ORIGINAL ARTICLE

AT_1 Receptors Prevent Salt-Induced Vascular Dysfunction in Isolated Middle Cerebral Arteries of 2 Kidney–1 Clip Hypertensive Rats

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BACKGROUND
Elevated blood pressure, elevated angiotensin II (ANG II), and ANG II suppression with high salt (HS) diet all contribute to vascular dysfunction. This study investigated the interplay of HS diet and vascular function in a high renin model of hypertension.

METHODS
Male Sprague-Dawley rats were subjected to 2 kidney–1 clip (2K1C) Goldblatt hypertension for 4 weeks and compared with sham-operated controls.

RESULTS
Middle cerebral arteries (MCA) of 2K1C rats and sham-operated controls fed normal salt (NS; 0.4% NaCl) diet dilated in response to acetylcholine (ACh) and reduced partial pressure of oxygen (PO_2). Switching to HS (4% NaCl) diet for 3 days to reduce plasma renin activity (PRA) eliminated vasodilation to ACh and reduced PO_2 in sham-operated controls, with no effect on vasodilation in 2K1C rats. AT_1 receptor blockade (losartan, 20 mg/kg/day; 1 week) eliminated vasodilator responses to ACh and reduced PO_2 in 2K1C rats fed NS or HS diet. ANG II infusion (5 ng/kg/min, intravenous) for 3 days to prevent salt-induced reductions in plasma ANG II restored vascular relaxation in MCA of sham-operated controls fed HS diet. Copper/zinc superoxide dismutase expression and total superoxide dismutase activity were significantly higher in arteries of 2K1C rats fed HS diet vs. sham-operated controls.

CONCLUSIONS
These results suggest that the sustained effects of elevated ANG II levels in 2K1C hypertension maintain endothelium-dependent vasodilatation via AT_1 receptor–mediated preservation of antioxidant defense mechanisms despite significant elevations in blood pressure and salt-induced suppression of PRA.

Keywords: angiotensin II; blood pressure; cerebral circulation; endothelial dysfunction; hypertension; oxidant stress; salt; sodium; superoxide.

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Two kidney–1 clip (2K1C) Goldblatt hypertension is an excellent experimental model to study renal vascular hypertension, a serious and prevalent cardiovascular disease. Animals with 2K1C hypertension exhibit substantial elevations in plasma renin activity (PRA) and circulating angiotensin II (ANG II) levels that reach peak levels around 4 weeks after clipping.[1,2] In contrast with 2K1C hypertension, several other forms of hypertension are characterized by low renin[3] concomitant with endothelial dysfunction.[4,5]

One of the most devastating consequences of hypertension and high salt (HS) diet is stroke. Dysregulation of cerebral vascular relaxation can have other disastrous consequences, and vascular-related factors (including oxidant stress) have been proposed to contribute to several varieties of cognitive dysfunction and dementia, including Alzheimer's disease.[6] Importantly, endothelial dysfunction has been shown to be a powerful prognostic indicator of adverse cardiovascular events, including stroke, independent of blood pressure.[7]

Although the ability of supraphysiological levels of ANG II to cause endothelial dysfunction and increase vascular oxidant stress is well known,[8] there is increasing evidence that salt-induced suppression of plasma ANG II also leads to endothelial dysfunction, vascular oxidant stress, and impaired vascular relaxation.[9–12] Dahl salt-sensitive (SS) rats, a rodent model of chronically lowered renin-angiotensin system (RAS) activity,[13] exhibit a similar vascular phenotype, even when they are normotensive and maintained on a normal salt (NS) diet.[14] In light of these disparate observations, this study addressed the following questions: (i) what is the effect of short-term exposure to HS diet on cerebral vascular function in rats with 2K1C Goldblatt hypertension; and (ii) do the elevated PRA and increased circulating ANG II levels in 2K1C hypertension exacerbate or ameliorate salt-induced vascular dysfunction in cerebral arteries?

