Low-Dose Angiotensin II Infusion Restores Vascular Function in Cerebral Arteries of High Salt–Fed Rats by Increasing Copper/Zinc Superoxide Dimutase Expression

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BACKGROUND
This study examined the vasoprotective role of circulating angiotensin II (ANG II) levels in the cerebral circulation of high salt (HS)–fed (SS.BN-(D13hmgc41-13hmgc23)/Mcwi) (Ren1-BN) congenic rats, which carry a normally functioning renin allele from the Brown Norway (BN) rat on the Dahl salt-sensitive genetic background.

METHODS
Ren1-BN rats were placed on an HS (4.0% NaCl) diet for 3 days. The vasodilator response to acetylcholine (ACh; $10^{-10} - 10^{-6}$ mol/L) was assessed in isolated middle cerebral arteries (MCAs), and Western blots were performed to assess the expression of the antioxidant enzymes copper (Cu)/zinc (Zn) superoxide dismutase (SOD) and manganese (Mn) SOD in cerebral resistance vessels. A separate group of HS-fed animals were infused with either a subpressor dose of ANG II (100 ng/kg/min) or saline vehicle via osmotic minipump for 3 days.

RESULTS
HS diet eliminated acetylcholine (ACh)-induced dilation in the MCAs of the congenic rats. Western blot analysis of antioxidant enzymes showed that Cu/Zn SOD and Mn SOD expression were significantly reduced in the cerebral resistance arteries of the HS-fed rats compared with control animals fed a normal salt diet. Infusion of ANG II restored the vasodilator response to ACh in the MCAs and increased Cu/Zn SOD (but not Mn SOD) expression compared with saline-infused animals.

CONCLUSIONS
These results indicate that prevention of salt-induced ANG II suppression prevents vascular dysfunction in the cerebral circulation by preventing the downregulation of Cu/Zn SOD and vascular oxidant stress that normally occurs with HS diet.

Endothelial dysfunction in salt-sensitive (SS) hypertensive patients is well characterized. However, recent studies have shown that short-term increases in dietary salt intake can also reduce vascular function in humans independent of changes in blood pressure.[1,2] Consistent with this observation, multiple studies in rats[3–10] and mice[11] have shown blood pressure–independent impairment of vascular relaxation with increased dietary salt intake in multiple vascular beds, including the cerebral circulation.[3,7,12] The loss of nitric oxide–mediated vasodilation in animals fed a high salt (HS) diet appears to be due to the suppression of the renin–angiotensin system because chronic intravenous infusion of a subpressor dose of angiotensin II (ANG II) to prevent salt-induced ANG II suppression[13–15] restores normal vascular relaxation mechanisms[7,12,16] and returns vascular superoxide levels to normal salt values in mesenteric resistance arteries.[4]

The goal of this study was to determine the effects of a short-term increase in dietary salt intake on endothelium-dependent vasodilation in response to acetylcholine (ACh) in middle cerebral arteries (MCAs) of the narrowed Ren1-BN congenic rat strain (SS.BN-(D13hmgc41-D13hmgc23)/Mcwi). Previous studies in this congenic rat strain have shown that these animals have genetically rescued vasodilator responses to ACh and higher expression of copper (Cu)/zinc (Zn) superoxide dismutase (SOD) in the cerebral vasculature compared with the Dahl SS parental strain because of the presence of the Brown Norway (BN) renin allele in the Dahl SS genetic background.[17] The use of this congenic rat strain provides a unique model to study the specific role of the renin–angiotensin system on vascular function because, aside from a 2.0 Mbp region of chromosome 13 that contains the BN renin gene, the rats are genetically identical to the Dahl SS strain. In this study, we hypothesized that salt-induced ANG II suppression would eliminate endothelium-dependent dilation in MCAs of the congenic rats due to downregulation of Cu/Zn SOD and manganese (Mn) SOD expression in the cerebral vasculature. We also hypothesized that restoring ANG II to physiological levels during HS intake would restore endothelium-dependent vascular relaxation by increasing SOD expression in the cerebral vasculature.

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MATERIALS AND METHODS

General procedures

All experiments were performed in age-matched (10–14 weeks old) male Ren1-BN congenic rats (SS.BN-(D13hmgc41-13hmgc23)/Mcwi) that were derived from backcrossing SS 13[BN] consomic rats to the parental Dahl SS rat strain, as previously described.[18] All animals were maintained on a normal salt (0.4% NaCl) diet (Dyets, Bethlehem, PA) from weaning, with water to drink ad libitum. Additional experimental groups (n = 5–9) were switched to an HS diet (4.0% NaCl) for 3–5 days. In a separate group of Ren1-BN congenic rats, osmotic minipumps (ALZET, Cupertino, CA) containing either a subpressor dose of ANG II (100 ng/kg/min) or saline vehicle were implanted subcutaneously on the day that the animals were switched to an HS diet. All procedures were approved by the Medical College of Wisconsin's Institutional Animal Care and Use Committee.

