Chronic Aldosterone Administration Causes NOX2-Mediated Increases In Reactive Oxygen Species Production and Endothelial Dysfunction in the Cerebral Circulation

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Abstract

Objective—An elevated plasma aldosterone level is an independent cardiovascular risk factor. Although excess aldosterone promotes cardiovascular disease, no studies have examined the effect of increased plasma aldosterone on the cerebral circulation. A major source of vascular reactive oxygen species (ROS) during cardiovascular disease is the NADPH oxidases. Because NOX2-containing NADPH oxidase (NOX2 oxidase) is highly expressed in cerebral endothelium, we postulated that it might contribute to ROS generation and vascular dysfunction in response to aldosterone. Here we examined the effect of aldosterone and NOX2 oxidase on ROS production and endothelial dysfunction in the cerebral circulation, and whether the effects of aldosterone are exacerbated in aged mice.

Methods and Results—In adult (average age ~24–25 wk) wild-type (WT) and Nox2-deficient (Nox2$^{-/y}$) mice, neither vehicle nor aldosterone (0.28 mg/kg/day for 14 days) affected blood pressure (measured using tail-cuff). By contrast, aldosterone treatment reduced dilation of the basilar artery (measured using myography) to the endothelium-dependent agonist acetylcholine in WT mice (P<0.05), but had no such effect in NOX2$^{-/y}$ mice (P>0.05). Aldosterone increased basal and phorbol-dibutyrate stimulated superoxide production (measured using L-012-enhanced chemiluminesence) in cerebral arteries from WT but not Nox2$^{-/y}$ mice. In aged WT mice (average age ~70 wk), aldosterone treatment increased blood pressure, but had a similar effect on cerebral artery superoxide levels as in adult WT mice.

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Conclusions—These data indicate that NOX2 oxidase mediates aldosterone-induced increases in ROS production and endothelial dysfunction in cerebral arteries from adult mice independently of blood pressure changes. Aldosterone-induced hypertension is augmented during aging.

Keywords

cerebral artery; cerebrovascular disease; endothelium; NOX2; aging

INTRODUCTION

Aldosterone acts on the mineralocorticoid receptor (MR) expressed in renal tubular epithelial cells to play an important role in sodium and water retention and potassium excretion, thus regulating fluid and electrolyte homeostasis and blood pressure [1]. Aldosterone synthesis and MR expression also occur in non-epithelial (non-renal) tissues including in the brain [2] and cerebral blood vessels [3].

Chronic hypertension exerts many adverse effects on the cerebral circulation and brain [4]. Patients with primary aldosteronism (characterized by an overproduction of aldosterone) suffer more strokes than patients with essential hypertension despite having lower blood pressure [5,6], and have much higher rates of stroke than age-, sex-, and blood pressure-matched essential hypertensives [7]. These observations suggest that elevated plasma aldosterone increases stroke risk in a blood pressure-independent manner. Such an effect could involve deleterious direct actions of aldosterone in the cerebral circulation. Thus, we first aimed to test the hypothesis that aldosterone increases cerebral vascular ROS production and causes endothelial dysfunction.

Aldosterone stimulates the production of reactive oxygen species (ROS) [8–12] and causes endothelial dysfunction [9, 11, 13–17] in systemic arteries. NADPH oxidases are a major source of vascular ROS, and aldosterone increases vascular NADPH oxidase activity [8, 11, 13] and expression of the NOX2 subunit [8]. However, the role of specific NADPH oxidases in mediating aldosterone-dependent vascular ROS production and dysfunction is not well characterized, and has received no study in the cerebral circulation. NOX2 is highly expressed in the endothelium of cerebral arteries [18, 19], and there is evidence for an involvement of NOX2 oxidase in mediating cerebrovascular dysfunction and oxidative stress in response to angiotensin II, and in models of diabetes, aging, hypercholesterolemia and Alzheimer's disease [20–22]. Thus, our second aim was to test whether NOX2 oxidase mediates the deleterious actions of aldosterone in cerebral arteries.

While the frequency of stroke and Alzheimer's disease increases with age [23], underlying age-dependent mechanisms leading to pathological changes in the cerebral circulation are poorly understood. Because there is evidence that mRNA expression of vascular MR is increased in aged (30 months) vs adult (8 months) rats [24], the third aim of our study was to test whether the cerebrovascular actions of aldosterone are exacerbated during aging.