METHODS

Experimental animal groups

Male Sprague-Dawley rats were anesthetized by intramuscular injection of ketamine (75 mg/kg), acepromazine (2.5 mg/kg), and anased (10 mg/kg). The left kidney was

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accessed through a left lateral incision, and a 0.20-mm silver clip was placed around the renal artery. 2K1C rats and their respective sham-operated controls in which the renal artery was exposed and cleared without clipping were aged 11–12 weeks at the time of the isolated vessel experiments and were studied 4 weeks after clipping or sham-operation. Other groups of rats anesthetized with the same anesthetic cocktail were fitted with indwelling catheters for blood sampling and arterial pressure measurement in the conscious undisturbed animal.[13–16] Rats used for chronic recording were allowed a 3-day recovery period before the experiment.

One group of sham-operated controls and a corresponding group of 2K1C rats maintained on an NS diet (0.4% NaCl; Dyets, Bethlehem, PA) were switched to an HS diet (4% NaCl; Dyets, Bethlehem PA) 3–5 days before the isolated vessel experiment to reduce plasma ANG II levels. To further test of the role of ANG II in modulating responses to vasodilator stimuli, other groups of 2K1C rats fed NS diet or switched to HS diet for 3 days were given the ATR_1 blocker losartan (20 mg/kg/day for 1 week) in the drinking water before the acute experiments. Because infusion of a low dose of ANG II (5 ng/kg/min intravenously) prevents salt-induced reductions in plasma ANG II levels,[16,17] another group of HS-fed sham-operated controls received a continuous intravenous infusion of a subpressor dose of ANG II (5 ng/kg/min) for 3 days before the experiment. All rats were housed with free access to food and water. All protocols were approved by the Medical College of Wisconsin Institutional Animal Care and Use Committee.

Cannulated middle cerebral artery (MCA) preparation

On the day of the experiment, animals were anesthetized with sodium pentobarbital (60 mg/kg, intraperitoneally) or a ketamine (75 mg/kg), acepromazine (2.5 mg/kg), and anased (10 mg/kg) cocktail. Mean arterial pressures (MAPs) in rats used for the isolated vessel experiments were evaluated by cannulating a carotid artery and were consistent with patterns obtained in the conscious rats (NS 2K1C = 154 ± 6 mm Hg (n = 6); HS 2K1C = 155 ± 6 mm Hg (n = 7); NS sham = 122 ± 5 mm Hg (n = 6); HS sham = 137 ± 5 mm Hg (n = 6)).

The brain was removed and immersed in physiological salt solution (PSS) with the following ionic composition: NaCl (119.0 mM), potassium chloride (4.7 mM), calcium chloride (1.6 mM), monosodium phosphate (1.18 mM), magnesium sulfate (1.17 mM), sodium bicarbonate (24.0 mM), D-glucose (5.5 mM), and ethylenediaminetetraacetic acid (EDTA) (0.03 mM). The MCA was carefully excised, cannulated with glass micropipettes, and extended to its approximate *in situ* length. Side branches were ligated to prevent leaks, and the artery was continuously perfused and superfused with PSS (37 °C) equilibrated with a 21% oxygen (O_2)/5% carbon dioxide (CO_2)/74% nitrogen (N_2) gas mixture. Intraluminal pressure was maintained at 80 mm Hg, and internal diameter was measured with video micrometer (model IV-550; FOR-A, Tokyo, Japan). Vessels lacking resting tone were not studied.

