) and aortic aneurysm ruptures (13% [2/16; DOCA and salt plus spironolactone] versus 18% [8 of 45; DOCA and salt]) was also seen in DOCA and salt mice administered spironolactone (Figure 6C).

It is well known that spironolactone cross-reacts with sex-steroid receptors.7 It was also noted that spironolactone only partially blunted aortic aneurysm formation (Figure 6A through 6C). To further define the role of mineralocorticoid receptor in aortic aneurysm, we determined the effect of eplerenone, a more specific mineralocorticoid receptor antagonist, on aldosterone and salt-induced aortic aneurysm. We used a low dose aldosterone (200 µg/kg per day), but not DOCA (50 mg; 21 day release) in attempting to increase the ratio of mineralocorticoid receptor antagonist to mineralocorticoid receptor agonist to achieve a better inhibition. Eplerenone treatment completely abolished aldosterone and salt-induced increases in maximal intraluminal diameters of abdominal aortas (eg, 2 weeks after treatment, 1.26 mm [aldosterone and salt plus eplerenone; n=12] versus 1.70 mm [aldosterone and salt; n=16]; P<0.001; Figure 6D) and maximal external diameters of abdominal (1.08 mm [aldosterone and salt plus eplerenone; n=16] versus 1.40 mm [aldosterone and salt; n=13]; P<0.001; Figure 6E) and thoracic aortas (0.84 mm [aldosterone and salt plus eplerenone; n=12] versus 1.34 mm [aldosterone and salt; n=13]; P<0.001; Figure 6E). Moreover, none of the 12 mice treated with eplerenone developed any aortic aneurysm in any part of aorta (Figure 6F). In contrast, control mice administered aldosterone and salt had significant abdominal and thoracic aortic dilations (Figure 6D and 6E) and dramatic incidences of AAAs, TAAs, and aortic aneurysm ruptures (65%, 45%, and 35%, respectively; Figure 6F).

**DOCA and Salt-Induced Hypertension Does Not Correlate With Aortic Aneurysms**

To investigate whether DOCA and salt-induced hypertension contributes to aortic aneurysm formation, we correlated DOCA and salt-induced hypertension (Figure 7A) to several quantified parameters of aortic aneurysm formation. In DOCA and salt mice, there was no significant correlation between systolic BP and maximal intraluminal diameters of suprarenal abdominal aortas (Figure 7B), and there was also no difference in systolic BP between the mice with and those without aortic aneurysms (Figure 7C). Moreover, DOCA and salt-induced aortic aneurysms were more severe in 10-month-old mice compared with 10-week-old mice (Figure 4), but DOCA and salt-induced hypertension was significantly less in 10-month-old mice compared with 10-week-old mice (Figure 7D).

There was also no correlation between the effectiveness of pharmacological inhibition of DOCA and salt-induced hypertension and aortic aneurysms. Administration of enalapril significantly decreased DOCA and salt-induced increases in systolic BP and maximal intraluminal diameters of suprarenal abdominal aortas (Figure 7B) and there was no difference in systolic BP between the mice with and those without aortic aneurysms (Figure 7C). Moreover, DOCA and salt-induced aortic aneurysms were more severe in 10-month-old mice compared with 10-week-old mice (Figure 4), but DOCA and salt-induced hypertension was significantly less in 10-month-old mice compared with 10-week-old mice (Figure 7D).
in systolic BP (Figure 7E) without affecting DOCA and salt-induced aortic aneurysms (Figure 5B–5D). In contrast, administration of spironolactone had no effect on DOCA and salt-induced hypertension (Figure 7F), but attenuated DOCA and salt-induced aortic aneurysms (Figure 6A–6C).

Discussion

The current study describes a novel mouse model of aortic aneurysms induced by administration of mineralocorticoid receptor agonist and high salt, and reveals a previously unrecognized, but potentially significant, role of aldosterone in the pathogenesis of aortic aneurysms. The major findings are as follows: (1) DOCA and salt or aldosterone and salt were able to induce AAAs and TAAs in 10-month-old male C57BL/6 mice.

The pathology of aortic aneurysms induced by DOCA and salt or aldosterone and salt resembled several features of human aneurysms, including elastin degradation, inflammatory cell infiltration, smooth muscle cell degeneration and apoptosis, and oxidative stress; (2) the incidence of aortic aneurysms and the severity of the vascular pathologies were age dependent; (3) aortic aneurysm formation did not correlate with BP increases; and (4) mineralocorticoid receptor antagonism, but not blockade of Ang II synthesis or the Ang II type 1 receptor, reduced DOCA and salt-induced aortic aneurysms.