Isolated vessel preparation and vasodilator stimuli

On the day of the experiment, animals were anesthetized with an intraperitoneal injection of a low dose of pentobarbital sodium (30 mg/kg; Ovation Pharmaceuticals, Lake Forest, IL) because of the enhanced sensitivity of SS rats to barbiturate anesthesia. Mean arterial pressure of the anesthetized animals was measured by carotid artery cannulation.

MCAs were isolated from the ventral surface of the brain, cannulated with tapered glass micropipettes, and maintained at 37 ºC in a heated chamber for 1 hour while they were perfused and superfused with physiological salt solution bubbled with a 21% oxygen, 5% carbon dioxide, 74% nitrogen gas mixture, as previously described.[19] The vessels were pressurized to 80 mm Hg to simulate in vivo conditions, and internal diameters were measured by television microscopy. The response of the arteries to the endothelium-dependent vasodilator ACh (10^{-10} – 10^{-6} mol/L) were assessed by measuring vessel diameters during cumulative addition of the agonists to the tissue bath.

Western blot analysis of endothelial nitric oxide synthase (eNOS), phosphorylated e-NOS (p-eNOS) (S1177), Mn-SOD and Cu/Zn SOD enzyme expression

Protein expression of the antioxidant enzymes Cu/Zn SOD and Mn SOD, total expression of eNOS, and relative phosphorylation of eNOS at the S1177 activator site were assessed by performing Western blots using cerebral resistance arteries dissected from the ventral surface of the brain after the MCAs were removed for study, as previously described.[17] Protein bands were quantified using the UNSCAN-IT software (Silk Scientific, Orem, UT), and final expression was normalized to β-actin. Relative phosphorylation of eNOS was normalized to total eNOS expression.

Statistical analysis

All data are summarized as mean value ± SEM. Because of limited availability of Ren1-BN rats, vessel responses to ACh in HS-fed control rats were compared with previously reported responses determined in congenic rats fed normal salt diet[17] using 2-way analysis of variance with a Holm–Sidak test post hoc, and responses to ACh from control diameter in HS-fed Ren1-BN rats were also evaluated using a paired t test with a Bonferroni correction for multiple comparisons. Differences in enzyme expression between 2 groups were assessed using an unpaired Student t test. A probability value of $P < 0.05$ was considered to be statistically significant.

RESULTS

Placing Ren1-BN congenic rats on an HS diet for 3–5 days eliminated the vasodilator response to acetylcholine (ACh) in isolated MCAs (Figure 1a). Chronic infusion of a low dose of ANG II (100 ng/kg/min) for 3 days ameliorated endothelial dysfunction in cerebral arteries of HS-fed Ren1-BN congenic rats (Figure 1b), as evidenced by the restoration of ACh-induced vasodilation in arteries of the ANG II-infused rats vs. saline-infused controls. ANG II infusion had no effect on mean arterial pressure in Ren1-BN rats fed an HS diet (113 ± 5.3 mm Hg in ANG II-infused rats (n = 8) vs. 111 ± 5.5 mm Hg in vehicle-infused controls (n = 7)), and vessel responses to the endothelium-independent vasodilator sodium nitroprusside were unaffected by ANG II infusion in HS-fed rats (data not shown). eNOS expression and phosphorylation of eNOS at S1177 in the cerebral vasculature were unaffected by increased dietary salt intake (Figure 2, a–c). In a similar fashion, ANG II infusion had no effect on eNOS expression or phosphorylation at S1177 in the cerebral arteries of congenic rats fed an HS diet (Figure 2, d–f). However, an HS diet caused a significant reduction in the expression of Cu/Zn SOD (Figure 3a,b) and Mn SOD (Figure 4a,b) in cerebral arteries compared with normal salt–fed controls. ANG II infusion caused a significant increase in Cu/Zn SOD expression in arteries of Ren1-BN rats fed an HS diet. (Figure 3c,d), whereas Mn-SOD expression was unaffected by ANG II infusion (Figure 4c,d).