Response to acetylcholine (ACh), reduced partial pressure of oxygen (PO_2), and calcium ion (Ca^{2+})–free solution

Diameter changes in response to a classic endothelium-dependent vasodilator agonist ACh (1 μM) and the physiological vasodilator stimulus of reduced PO_2 were assessed in each group. The single dose of ACh was used to minimize the duration of the experiment and was identical to that previously used to demonstrate salt-induced endothelial dysfunction.[10,11] However previous studies have shown that HS diet eliminates vasodilator responses to multiple doses of ACh in Sprague-Dawley rats[9] and congenic rats carrying a normally functioning renin allele from the Brown Norway rat in the Dahl SS genetic background.[18]

To evaluate vessel responses to reduced PO_2, the artery was allowed a minimum 30-minute equilibration period at 21% O_2, after which the perfusion and superfusion solutions were simultaneously equilibrated with a 0% O_2/5% CO_2/95% N_2 gas mixture for 10 minutes. Under these conditions, the PO_2 of PSS equilibrated with 21% O_2 is approximately 140 mm Hg, and PO_2 in the perfusate and superfusate decreases to 35–45 mm Hg during equilibration with 0% O_2. At the end of the experiment, resting tone and maximum diameter were assessed by superfusion with Ca^{2+}-free PSS.[9–11,19]

Plasma renin activity (PRA)

For measurement of PRA, arterial blood (2 ml) was withdrawn by spontaneous bleeding from the arterial catheter in the undisturbed, chronically cannulated rats. The blood was collected in chilled tubes containing potassium EDTA 50 μl/ml and 300 mmol/l Na_4EDTA. Samples were centrifuged at 4 °C, and the plasma was frozen and stored at −80 °C. PRA (nanograms angiotensin I formed per milliliter per sample per hour) was measured in the Physiology Department Assay Core facility as described previously.[13]

Western blots and superoxide dismutase (SOD) activity

In addition to removing cerebral arteries to evaluate vessel responses to vasodilator stimuli, resistance arteries (100–300 μm) supplying the small intestine of the same rats were isolated to provide tissue to evaluate the expression of copper (Cu)/zinc (Zn) SOD, manganese SOD, endothelial nitric oxide synthase (eNOS), and phosphorylated eNOS (Ser-1177) by Western blotting.[15,20] All values were normalized as percentage β-actin. As a complement to SOD expression, total SOD activity was measured in mesenteric arteries of 2K1C rats and sham-operated controls fed HS diet using a Cayman Chemical SOD-KIT (Cayman Chemical, Ann Arbor, MI) according to the manufacturer's instructions.

Statistical methods

Data are presented as mean ±SEM. Differences between multiple means were determined using analysis of variance with a Newman–Keuls test *post hoc*. $P < 0.05$ was considered to be statistically significant.

RESULTS

Arterial blood pressure and PRA

Table 1 compares MAP and PRA in sham-operated controls and 2K1C rats fed NS and HS diets. MAP, measured by chronic in-dwelling catheters in conscious rats, was significantly elevated ($P < 0.05$) in 2K1C rats fed an NS or HS diet vs. corresponding sham-operated controls. PRA was significantly elevated in 2K1C vs. sham-operated controls fed an NS diet. Short-term HS diet caused a significant reduction of PRA in 2K1C rats vs. 2K1C rats fed an NS diet and also reduced PRA by approximately 47% in sham-operated controls.

Response of MCA to ACh and reduced PO_2

MCA of sham-operated controls fed an NS diet dilated in response to ACh and reduced PO_2. These responses were converted to a paradoxical constriction in animals fed an HS diet (Figure 1). MCA of 2K1C rats fed an NS diet dilated in response to ACh and reduced PO_2, despite the elevated blood pressure. However, in contrast with sham-operated controls, short-term HS diet did not eliminate vascular relaxation in response to ACh or reduced PO_2 in 2K1C rats, despite the significant elevation in arterial pressure. Active resting tone, calculated as $[(D_{max} - D_{rest})/D_{max}] \times 100$, where D_{max} is maximum diameter in Ca^{2+}-free solution and D_{rest} is resting control diameter, was similar in isolated MCA from all the groups (NS sham-operated controls = 46 ± 3% (n = 6); NS 2K1C = 48 ± 2% (n = 6); HS sham-operated controls = 51 ± 2% (n = 6); HS 2K1C = 47 ± 3% (n = 6)), showing that any differences in vascular relaxation were not due to differences in initial tone of the artery.