In humans, elevated plasma aldosterone levels are associated with essential hypertension, primary aldosteronism, and congestive heart failure, suggesting that aldosterone is emerging as a major independent cardiovascular risk factor.
factor. The normal plasma aldosterone concentration range in humans (0.139–0.416 nmol/L) can increase up to 58- to 173-fold (24 nmol/L) in patients with congestive heart failure. In the current study, infusions of 200, 500, or 700 μg/kg per day, respectively, resulted in plasma aldosterone concentrations (10.58, 21.21, and 27.36 nmol/L, respectively) that are comparable with those observed under pathological conditions. Whether aortic aneurysms are present in patients with congestive heart failure with high plasma aldosterone concentrations is not known. However, aortic dissection has been demonstrated in patients with primary aldosteronism. In addition, patients with glucocorticoid-remediable aldosteronism, an inherited form of primary hyperaldosteronism, often die at a young age because of rupture of cerebral aneurysms.

Little is known regarding the level of NR3C2 mRNA and protein in human aortic aneurysm. Lenk et al performed a genome-wide microarray-based expression study and reported that NR3C2 mRNA level was significantly downregulated in human AAA tissue samples compared with age-, sex-, ethnicity-, and location-matched controls of the infrarenal abdominal aorta. Although it remains elusive that this significant downregulation of NR3C2 mRNA (P=0.0004) alters the level of NR3C2 protein and function, an analysis of drug modulation of AAA growth through 25 years of surveillance in 1269 patients demonstrated that there is a strong association between mineralocorticoid receptor blockers and slowed AAA progression, implicating mineralocorticoid receptor in human AAAs.

Given that DOCA and salt- or aldosterone and salt-induced hypertension has been used extensively as an experimental model of low-renin hypertension, it is surprising that aortic aneurysms have not been reported in previous studies. Although exact reasons for this discrepancy are unclear, our results suggest that the age of mice may be critical to aneurysm formation. Specifically, 10-month-old mice exhibited more severe aneurysms at a higher incidence compared with 10-week-old mice, an age (or even younger) commonly used in previous studies studying DOCA and salt or aldosterone and salt hypertension. Moreover, previous studies demonstrated that mineralocorticoid receptor expression in aorta increases with age, as does the functional response to mineralocorticoid receptor stimulation (eg, mice with smooth muscle–specific deficiency of the mineralocorticoid receptor decreased BP as they aged). Because age is a risk factor for human AAAs, the ability to induce AAAs by DOCA and salt
or aldosterone and salt with increasing age may have contributed to an inability to detect aneurysms in young mice.

An important finding of the present study is that administration of 2 distinct mineralocorticoid receptor antagonists, spironolactone and eplerenone, significantly attenuated DOCA and salt- or aldosterone and salt-induced aortic aneurysm. This suggests that the mineralocorticoid receptor mediates DOCA and salt- or aldosterone and salt-induced aortic aneurysm. Compared with an inability of spironolactone to completely abolish DOCA and salt-induced aortic aneurysms, the inhibitory effect of eplerenone on aldosterone and salt-induced aortic aneurysm was much more dramatic. The difference between spironolactone and eplerenone in their ability to inhibit aortic aneurysm is likely attributed to the doses of the mineralocorticoid receptor agonists versus antagonists used, because the molecular ratio of aldosterone to eplerenone and DOCA to spironolactone used was 1:900 and 1:2, respectively. Spironolactone, but not eplerenone, exerts antiandrogen and proestrogen effects, which may have contributed to the ability of spironolactone to inhibit DOCA and salt-induced aneurysms, because it has been shown that androgen increases Ang II–induced AAAs, whereas exogenous estrogen decreases AAAs.

Although the molecular mechanism that links activation of the mineralocorticoid receptor and aortic aneurysm formation remains elusive, our results suggest that elastin degradation and collagen suppression are likely involved in DOCA and salt- or aldosterone and salt-induced aortic aneurysm. These findings seem to contradict the result by Bunda et al who found that aldosterone induced elastin and collagen production when they studied cultured cardiac fibroblasts. However, these findings do not necessarily contradict each other because elastin and collagen degradation may be dominant over their production in aortic aneurysm because of smooth muscle cell degeneration and activation of MMPs by vascular cells and inflammatory cells. Obviously, these 2 factors are absent in cultured cardiac fibroblasts.