DISCUSSION

The results of this study show that a short-term increase in dietary salt intake reduces the expression of the crucial antioxidant enzymes Cu/Zn SOD and Mn SOD in the cerebral circulation of congenic rats carrying the BN renin allele, concomitant with the development of endothelial dysfunction. Infusion of a subpressor dose of ANG II during the period of HS intake restored the vasodilator response to ACh in isolated MCAs and prevented the reduction of Cu/Zn SOD expression (but not Mn SOD expression) that occurred in HS-fed control animals.

A recent study by Tzemos and colleagues showed that nitric oxide–dependent vasodilation to ACh was severely impaired in young, normotensive humans placed on a short-term HS diet.[20] In another study, Dickinson et al.[21] reported that reducing sodium intake in obese, normotensive individuals dramatically improved flow-mediated vasodilation in the brachial artery. The exact mechanism by which increased dietary salt intake causes vascular dysfunction in humans
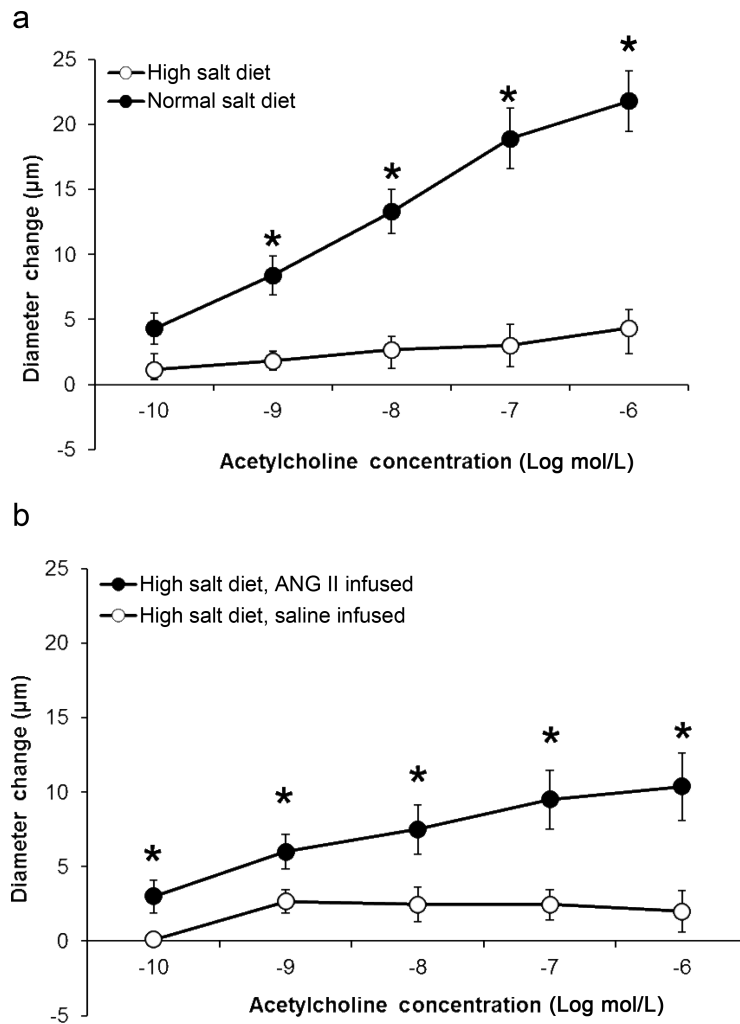
Figure 1. Evaluation of endothelium-dependent dilation to acetylcholine in cerebral arteries of Ren1-BN congenic rats. (**a**) Response to acetylcholine ($10^{-10} – 10^{-6}$ mol/L) in isolated middle cerebral arteries from Ren1-BN congenic rats fed either a normal salt (n = 10) or high salt (n = 6) diet. Because of the limited availability of Ren1-BN rats, the normal salt response is replotted from Durand et al.[17] (**b**) Response to acetylcholine ($10^{-10} – 10^{-6}$ mol/L) in isolated middle cerebral arteries from high salt–fed Ren1-BN congenic rats that received an infusion of either angiotensin II (100 ng/kg/min; n = 8) or saline vehicle (n = 9) by subcutaneously implanted osmotic minipump. Data are expressed as mean change from baseline diameter (µm). *Significant difference (*P* < 0.05) in high salt–fed rats vs. normal salt–fed controls (**a**) or in saline-infused rats vs. angiotensin II–infused rats fed a high salt diet (**b**). Acetylcholine had no significant effect on vessel diameter in high salt–fed Ren1-BN congenic rats.

remains to be elucidated. However, studies in normotensive rodents have shown that the vascular dysfunction induced by feeding the animals an HS diet is due to salt-induced suppression of the renin–angiotensin system because restoring plasma ANG II to normal physiological levels restores vascular reactivity in multiple vascular beds.[16,17,22–24]