Effect of 2K1C hypertension and dietary salt intake on SOD expression, eNOS expression, eNOS phosphorylation, and total SOD activity

HS diet downregulated Cu/Zn SOD expression significantly in vessels of sham-operated controls but not 2K1C rats, and Cu/Zn SOD expression was significantly higher in arteries of 2K1C rats fed an HS diet vs. sham-operated controls fed an HS diet (Figure 2). Consistent with the latter observation, total SOD activity was significantly higher in 2K1C rats fed an HS diet (39.8 ± 3.80 U/mg protein; n = 8) compared with sham-operated controls fed an HS diet (25.4 ± 1.80 U/mg protein; n = 10). There were no significant differences in magnesium SOD expression, eNOS expression, or phosphorylation of eNOS (S1177) between any of the groups (not shown).

Table 1. Mean arterial blood pressure (MAP) and plasma renin activity (PRA) during normal salt (NS) or high salt (HS) diet in sham operated controls and rats with 2 kidney–1 clip (2K1C) hypertension

Experimental Group	MAP (mm Hg)	PRA (ng angiotensin I/ml/h)
NS sham	110 ± 1 (7)	2.92 ± 0.48 (9)
HS sham	121 ± 2 (8)	1.54 ± 0.38 (11)
NS 2K1C	133 ± 4 (10)*	4.51 ± 0.54 (14)*
HS 2K1C	143 ± 7 (10)*	0.92 ± 0.20 (8)**

*Significantly different ($P < 0.05$) vs. sham-operated controls on the same diet. **Significantly different ($P < 0.05$) from NS diet within the same experimental group. Numbers in parentheses indicate the number of animals.

Effect of losartan and low-dose ANG II infusion on vasodilator responses

AT_1 receptor blockade with losartan eliminated vascular relaxation in response to ACh and reduced PO_2 in 2K1C rats fed either an NS or HS diet (Figure 3). MAP was significantly lower ($P < 0.05$) in losartan-treated 2K1C rats fed an NS diet (96 ± 2 mm Hg; n = 5) vs. untreated 2K1C rats (133 ± 3 mm Hg; n = 10), but not in losartan-treated 2K1C rats fed an HS diet (126 ± 6 mm Hg; n = 11) vs. nontreated 2K1C rats fed an HS diet (143 ± 7 mm Hg; n = 10). As previously reported in intact Sprague-Dawley rats,[9,11,12,19] continuous intravenous infusion of ANG II to prevent salt-induced reductions in plasma ANG II levels[16,17] restored vasodilator responses to ACh and reduced PO_2 that were absent in noninfused