Intriguingly, the current study also suggests that angiotensin-converting enzyme and Ang II type 1 receptor unlikely play a major role in DOCA and salt aortic aneurysm model. These results, however, do not rule out that stimulation of aldosterone synthesis by Ang II still plays an important role in pathogenesis of aortic aneurysms under pathological conditions (eg, in patients with elevated renin angiotensin system and sodium intake). Our results also suggest that hypertension is not a contributory mechanism to aldosterone and salt- or DOCA and salt-induced aortic aneurysms. These results are consistent with previous studies that showed Ang II infusion promoting AAAs was independent of increased BP. More importantly, we demonstrated that DOCA and salt-induced vascular oxidative stress preceded aortic aneurysm formation and, specifically, we showed that Ncf1 mRNA expression was upregulated by DOCA-salt in thoracic and abdominal aortas in a time-dependent manner. These results fit well with previous studies that Ncf1 protein expression was increased in human AAA segments compared with non-AAA segments, and genetic deletion of Ncf1 attenuated Ang II–induced AAA formation in mice, supporting a key role of NAD(P)H oxidase in vascular oxidative stress and the pathogenesis of AAAs.

Several aortic aneurysm animal models have been developed. Among them, the Ang II-infusion–induced aortic aneurysm mouse model is mostly used. Similar to the Ang II-infusion–induced aortic aneurysm mouse model, DOCA and salt- or aldosterone and salt-induced mouse aortic aneurysms were only found in suprarenal aortas but not infrarenal aorta. Although the exact reasons why mice developed no aortic aneurysms in the infrarenal aorta that are different from human remain elusive, Rush et al performed a whole genome expression analysis in the Ang II–induced aortic aneurysm. Interestingly, they found that 304 transcripts were differentially expressed between suprarenal and infrarenal aortas. It is noted that a number of genes that may be relevant to the predilection of the suprarenal aorta to aortic aneurysm formation were downregulated. These differentially expressed genes may potentially account for why mice developed aortic aneurysm in suprarenal aorta but not infrarenal aorta.

Compared with the Ang II infusion model, the DOCA or aldosterone plus high-salt model described here had several unique features, including using aged wild-type mice, requiring high salt, and exhibiting more severe elastin degradation and aortic aneurysm formation. Ang II is the primary stimulator of adrenal aldosterone synthesis, but Ang II–stimulated aldosterone synthesis is involved in aortic aneurysm formation only in the presence of high salt. It has also been shown that spironolactone had no significant effect on Ang II–induced aortic aneurysm formation in the absence of high salt. It has also been shown that in hypertensive angiotensinogen and renin transgenic mice that overproduce Ang II, aortic aneurysms did not occur unless transgenic mice were fed a high-salt diet. Consistent with these findings, we demonstrated that high salt was required for DOCA-induced aortic aneurysm formation. According to the National Health and Nutrition Examination Survey (NHANES), sodium intake has increased among all age groups since 1970, and only 32% of females and 9% of males meet the recommendation of sodium intake (2400 mg per day). Under the circumstances that the majority of Americans are under the risk of high salt intake, the finding that activation of mineralocorticoid receptor by aldosterone plus high salt induced aortic aneurysm may have a potentially important impact on the current understanding regarding the pathogenesis of aortic aneurysm.

Based on the current study and literature on the function of mineralocorticoid receptor, it is tentative to propose a potential mechanism that may underlie DOCA and salt- or aldosterone and salt-induced aortic aneurysms. Activation of mineralocorticoid receptor in circulating inflammatory cells and vascular cells by DOCA or aldosterone in the presence of high salt promotes monocyte/macrophage, neutrophil, and T cell infiltration into aorta to produce MMPs and oxidative stress, leading to a loss of structural integrity (eg, elastin degradation and vascular smooth cell degeneration) and aortic aneurysm formation and rupture. It should be pointed out that this is an oversimplified mechanism that needs to be further studied.
In conclusion, results from this study demonstrate that mineralocorticoid receptor agonism via DOCA or aldosterone, coupled with increased salt consumption, results in severe aneurysms in the thoracic and abdominal aortas of mice; and age, but not BP, is a contributory mechanism to mineralocorticoid receptor and salt-induced aneurysms. In addition, mineralocorticoid receptor antagonism, but not Ang II synthesis or Ang II type 1 receptor antagonism, attenuates mineralocorticoid receptor and salt-induced aortic aneurysms. Given that there are no effective drug therapies for aortic aneurysms, our results may lead to mineralocorticoid receptor antagonism as a novel therapy to potentially prevent and attenuate aortic aneurysm formation.