METHODS

Experimental animals

Male mice were studied. $Nox2^{-/y}$ mice were originally generated in the laboratory of Professor Mary Dinauer [25] and have been backcrossed to the C57Bl/6J strain for at least 10 generations. Littermates and age-matched C57Bl/6J mice were used as wild-type (WT; i.e. $Nox2^{+/y}$) controls. Mice had access to regular chow and water ad libitum. All protocols and procedures were approved by the Animal Ethics Committee at Monash University. Adult WT (average age: 24.1±0.8 wk, n=62), $Nox2^{-/y}$ (average age: 25.4±0.6 wk, n=27) and aged WT (average age: 69.8±2.4 wk, n=18) mice were used in this study.

Aldosterone administration and measurement of blood pressure

Following anesthesia with ketamine/xylazine, an osmotic minipump (Alzet, model 1004) was placed subcutaneously in the mid-scapular region to administer vehicle (87% propylene glycol; 9% ethanol; 4% distilled H_2O) or aldosterone (0.28 mg/kg/d for 14 days). Systolic blood pressure was measured using an automated tail-cuff device (MC 4000, Hatteras Instruments Inc.). Prior to surgery, mice were trained for 2–5 days and baseline blood pressure was recorded, followed by implantation of pumps and subsequent measurements of arterial pressure.

Functional study of basilar artery

Isolation and preparation of cerebral arteries was performed as described previously [18, 21]. Basilar arteries were mounted between two microcannulae in a myograph (Living Systems Instrumentation Inc.) and superfused with carbogen-bubbled (95% O_2, 5% CO_2) Krebs-bicarbonate solution (composition in mmol/L; NaCl 118, KCl 4.5, $MgSO_4$ 0.45, KH_2PO_4 1.03, $NaHCO_3$ 25, glucose 11.1, $CaCl_2$ 2.5) maintained at 37°C. Intraluminal pressure was gradually increased to 60 mmHg and maintained at this level with a pressure servo unit without further intraluminal perfusion. Baseline diameters were recorded, and changes in artery diameter in response to KCl (50 mmol/L) were then measured. To examine responses to the endothelium-dependent vasodilator, acetylcholine, arteries were constricted submaximally (~40% of the response to KCl) using the thromboxane A_2 analog, U46619 (100 – 400 nmol/L). The approach of constricting isolated vessels submaximally prior to examination of endothelium-dependent dilator response is very common in the literature.[21, 26–31] This approach places the vessels in a state where dilation or constriction (additional constriction) can be detected. In that sense, it approximates a level of tone that would be seen commonly in vivo. After development of a stable baseline diameter, concentration-response curves to acetylcholine were obtained. Papaverine (100 μmol/L) was used as a control (i.e. endothelium-independent) vasodilator and its effect was assessed at the end of each experiment. In some cases, full dose response curves were tested for papaverine.

Measurement of ROS

L-012 (100 μmol/L) -enhanced chemiluminesence was used to measure superoxide levels in cerebral arteries (pooled samples of basilar artery, middle cerebral arteries, and the circle of

Willis) in WT and Nox2$^{-/y}$ mice, as described previously [21]. Measurements were made under basal conditions and in the presence of phorbol-12,13-dibutyrate (PdB,10 μmol/L). PdB was used to compare the effect of vehicle and aldosterone chronic treatments on the capacity of NOX2 oxidase to acutely generate superoxide. All readings were corrected for background.

Drugs

Acetylcholine and papaverine were obtained from Sigma and were dissolved in saline. Aldosterone was obtained from Sigma and dissolved in 87% propylene glycol, 9% ethanol, 4% distilled H_2O. U46619 was obtained from Cayman Chemical and dissolved in 100% ethanol, with subsequent dilutions being made with Krebs-bicarbonate buffer. PdB was purchased from Calbiochem and prepared at 10 mmol/L in dimethyl sulphoxide and diluted in Krebs-HEPES solution (composition in mmol/L; NaCl 99, KCl 4.7, KH_2PO_4 10, $MgSO_4$ 1.2, $NaHCO_3$ 25, glucose 11, $CaCl_2$ 2.5), such that the final concentration of dimethyl sulfoxide was 0.1%. L-012 was purchased from Wako Pure Chemicals and was prepared at 100 mmol/L in dimethyl sulfoxide and diluted in Krebs-HEPES solution such that the final concentration of dimethyl sulfoxide was 0.1%.