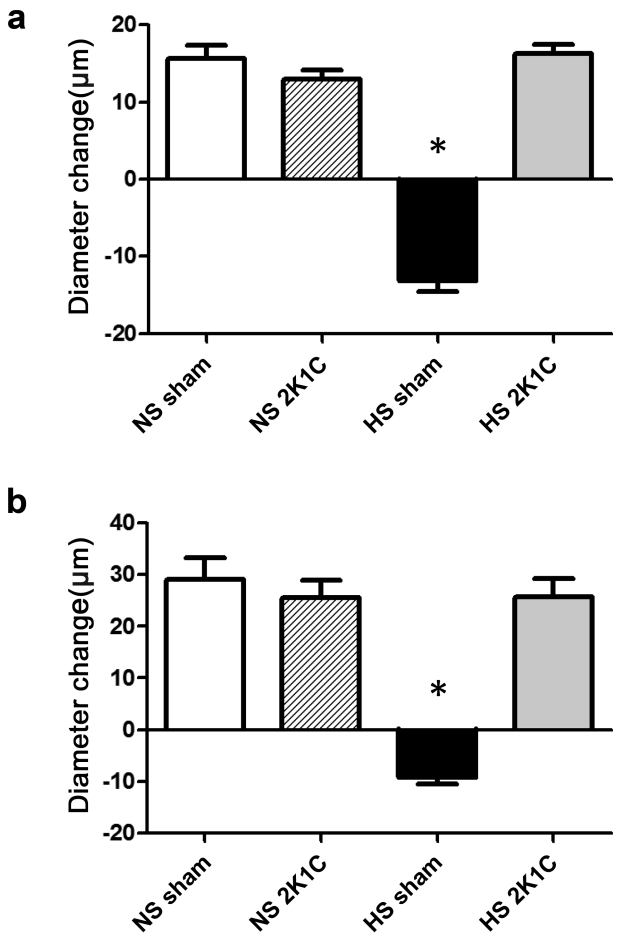

Figure 1. Responses to acetylcholine (ACh; 1 µM) (**a**) and reduced partial pressure of oxygen (PO_2) (**b**) in middle cerebral arteries of 2 kidney–1 clip (2K1C) rats and sham-operated controls fed normal salt (NS) or high salt (HS) diet. Data are summarized as mean change from resting diameter ± SEM for n = 6–8 per group. *$P < 0.05$ vs. NS sham and HS 2K1C rats.

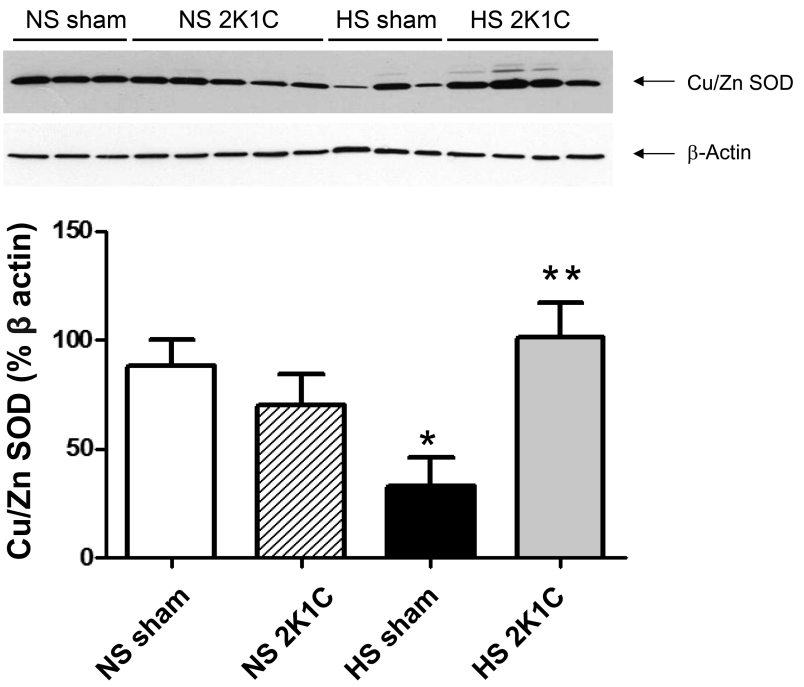

Figure 2. Copper/zinc superoxide dismutase (Cu/Zn SOD) expression (% β-actin) in mesenteric arteries of 2 kidney–1 clip (2K1C rats) and sham-operated controls fed a normal salt (NS) or high salt (HS) diet. Data are summarized as mean ± SEM for n = 6–8 per group. *$P < 0.05$ vs. sham-operated controls fed an NS diet; **$P < 0.05$ vs. sham-operated control fed an HS diet.

controls fed an HS diet (Figure 4) without an increase in arterial blood pressure (anesthetized MAP = 104 ± 4 mm Hg; n = 7).

DISCUSSION

Vascular relaxation is impaired not only in human hypertension[21] but also in response to elevated intravascular pressure in normotensive animals.[22] An HS diet also leads to impaired vascular relaxation and endothelial dysfunction in normotensive rats,[9–12,19] mice,[23] and healthy human volunteers.[24] An HS diet reduces circulating ANG II levels in humans[25] and other species,[16,17,26,27] and salt-induced ANG II suppression is a crucial factor contributing to loss of vascular relaxation in normotensive rats fed an HS diet.[9–12,19] However, to our knowledge, there have been no studies of the effects of short-term elevations in dietary salt intake on PRA and vascular reactivity in 2K1C hypertension.