Acknowledgments
We thank Dr Katz Wendy for her excellent technical assistance in preparing the histology samples.

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Disclosures
None.

References
Aortic aneurysm is a devastating disease, but currently no drug has been approved for its treatment. This study describes a novel mouse model of aortic aneurysms induced by administration of mineralocorticoid receptor agonist and high salt, which mimics human aortic aneurysm in many aspects. This study reveals a previously unrecognized but potentially significant role of aldosterone/mineralocorticoid receptor and high salt intake in the pathogenesis of aortic aneurysms. The results identify the high salt intake as a potential new risk factor for aortic aneurysm formation. Moreover, the results implicate that spironolactone and eplerenone, 2 Food and Drug Administration (FDA) approved and widely used mineralocorticoid receptor antagonists, may be effective in the prevention and attenuation of some aortic aneurysm formation.

Significance
Mineralocorticoid Receptor Agonists Induce Mouse Aortic Aneurysm Formation and Rupture in the Presence of High Salt
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Mineralocorticoid Receptor Agonists Induce Mouse Aortic Aneurysm Formation and Rupture in the Presence of High Salt

Running title: Aortic aneurysms in DOCA and salt mouse model

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Supplemental Table I. Antibodies used in the current study. The antibody specificity (+++ is based on the information from companies and/or our data showing that no immunostaining was detected in control antibodies but the increased immunostaining was detected in sections from mice administrated DOCA and salt or aldosterone and salt.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Company&amp; catalog number</th>
<th>Species</th>
<th>Dilution used</th>
<th>Specificity</th>
<th>Protein Size in kD</th>
<th>Cited References</th>
</tr>
</thead>
<tbody>
<tr>
<td>MMP2</td>
<td>IHC world, Woodstock, MD IW-PA1122</td>
<td>Human Rat mouse</td>
<td>Working solution</td>
<td>+++</td>
<td>72-kD</td>
<td>None</td>
</tr>
<tr>
<td>CD90.2</td>
<td>BD Biosciences San Jose, CA 550543</td>
<td>Mouse</td>
<td>1:100</td>
<td>+++</td>
<td>18-kD</td>
<td>Circulation 2009 119(3):426-35</td>
</tr>
<tr>
<td>Caldesmon</td>
<td>Sigma-Aldrich St. Louis, MO C4562</td>
<td>Human Rat Mouse</td>
<td>1:500</td>
<td>+++</td>
<td>150-kD</td>
<td>J Mol Cell Cardiol 2011 50(4):621-33</td>
</tr>
</tbody>
</table>
### Supplemental Table II. Effect of aldosterone and salt on intraluminal diameters of abdominal aortas.

<table>
<thead>
<tr>
<th>Time after aldosterone and salt</th>
<th>Control</th>
<th>Aldosterone 200 µg/kg/day</th>
<th>Aldosterone 500 µg/kg/day</th>
<th>Aldosterone 700 µg/kg/day</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 week</td>
<td>1.12±0.03 (N=12)</td>
<td>1.24±0.03 (N=20)</td>
<td>1.28±0.02 (N=10)</td>
<td>1.21±0.03 (N=9)</td>
</tr>
<tr>
<td>1 week</td>
<td>1.06±0.06 (N=6)</td>
<td>1.42±0.03** (N=20)</td>
<td>1.44±0.02 (N=10)</td>
<td>1.21±0.05 (N=9)</td>
</tr>
<tr>
<td>2 week</td>
<td>1.09±0.03 (N=11)</td>
<td>1.62±0.03*** (N=16)</td>
<td>1.70±0.12*** (N=10)</td>
<td>1.60±0.09** (N=8)</td>
</tr>
<tr>
<td>3 week</td>
<td>1.05±0.05 (N=11)</td>
<td>1.61±0.08*** (N=14)</td>
<td>1.82±0.12*** (N=7)</td>
<td>1.56±0.15* (N=6)</td>
</tr>
<tr>
<td>4 week</td>
<td>1.19±0.04 (N=6)</td>
<td>1.58±0.08*** (N=13)</td>
<td>1.80±0.15*** (N=7)</td>
<td>1.53±0.13* (N=5)</td>
</tr>
</tbody>
</table>

*P < 0.05; **P < 0.01; ***P < 0.001 vs. 0 week in each column.