It is well known that supraphysiological levels of ANG II cause vascular dysfunction and increase superoxide levels in the vasculature.[25,26] However, there is increasing evidence that similar pathological conditions occur when circulating ANG II levels are suppressed in normotensive rodents either pharmacologically[17,27,28] or because of an increased dietary salt intake.[3,6,9–11,29–31] Studies in Sprague–Dawley rats fed an HS diet have shown that reduced expression or activity of the crucial antioxidant enzyme Cu/Zn SOD leads to vascular dysfunction and increased levels of superoxide in the vasculature.[8,12] Consistent with these studies, chronic

pharmacological inhibition of the renin–angiotensin system with angiotensin converting enzyme inhibitors or angiotensin type 1 receptor antagonists reduces nitric oxide–mediated vasodilation in different vascular beds of normotensive animals,[16,17,27,28,32] providing further support for the hypothesis that normal physiological levels of ANG II are necessary to maintain vascular homeostasis.

Previous studies have shown that endothelium-dependent dilation is intact in isolated MCAs of Ren1-BN rats but absent in Dahl SS rats and in narrowed congenic strains carrying closely adjacent segments of BN chromosome 13 on either side of the renin locus (but still retaining the Dahl SS renin allele).[17] The most novel and important observation of the present study is that an HS diet downregulates the expression of Cu/Zn SOD and Mn SOD concomitant with the development of endothelial dysfunction in the cerebral circulation in the Ren1-BN congenic strain.
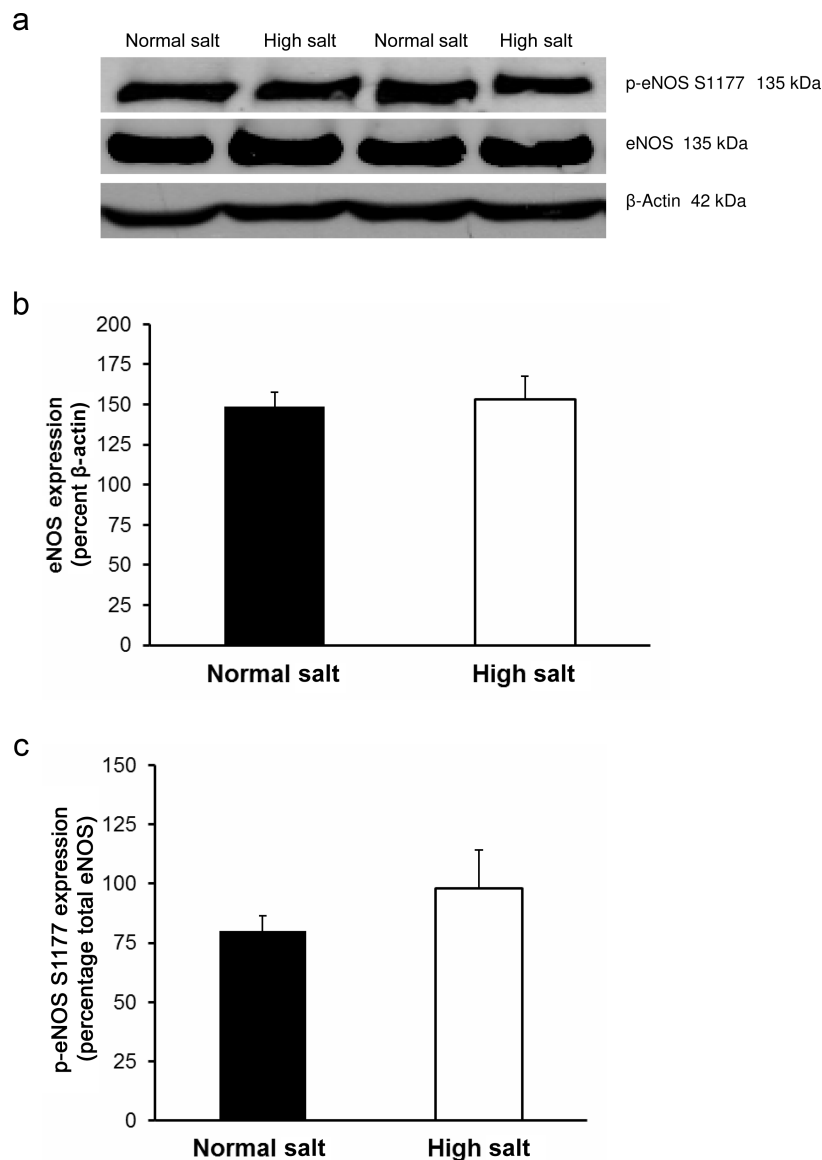
Figure 2. Endothelial nitric oxide synthase expression and phosphorylation at the S1177 activator site in cerebral arteries from normal salt–fed (n = 8) and high salt–fed (n = 6) Ren1-BN congenic rats **(a–c)** and high salt–fed Ren1-BN congenic rats receiving an infusion of either angiotensin II (ANG II; 100 ng/kg/min; n = 4) or isotonic saline vehicle (n = 5) by subcutaneously implanted osmotic minipump **(d–f)**. Panels **a** and **d** show representative Western blots of eNOS and phospho-eNOS (S1177). Panels **b** and **e** show total eNOS expression normalized to β-actin. Panels **c** and **f** show phosphorylation of eNOS at S1177, normalized to total eNOS. All values are summarized as mean ± SEM.