Similar to existing studies investigating the effect of a long-term HS diet on PRA in 2K1C Goldblatt hypertension,[28,29] we found that a short-term HS diet reduced PRA in 2K1C rats. Consistent with existing studies of intact Sprague-Dawley rats, an HS diet also eliminated vascular relaxation in response to ACh and reduced PO_2 in MCA of the normotensive sham-operated controls. By contrast, MCA of NS- and HS-fed 2K1C rats dilated to a similar extent in response to ACh and reduced PO_2. Thus, HS-induced vascular defects were not present in 2K1C rats despite a substantial elevation of blood pressure, prolonged exposure to elevated plasma renin activity[2] (Table 1) and elevated ANG II levels,[1] and an HS diet—all of which are well-known to cause severe endothelial dysfunction.

At the integrative level, the most likely mechanism for the preservation of vascular relaxation in the hypertensive 2K1C rats fed either an NS or HS diet is activation of the AT_1 receptor because administration of losartan eliminated the restored relaxation to both ACh and reduced PO_2 in both groups. By contrast, vasodilator responses to ACh and reduced PO_2 that were absent in sham-operated controls fed an HS diet were restored by infusion of ANG II at a dose previously shown to prevent salt-induced ANG II suppression.[16,17,30]

Because administration of losartan before the diet change eliminated vascular relaxation in 2K1C rats fed an HS diet, the maintenance of vasodilator responses to ACh and reduced PO_2 in HS-fed 2K1C rats is most likely related to the persisting effects of AT_1 receptor activation to upregulate antioxidant defenses (e.g., Cu/Zn SOD) (Figure 2) in response to the elevated ANG II levels after unilateral renal artery clipping. Those findings provide additional support for the hypothesis that tonic interaction of ANG II with the AT_1 receptor plays an important role in maintaining normal vascular relaxation mechanisms. The most novel and important finding of this study is that these protective effects of AT_1 receptor activation are manifest in the context of the prolonged elevations of ANG II levels with 2K1C hypertension[1] and also during exposure to an HS diet.

The mechanisms by which PRA and ANG II maintain vascular relaxation in 2K1C rats fed an HS diet are most likely related to a paradoxical reduction in vascular oxidant stress. Continuous intravenous infusion of a low dose of ANG II to prevent the salt-induced decrease in plasma ANG II levels not only restores ACh-induced dilation that is lost in cerebral arteries of HS-fed Sprague-Dawley rats[9–11,19] but also reduces