### Supplemental Table III. Effect of aldosterone and salt on maximal external diameters of abdominal and thoracic aortas.

<table>
<thead>
<tr>
<th>Type of aorta</th>
<th>Control</th>
<th>Aldosterone 200 µg/kg/day</th>
<th>Aldosterone 500 µg/kg/day</th>
<th>Aldosterone 700 µg/kg/day</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thoracic aorta</td>
<td>0.88±0.18 (N=12)</td>
<td>1.48±0.21** (N=13)</td>
<td>1.42±0.16** (N=9)</td>
<td>1.33±0.09* (N=5)</td>
</tr>
<tr>
<td>Abdominal aorta</td>
<td>0.76±0.03 (N=12)</td>
<td>1.56±0.26* (N=13)</td>
<td>1.61±0.22** (N=9)</td>
<td>1.46±0.34* (N=5)</td>
</tr>
</tbody>
</table>

*P < 0.05; **P < 0.01 vs. control in each row.
Supplemental Figure I. DOCA and salt induced aortic ruptures are associated with aortic dilations. (A) The time course of aortic ruptures induced by DOCA and salt. (B) Quantification of ultrasound data of ruptured aortas from control mice and mice administrated DOCA and salt for 2 weeks.
Supplemental Figure II. High salt alone has no effect on aortic aneurysm. (A) Quantification of ultrasound data of aortas from mice administrated high salt (N=14). (B) ex vivo aortic diameter quantification from control mice (N=12) or mice administrated high salt (N=14).
Supplemental Figure III. DMSO and high salt do not dilate mouse aorta. (A) Quantification of ultrasound data of aortas from mice administrated 50% DMSO and high salt (N=10). (B) ex vivo aortic diameter quantification from control mice (N=12) and mice administrated 50% DMSO and high salt (N=10).
Supplemental Figure IV. Schematic diagram of a serial of sequential aortic cross sections. Suture knots were tied at 2 mm from descending aorta or above right renal artery as section markers. Aortas were cut equally into two parts as indicated by dashed white line. Each part of aorta was embedded vertically into paraffin with suture knots on the top. Arrow lines indicate the beginning and ending sites of aortic cross sections. Ald (Aldosterone).
Supplemental Figure V. Representative images of aortic elastic staining from control mice. Red borders indicate aortic cross-sections that are anatomically comparable in Figure 5.
Supplemental Figure VI. Representative images of aortic elastic staining from mice administrated DOCA and salt. Red borders indicate aortic cross-sections containing elastin degradation.
Supplemental Figure VII. Effect of DOCA and slat or aldosterone (Ald) and salt on aortic dissections and elastin degradation. (A) The incidence of aortic dissections induced by DOCA and salt or aldosterone (Ald) and salt. Of note, aortic dissections were identified by double channel aortas in cross-sections of elastin staining. (B) Quantification of elastin degradation. Elastin breaks per field under 40× magnification in mice were counted in aortic sections from three groups of mice: 1) Control (Ctrl); 2) DOCA and salt; 3) Aldosterone (Ald) and salt. One number in the figure represents an average of 5-field counts in one cross-section from one mouse.
Supplemental Figure VIII. Localization of total MMP activity in abdominal aortas by in situ zymography. Representative in situ zymograph of abdominal aortas from control mice (a and b) and mice administrated DOCA and salt (c, d, g, and h) or aldosterone and salt (e and f). Proteolysis of DC gelatin fluorescein conjugate by MMPs yields green fluorescence representing their enzymatic activity. Cells’ nuclei were stained with 4’-6-Diamidino-2-phenylindole (DAPI, blue color). Notably, DOCA and salt induced MMP activity was abolished by MMP inhibitor (g and h). The white rectangles in low magnification images (a, c, e, and g) indicate the regions that were further examined in higher magnification images (b, d, f, and h).
Supplemental Figure IX. T cells presents in adventitial layers of abdominal aortas from mice administrated DOCA and salt or aldosterone and salt. Representative CD90.2 immunostaining of paraffin-embedded sections of abdominal aortas. Notably, these aortic sections are the same sections shown in Figure 3v through 3x. Black rectangles in low magnification images (a and b) indicate the regions that were further examined in higher magnification images (c and d).
Supplemental Figure X. Effect of DOCA and salt on vascular \textit{Vcam-1}, \textit{Ccl2}, and \textit{Tnf} mRNA expression. Thoracic aorta (A, B, and C) and Abdominal aorta (D, E, and F) were isolated from control mice or mice administrated DOCA and salt for 9 days and then subjected to real-time PCR analysis.
Supplemental Figure XI. Quantification of TUNEL positive cells. Total TUNEL positive cells in the tunica media of abdominal aortic cross-section were counted. One number in the figure represents one cross-section from one control mouse or one mouse administrated DOCA and salt or aldosterone (Ald) and salt.
Supplemental Figure XII. DOCA and salt increases superoxide production and Ncf1 mRNA expression. (A) Abdominal aortas were isolated from control mice (left panel) or mice administered DOCA and salt for 3 weeks (right panel). Cryosections were stained with DHE for superoxide detection. Of note, elastin had auto-fluorescence that appeared in both groups of mice but DHE staining was more intensive in DOCA and salt mice. (B) Time course of Ncf1 mRNA expression in thoracic aortas from mice administrated DOCA and salt. (C) Time course of Ncf1 mRNA expression in abdominal aorta from mice administrated DOCA and salt. Samples at each time point contain 3 to 4 aortas.
MATERIALS AND METHODS