The observation that Cu/Zn SOD expression, but not Mn SOD expression, was higher in the cerebral vasculature of HS-fed congenic rats receiving a low-dose ANG II infusion is interesting because it suggests that ANG II may regulate expression of the cytosolic isoform of SOD (Cu/Zn SOD) but not the mitochondrial form (Mn SOD). This hypothesis is supported by the observation that Ren1-BN congenic rats (which have a normally functioning renin allele) have significantly elevated Cu/Zn SOD expression but equal expression of Mn SOD in the cerebral vasculature compared with Dahl SS rats under normal salt diet conditions.[33]

The complex nature of the regulation of vascular SOD isoform expression and activity is highlighted in a recent study[34] that showed that ANG II infusion increases Cu/Zn SOD expression in mesenteric arteries of wild-type mice but not in extracellular superoxide dismutase (ecSOD) knockout mice. By contrast, ANG II infusion caused a significant decrease in Mn SOD expression in mesenteric arteries of ecSOD knockout mice but not in wild-type mice. A particularly intriguing observation in that study was that ANG II infusion caused a significant increase in Cu/Zn SOD activity in the aorta (but not mesenteric arteries) of ecSOD knockout mice by upregulating copper chaperone protein for SOD and did so in the absence of a change in Cu/Zn SOD expression.

We can only speculate as to underlying mechanisms behind the differential effects of ANG II infusion on Cu/Zn SOD
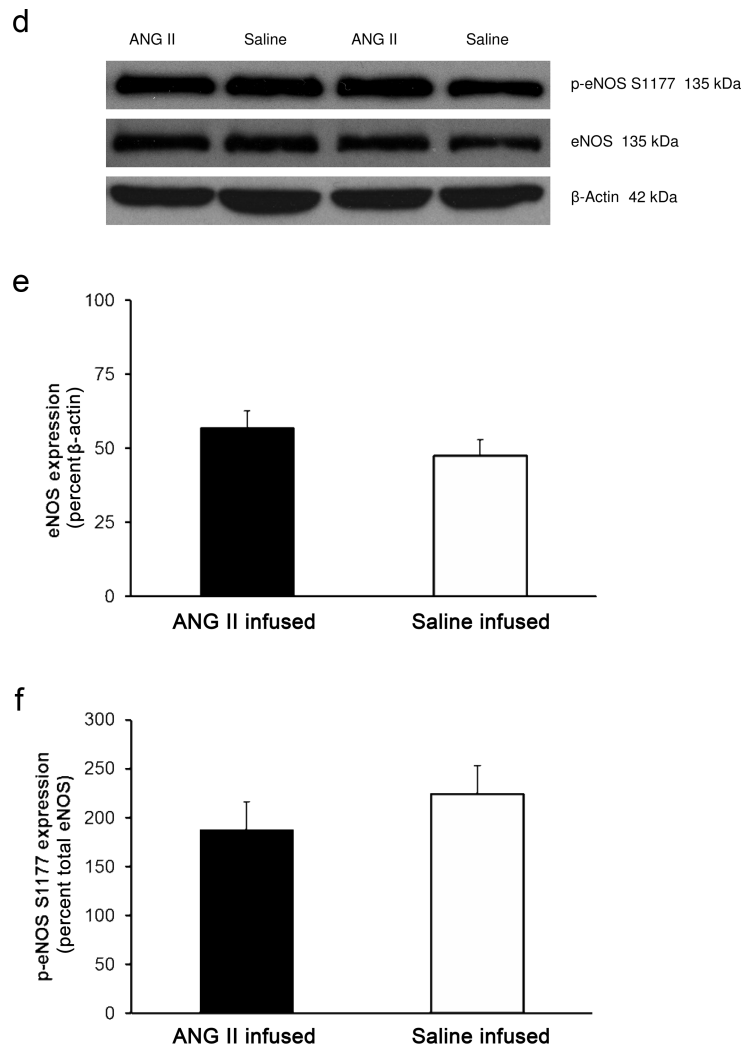
d