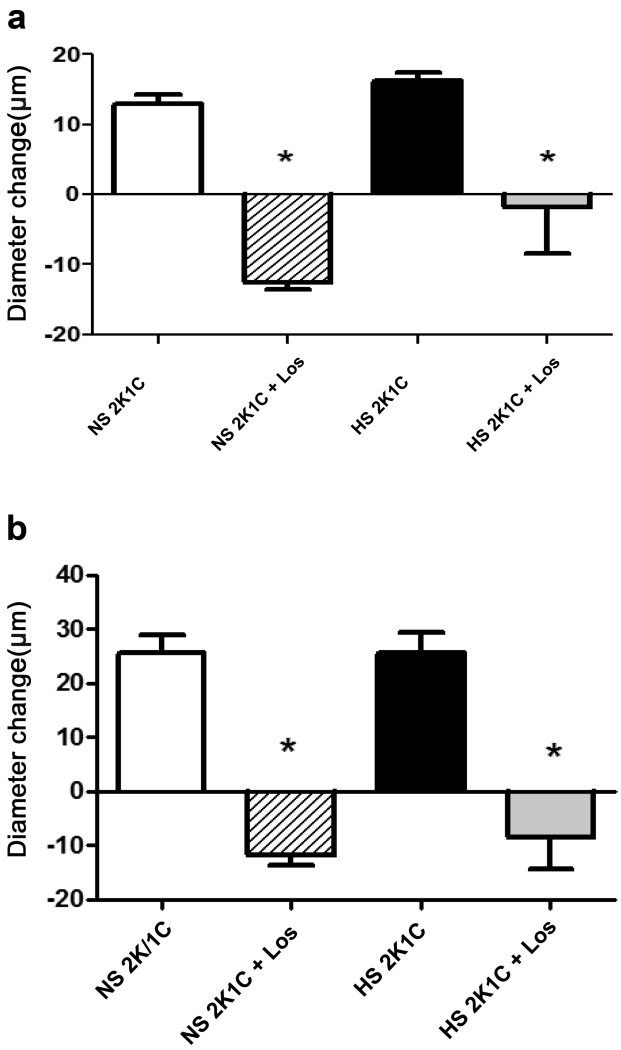

Figure 3. Effect of losartan (Los) on responses to acetylcholine (ACh; 1 μM) (**a**) and reduced partial pressure of oxygen (PO_2) (**b**) in middle cerebral arteries of 2 kidney–1 clip (2K1C) rats fed a normal salt (NS) or high salt (HS) diet. Data are summarized as mean change from resting diameter ± SEM for n = 6–8 per group. *$P < 0.05$ vs. untreated 2K1C rats fed same diet.

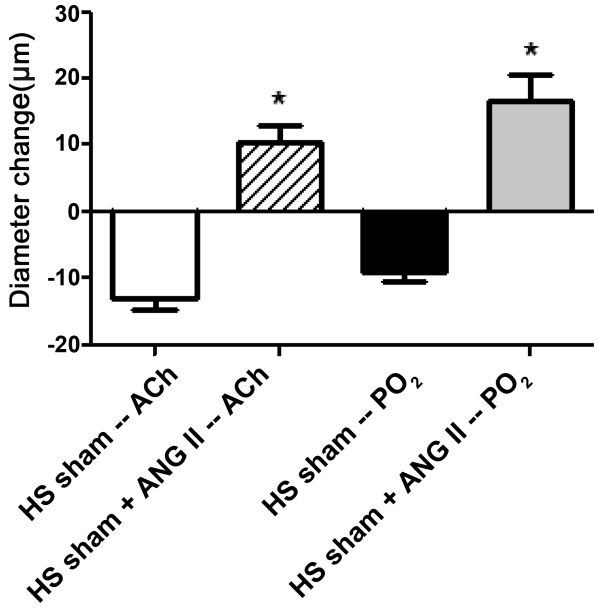

Figure 4. Restoration of responses to acetylcholine (ACh; 1 μM) and reduced partial pressure of oxygen (PO_2) in middle cerebral arteries of high salt (HS)–fed sham-operated control rats receiving continuous intravenous infusion of a low dose of angiotensin II (ANG II). Data are summarized as mean change from resting diameter ± SEM for n = 6–8 per group. *$P < 0.05$ vs. noninfused sham-operated controls fed an HS diet.

vascular superoxide levels to NS values and maintains nitric oxide availability in small mesenteric arteries[12] and aortas[30] of HS-fed Sprague-Dawley rats. Low-dose ANG II infusion also prevents the downregulation of Cu/Zn SOD in cerebral arteries of Sprague-Dawley rats fed an HS diet.[31] Consistent with the hypothesis that AT_1 receptor activation maintains antioxidant defenses are recent findings that losartan blocks the protective effect of ANG II infusion to reduce vascular superoxide levels and restore arteriolar dilation in hamsters fed an HS diet.[32]