Mineralocorticoid Receptor Agonists Induce Mouse Aortic Aneurysm Formation and Rupture in the Presence of High Salt

Running title: Aortic aneurysms in DOCA and salt mouse model

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Materials and Methods

Animals

Ten-month-old male C57BL/6 mice (N = 205) were either purchased from colonies of the National Institute on Aging (Charles River, Raleigh, NC) or from the Jackson Laboratory (Bar Harbor, ME) as retired breeders. Ten-week-old male C57BL/6 mice (N = 12) were purchased from the Jackson Laboratory. All protocols were approved by the committee on animal research care and use at University of Kentucky.

General Experimental Protocol

DOCA was delivered by pellet implantation for 3 weeks; aldosterone, losartan, and enalapril were delivered by osmotic minipumps for 4 weeks; spironolactone was delivered by both intraperitoneal (IP) injections for 4 days and pellets implantation for 3 weeks; salt (0.9% NaCl plus 0.2% KCl) was delivered in drinking water upon DOCA or aldosterone administration. Eplerenone was delivered by feeding mice with custom diets [chow supplemented with eplerenone, Research Diets, Inc., New Brunswick, NJ] at 2.5 mg/g for 5 weeks.

One week before DOCA and aldosterone administration, we quantified blood pressure (BP) by tail cuff and aortic lumen diameters by ultrasound. For studies using ACE inhibitors and ARBs, mice were implanted with minipumps to deliver enalapril or losartan prior to BP measurements. For studies using mineralocorticoid receptor antagonists, mice received daily single IP injections of spironolactone for 4 consecutive days immediately followed by co-implantations of DOCA and spironolactone pellets. Upon DOCA or aldosterone administration, ultrasound imaging was performed weekly, and BP was quantified during the last week of DOCA and salt or aldosterone and salt administration. At study endpoint, mice were anesthetized for exsanguination and tissue harvest. Aortas were isolated for ex vivo aortic diameter quantification, and pathological and immunohistological analysis as described in detail in Supplemental Materials.

DOCA Pellet and Aldosterone Minipump Implantation

Mice were anesthetized by intraperitoneal injection of ketamine (100 mg/kg) and xylazine (10 mg/kg). DOCA pellets (50 mg, 21-day release, Innovative Research of America, Sarasota, FL) were subcutaneously implanted in the lateral dorsal area as described. Osmotic minipumps (Alzet model 2004, 28-day release, Alza Co., Palo Alto, CA) containing either D-aldosterone [Sigma-Aldrich (St. Louis, MO); 200, 500, or 700 μg/kg/day] or vehicle (50% DMSO) were subcutaneously implanted on the right flank via an incision in the scapular region.