e

f

Figure 2. (Continued)

vs. Mn SOD expression in our study (and others as well).[34] For example, regulation of Cu/Zn SOD and Mn SOD may involve different transcription factors that have different sensitivities to plasma ANG II levels. In addition, recent studies by Reed et al.[35] showed that the ultimate effect of ANG II on specific signal transduction pathways and on coronary collateral development after transient ischemic episodes depends on existing levels of oxidant stress in the tissue, which may be affected by the plasma ANG II levels reached under different experimental conditions. Regardless of these considerations, it is important to note that because rats have a point mutation in the heparin-binding domain of ecSOD[36] that is thought to render it dysfunctional at the endothelial–vascular smooth muscle boundary, Cu/Zn SOD contributes to the vast majority of total SOD activity in the vasculature of this species.[37] This is further evidenced by multiple studies showing that acute inhibition of Cu/Zn SOD with diethyldithiocarbamate (DETC) increases superoxide levels and causes vascular dysfunction in multiple vascular beds.[17,38,39]

There are several limitations to this study. First, ANG II infusion did not completely restore the vasodilator response in MCAs of HS-fed rats. One possible explanation for this observation is that consumption of an HS diet may affect muscarinic receptor signaling, similar to altered prostacyclin receptor signaling observed in HS-fed Sprague–Dawley rats[24] but in an ANG II-independent manner. Another possible reason for the partial restoration of Ach-induced dilation by ANG II infusion in HS-fed Ren1-BN rats is that the final plasma ANG II levels obtained with subcutaneous infusion may be lower than those obtained with intravenous infusion in other studies[13–15] and could, therefore, affect different signal transduction pathways relevant to vascular oxidant stress.[35]

Another limitation of this study is that we were unable to directly evaluate vascular and mitochondrial superoxide levels in the cerebral vasculature because of limited availability of tissues. However, previous studies from our laboratory[4,5,14] and others[8–11] have shown that short-term dietary

a

Normal salt   High salt   Normal salt   High salt

Cu/Zn SOD: 19 kDa

β-Actin: 42 kDa

b

c

ANG II   Saline   ANG II   Saline
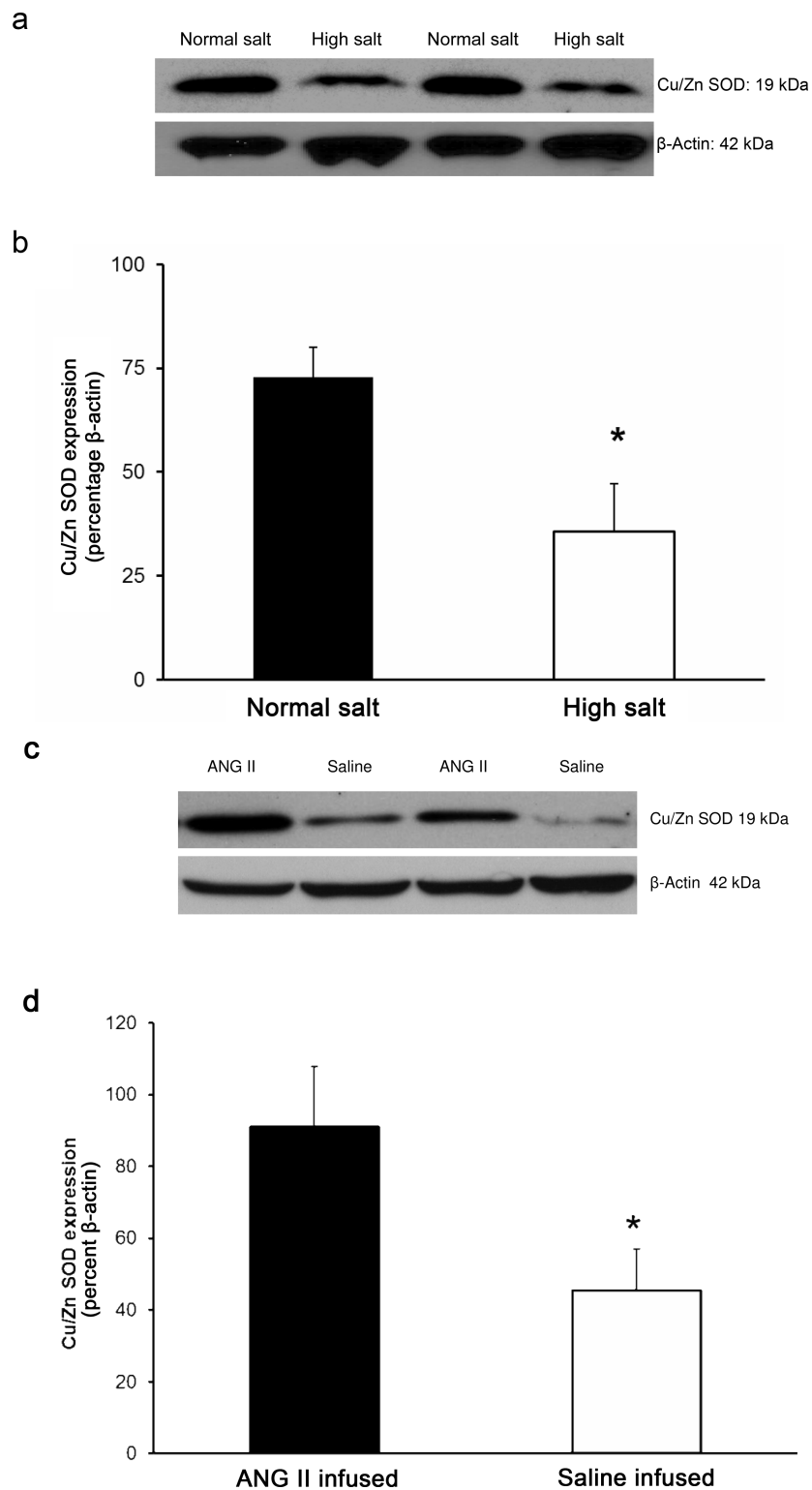
Cu/Zn SOD 19 kDa