In this study, Cu/Zn SOD expression was significantly higher in arteries of 2K1C rats fed an HS diet vs. sham-operated controls fed an HS diet and was not downregulated by an HS diet in the 2K1C rats. Those observations are consistent with the hypothesis that earlier exposure to elevated PRA and ANG II in HS-fed 2K1C rats preserves vascular relaxation by preventing salt-induced downregulation of Cu/Zn SOD (and possibly other antioxidant enzymes). A role for normal plasma ANG II levels in maintaining vascular antioxidant defenses is consistent with reports that ANG II upregulates extracellular SOD expression in mouse aorta and human aortic smooth muscle cells[33] and increases Cu/Zn SOD activity in aortas of extracellular SOD knockout mice.[34] Consistent with the latter report, we also found that total SOD activity in arteries of 2K1C rats fed an HS diet was significantly higher than that in arteries of sham-operated controls fed an HS diet.

One limitation of our study was the use of small mesenteric arteries as a surrogate for SOD expression in the cerebral arteries. We sampled mesenteric arteries because they provided ample amounts of tissue for Western blots while minimizing animal use and conserving resources. However, previous studies have shown that an HS diet reduces nitric oxide levels and increases vascular superoxide levels in aortas[30] and small mesenteric arteries[12] and that these changes are prevented by low-dose ANG II infusion. Because the actions of an HS diet (±ANG II infusion) in mesenteric arteries[12] are similar to those in cerebral arteries,[9,18] those vessels should be representative of changes occurring in the cerebral vasculature. Nonetheless, verification of these changes in future studies of cerebral arteries is clearly warranted.

Another limitation of this study is that mechanisms other than preservation of antioxidant defenses could also contribute to the ability of AT_1 receptor activation to preserve vascular function. The mechanisms by which AT_1 receptor activation exerts its protective effect on vascular function in 2K1C rats fed an HS diet at a more reductionist (cellular and molecular) level also remain to be determined and are clearly worthy of further investigation.

An HS diet is a risk factor not only for hypertension but also for vascular dysfunction in normotensive individuals.

Even short-term elevations in dietary salt intake lead to significant reductions in endothelium-dependent vasodilation in healthy young human volunteers.[24] An HS diet not only increases the risk for hypertension but also increases the risk for other adverse cardiovascular events, including death, even in the absence of an elevated blood pressure.[35] The MCA investigated in this study are highly relevant to clinical problems in humans because an HS diet and endothelial dysfunction have been implicated in the pathogenesis of stroke and other cardiovascular pathologies in humans.[7,35]

The pathophysiological effects of high levels of ANG II in promoting oxidant stress and tissue damage are well known, and the therapeutic benefits of angiotensin-converting enzyme inhibitors and AT_1 receptor blockers are indisputable. However, unexpected beneficial effects of ANG II infusion and detrimental effects of AT_1 receptor blockade have been reported. For example, Takazawa *et al.*[36] reported that ANG II infusion reduces glomerular injury in the early phase of anti-Thy-1.1 nephritis; and Maitland *et al.*[37] reported that AT_1 receptor blockers treatment increases tissue injury in the Dahl salt-sensitive model of low renin hypertension. In another study, Reed *et al.*[38] reported that the effects of ANG II on the p38 and Akt signal transduction pathways and on ischemia-induced coronary collateral growth differed in WKY rats vs. JCR rats (a rodent model of metabolic syndrome), depending on the level of tissue oxidant stress and the dose of ANG II that was administered. In that study, low doses of ANG II were beneficial for coronary collateral growth, and high doses of ANG II were detrimental for coronary collateral growth in WKY.

The paradoxical effect of AT_1 receptor activation to prevent salt-induced vascular dysfunction that we observed in a high renin model of hypertension is highly novel and can provide valuable insight into an unexpected role of ANG II in maintaining normal vascular reactivity under certain physiological and pathophysiological conditions. Future studies to determine how elevations in blood pressure and ANG II interact with the long-term effects of HS diet on vascular function in the 2K1C model Goldblatt hypertension could be of substantial benefit in understanding the relationships between salt, vascular function, and hypertension, especially in light of the well-known difficulties of remaining on a salt-restricted diet in humans.[39]

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DISCLOSURE

The authors declared no conflict of interest.

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