Ultrasound Imaging

A high-resolution ultrasound imaging system (Vevo 2100, Visualsonics, Toronto,
Canada) was used to image and quantify intraluminal diameters of mouse suprarenal abdominal aortas. Briefly, mice were anesthetized by inhalation of isoflurane mixed with O2 (3-5% isoflurane/97% O2) and maintained by inhalation of isoflurane mixed with O2 (1-3% isoflurane/97% O2) throughout the procedure using a VIP 3000 Isoflurane Matrix. Mouse hair was removed from the abdomen by using a depilatory cream (Nair; Church & Dwight Co, Inc; Princeton, NJ). Mice were laid supine on a heated table. Warmed ultrasound transmission gel (Aquasonic 100; Parker Laboratories, Orange, NJ) was placed on the abdomen. Cine loops of 300 frames were acquired throughout the renal region of the abdominal aorta and used to determine the maximal diameters of the abdominal aorta in the suprarenal region. “Portal Triad” (hepatic artery, hepatic vein, and bile duct) was used as anatomic marker to assess transverse images in the same location in each mouse. Ultrasound images were acquired 1 week before and weekly after DOCA or aldosterone administration, and were independently validated by two different operators.

**Determination of Plasma Aldosterone Concentration**

At the end of the 4 weeks of aldosterone-salt administration, mice were anesthetized and blood was collected via heart puncture. Plasma aldosterone concentrations were determined using a commercial EIA kit (EnZo Life Science, Plymouth Meeting, PA) according to the manufacturer’s instructions.

**Definition and Quantification of Aortic Aneurysms**

Based on the definition of human aortic aneurysm, AAA and TAA were defined in the current study as having at least a 50% increase in maximal intraluminal and external diameters compared with the same region of aorta in control mice. Maximal intraluminal diameters of suprarenal abdominal aortas were quantified **in vivo** by ultrasound imaging. Maximal external diameters of thoracic and abdominal aortas were quantified **ex vivo** by microscopy as described.

**Drug Delivery**

Losartan and enalapril were purchased from Sigma-Aldrich and dissolved in phosphate buffered saline (PBS). Losartan or enalapril were delivered by subcutaneously implanted osmotic minipumps (Alzet model 2004, 28-day release, Alza Co., Palo Alto, CA). Mice received losartan (25 mg/kg/day) or enalapril (2.5 mg/kg/day) 1 week prior to and 3 weeks during DOCA and salt administration.

Spironolactone was delivered by both intraperitoneal injection and by pellet. Spironolactone powder was purchased from Sigma-Aldrich and was dissolved in sesame oil (Sigma-Aldrich). Mice received spironolactone (700 μg/kg) or vehicle (sesame oil) via daily single intraperitoneal injection for 4 consecutive days prior to DOCA and salt administration immediately followed by coimplantation of spironolactone (100 mg, 21-day release, Innovative Research of America, Sarasota, FL) and DOCA pellets (50 mg, 21-day release) plus high salt for 3 weeks.
Eplerenone (Inspira) was purchased from Pfizer (New York, NY) and was delivered by feeding mice with custom diets [chow supplemented with eplerenone, Research Diets, Inc., New Brunswick, NJ] at 2.5 mg/g for 5 weeks. 1 week after custom diet, mice were implanted osmotic minipumps (Alzet model 2004, 28-day release, Alza Co., Palo Alto, CA; 200 µg/kg/day). Mice received aldosterone and salt for 4 weeks.

**Blood Pressure Measurement**

Systolic blood pressure (SBP) was measured using a non-invasive tail cuff system (Coda 6; Kent Scientific Corp., Torrington, CT). Measurements were performed for 5 consecutive days for determination of weekly average measures as described.3 SBP was measured 1 week before DOCA or aldosterone administration (basal) and again during the third week after DOCA and salt administration or during the fourth week after aldosterone-salt administration.

**Histological and Immunohistochemical Staining**

At the end of the study, mice were perfusion-fixed at approximately 100 mm Hg. Aorta, heart, and kidney were harvested and fixed in formaldehyde as described.5 Extraneous tissues (e.g. fat) surrounding aortas were removed as much as possible. Aorta, heart, and kidney were then photographed by Nikon SMZ800 Stereo Microscope with Digital Imaging. Maximal external diameters of thoracic and abdominal aortas were measured by the NIS-Elements software. Aortas were tied with suture knots at 2 mm from the descending aorta or above the right renal artery as section markers (Supplemental Figure IIIA). Sequential paraffin-embedded cross-sections (5 µm thick) that encompassed the aortic segment between the two suture knots were collected and mounted on microscope slides. Sections were stained using an Elastic Stain Kit (EVG, Fisher Scientific Co.) and Masson Trichrome Kit (Fisher Scientific Co., Pittsburgh, PA) for histochemical examination of elastin and collagen, respectively. Elastin degradation was quantified by per field under 40X magnification (Olympus IX70 microscope) as described.6 7