β-Actin 42 kDa

d

Figure 3. Copper (Cu)/zinc (Zn) superoxide dismutase (SOD) expression in cerebral arteries from Ren1-BN congenic rats fed a normal salt (n = 4) or high salt (n = 5) diet (**a,b**) and Ren1-BN congenic rats fed a high salt diet and receiving infusion of either angiotensin II (ANG II; 100 ng/kg/min; n = 4) or isotonic saline vehicle (n = 6) by subcutaneously implanted osmotic minipump (**c,d**). Cu/Zn SOD expression was normalized to β-actin, and all values are summarized as mean ± SEM. *Significant difference ($P < 0.05$) in high salt–fed rats vs. normal salt–fed rats or in high salt–fed + angiotensin II–infused rats vs. high salt–fed + saline-infused rats.
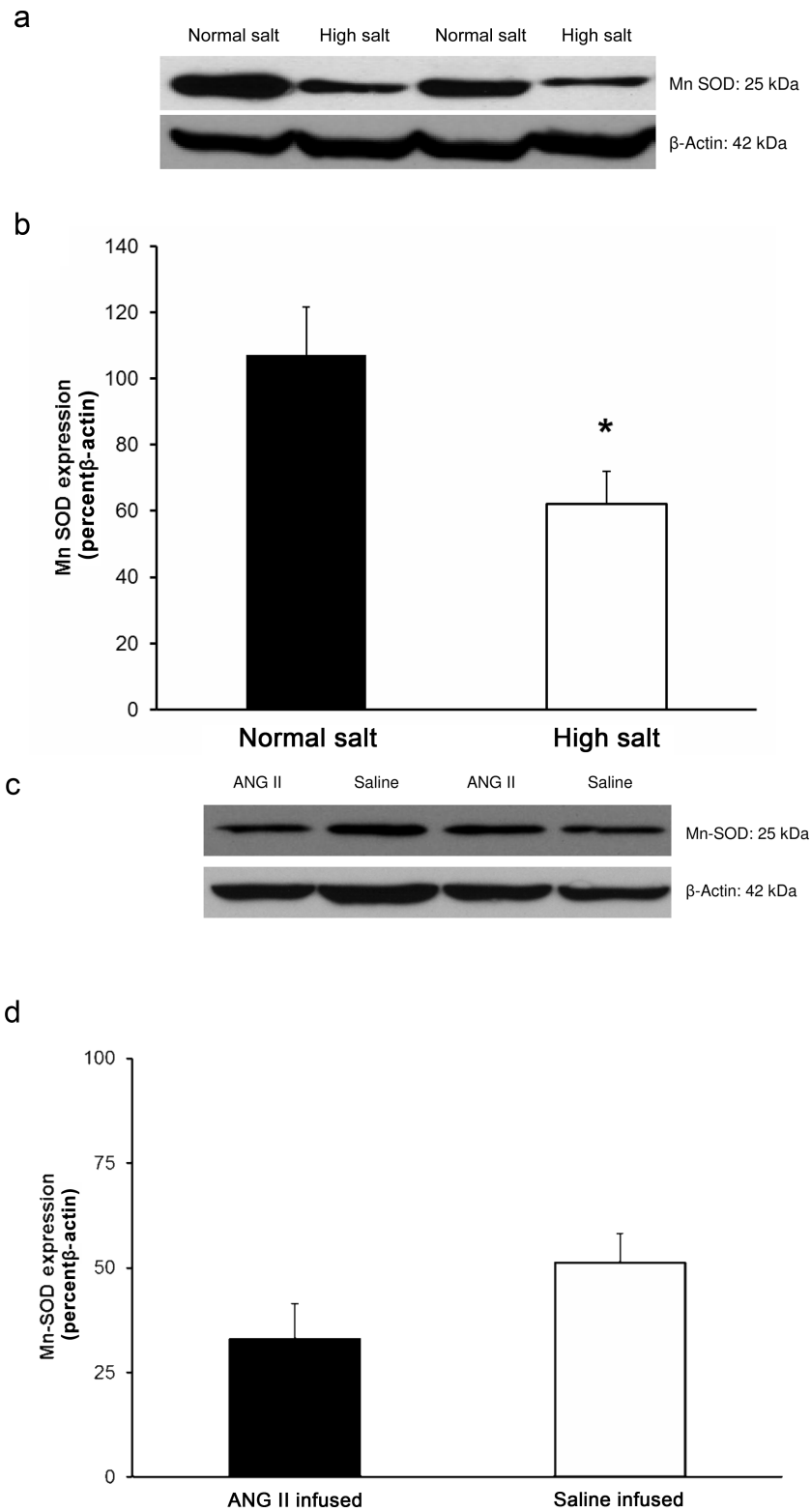
Figure 4. Manganese (Mn) superoxide dismutase (SOD) expression in cerebral arteries from Ren1-BN congenic rats fed a normal salt (n = 8) or high salt (n = 7) diet (**a,b**) and Ren1-BN congenic rats fed a high salt diet and receiving infusion of either angiotensin II (ANG II; 100 ng/kg/min; n = 4) or saline vehicle (n = 6) by subcutaneously implanted osmotic minipump (**c,d**). Mn SOD expression was normalized to β-actin, and all values are summarized as mean ± SEM. *Significant difference ($P < 0.05$) in high salt–fed rats vs. normal salt–fed rats.