Sections containing elastin degradation were deparaffinized, rehydrated, and treated with low pH antigen retrieve buffer (Vector Laboratory, Burlingame, CA) for antigen retrieval. Sections were treated with 3% H2O2 for 20 min to quench endogenous peroxidases. After blocking endogenous biotin with Avidin/Biotin blocking kit (Vector Laboratories) and non-specific binding (normal goat serum, Vectastain ABC Kit), slides were incubated with the following primary antibodies overnight at 4°C (Supplemental Table I). Slides were then subjected to the procedure of VECTASTAIN Elite ABC system (Vector Laboratories). Immunoreactivity was visualized by DAB (DAKO North America Inc, Carpinteria, CA) or AEC (Biomedia Corporation Foster City, CA), followed by counterstaining with hematoxylin. To assess apoptosis, the terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL) assay was performed by using the ApopTag kit (ApopTag Peroxidase In Situ Oligo Ligation Apoptosis Detection Kit; EMD Millipore, Billerica, MA) according to the manufacturer’s instructions. Images were captured by Olympus IX70 microscope equipped with Olympus DP70 digital camera.
**In situ zymography**

Abdominal aortas from control mice or mice administrated DOCA and salt were snap frozen in liquid nitrogen and mounted in Optimal Cutting Temperature compound (OCT; Tissue-Tek, Torrance, CA). Freshly cut 10-μm-thick sections were incubated with a DQ gelatin fluorescein conjugate (Life Technologies, Grand Island, NY) according to the manufacture’s protocol and literature. DQ gelatin fluorescein conjugate is heavily labeled with fluorescein that fluorescence is quenched. Proteolysis by MMPs yields fluorescence that is proportional to proteolytic activity. Fluorescence was recorded by Olympus IX70 microscope equipped with Olympus DP70 digital camera. To ensure that detected fluorescence comes from MMPs, aortic sections were also incubated with DQ gelatin fluorescein conjugate in the presence of 0.4 mM 1,10 phenanthroline monohydrate, a general MMP inhibitor.

**Real-Time PCR**

Aortas were harvested from mice administrated with DOCA and salt for 0, 2, 5, and 9 days and divided into thoracic aorta (2 mm from descending aorta to diaphragm level) and abdominal aorta (from diaphragm level to just above right renal artery). The aortas were placed in RNeAlter® Solution (Life Technologies, Grand Island, NY) and mRNA level was quantified by real-time PCR as described previously. Primers used for quantification of mouse Ncf1 (Gene ID: 17969) are: 5'–CTGGTGCGGTGTCAGGAAG–3' (forward) and 5'–TCTCCTCCCCAGCCTCTG–3' (reverse). Primers used for quantification of mouse Vcam1 (gene ID: 22329), are: 5'–AGTTGGGGATTCGGTTGTTCT–3' (forward) and 5'–CCCCTCATTCCTTACCACC–3' (reverse). Primers used for quantification of mouse Ccl2 (gene ID: 20296) and Tnf (gene ID: 21926) were described previously.

**Superoxide detection**

Superoxide concentrations were measured in situ in aortic cryosections with the oxidative fluorescent dye dihydroethidium (DHE; Life Technologies, Grand Island, NY) as described. OCT-embedded cryosections (10 μm) of aortas were obtained from 10-month-old C57B/6 male mice administrated DOCA and salt for 3 weeks or from control mice. Cryosections were incubated with DHE (10 μmol/L) in PBS for 30 minutes at 37°C. Fluorescence images were photographed using an Olympus IX70 microscope equipped with an Olympus DP70 digital camera using identical imaging parameters.

**Statistics**

Data were pooled prior to analyses. Data are illustrated as mean ± SEM and statistical analyses were carried out using GraphPad, Prism 4 (San Diego, CA). One-way ANOVA was used to compare the maximal external diameter of abdominal and thoracic aortas and blood pressure. Two-way ANOVA followed by Bonferroni post-tests was used to analyze the time course of various treatments. Fisher’s exact test was used to analyze the incidence of AAA, TAA, and aortic aneurysm rupture. A p<0.05 was deemed statistically
significant.

References