salt intake increases superoxide levels in multiple vascular beds, including the cerebral circulation, independent of an increase in blood pressure. Our laboratory has also shown that infusing HS-fed animals with a subpressor dose of ANG II restores superoxide levels to normal salt control values.[4]

The direct contributions of specific pro-oxidant enzymes to superoxide levels were also not directly evaluated in this study. However, a reduced capacity to scavenge vascular superoxide will inevitably lead to oxidative stress and reduced endothelial function, regardless of whether pro-oxidant enzyme expression and/or activity are increased.[9–10]

Despite these limitations, the results of this study provide additional support for the hypothesis that physiological levels of ANG II are necessary to maintain the expression of the crucial antioxidant enzyme Cu/Zn SOD in the cerebral circulation and emphasize the emerging role of physiological levels of ANG II in maintaining antioxidant defenses in the vasculature. However, several important questions remain that are clearly worthy of additional investigation. First, what is the relative role of mitochondrial superoxide levels in contributing to vascular dysfunction in the Dahl SS rat vs. other experimental models that show normal regulation of the renin–angiotensin system? Second, do similar mechanisms regulate Mn SOD expression in the Dahl SS genetic model of SS hypertension and in experimental models that regulate plasma renin activity normally? Finally, and most important, does the apparent absence of a role for plasma ANG II in regulating Mn SOD expression in HS-fed Ren1-BN congenic rats apply to other experimental models and pathophysiological conditions, such as low renin SS hypertension in humans, which is characterized by low levels of circulating ANG II? These intriguing questions are directly relevant to a recent editorial commentary by Wolin,[40] emphasizing that the localized activities of different SOD isoforms in the arterial wall are crucial determinants of vascular function in experimental models of vascular disease and that differences in the influence of ANG II on the expression and activity of various forms of SOD in the vessel wall underscores the incomplete understanding that we have regarding the control of these crucial enzymes in health and disease.

DISCLOSURE

The authors declared no conflict of interest.

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