Statistical analysis

All data are expressed as mean±SE. Changes in blood pressure were calculated by subtracting baseline blood pressure (i.e. at Day 0) from the average blood pressure recorded over Days 5–7 (representing 1 week) and Days 12–14 (representing 2 weeks) of treatment. Kidney weight:body weight was expressed as kidney weight (mg)/body weight (g) at the end of the study. Vasoconstriction in response to KCl is expressed as % decrease in diameter from baseline. Vasodilator responses are expressed as the % increase in diameter from the pre-constricted level towards the resting artery diameter, which was equivalent to 100%. For measurements of superoxide, data were normalized to dry tissue weight, and expressed as counts/s/mg. Comparisons of vasodilator and vasoconstrictor responses, superoxide levels, and blood pressure were made using two-way ANOVA, or Students *t*-test, as appropriate. Statistical significance was accepted at P<0.05.

RESULTS

ADULT MICE

Blood pressure and kidney weights—Consistent with previous findings [21, 32], initial blood pressure was similar in WT and Nox2$^{-/y}$ mice (Table 1). In WT and Nox2$^{-/y}$ mice, two weeks of treatment with either vehicle or aldosterone had no significant effect on blood pressure (Table 1). Kidney weight:body weight ratio was markedly increased in aldosterone- vs vehicle-treated mice in both WT and Nox2$^{-/y}$ mice (Table 1).

Functional responses—Baseline internal diameter of the basilar artery at 60 mmHg in the absence of any pharmacologically-induced tone was similar in WT and Nox2$^{-/y}$ mice, whether treated with either vehicle or aldosterone (Table 1). In WT mice, treatment with aldosterone resulted in marked and selective impairment of vasodilator responses to acetylcholine compared with vehicle treatment (Figure 1A), whereas vasomotor responses to

papaverine (Figure 1B) and KCl (Figure 1C) were unaffected by aldosterone treatment. In vehicle-treated $Nox2^{-/y}$ mice, responses to acetylcholine were similar to responses in vehicle-treated WT mice (Figures 1A & 2A, P>0.05). By contrast, in $Nox2^{-/y}$ mice treatment with aldosterone had no effect on vasodilator responses to acetylcholine compared to vehicle treatment (Figure 2A). Responses to papaverine (Figure 2B) and KCl (Figure 2C) were also unaffected.

Superoxide levels—Under basal conditions, there was a small (~1.4-fold) increase in superoxide levels in arteries from aldosterone- versus vehicle-treated WT mice (P=0.056), whereas there was no such effect of aldosterone treatment in $Nox2^{-/y}$ mice (Figure 3). In cerebral arteries from vehicle-treated WT mice, superoxide levels were increased by ~7.5-fold following stimulation with PdB (Figure 3). Superoxide levels were found to be further increased (by ~1.3-fold) in cerebral arteries from aldosterone-treated WT mice (Figure 3). In contrast, PdB had little or no effect on superoxide levels produced in arteries from $Nox2^{-/y}$ mice treated with either vehicle or aldosterone, suggesting that effects of aldosterone on cerebrovascular superoxide levels occur in a NOX2-dependent manner.

AGED WT MICE

Blood pressure and kidney weights—Initial blood pressure was ~10 mmHg lower in aged WT mice than in adult WT mice (Tables 1 & 2). Whereas treatment with vehicle had no effect on blood pressure, treatment with aldosterone increased blood pressure by ~20 mmHg over 2 weeks (Table 2). Kidney weight:body weight ratio was markedly increased by aldosterone treatment in aged mice, in a similar manner to that observed in adult mice (Tables 1 & 2).

Superoxide levels—Under basal conditions, there was no difference in superoxide levels of cerebral arteries from aldosterone- versus vehicle-treated WT aged mice (Figure 4). Similar to adult mice, superoxide levels in cerebral arteries from WT aged mice were acutely increased by stimulation with PdB, and were ~1.4 fold greater following aldosterone versus vehicle treatment, although this difference did not reach statistical significance (Figure 4).

DISCUSSION

To our knowledge, this is the first study to report the effects of chronic aldosterone administration on cerebrovascular levels of ROS or endothelial function. Moreover, this is the first study to examine effects of aldosterone in any vascular bed using aged mice. There are four major new findings of this study. First, chronically increased systemic levels of aldosterone lead to increased ROS production and endothelial dysfunction in cerebral arteries. Second, these detrimental effects of aldosterone in the cerebral circulation occurred in the absence of a significant change in blood pressure. Third, aldosterone causes increased cerebral vascular ROS production and endothelial impairment in a NOX2-dependent manner. Fourth, we found no evidence for any further exacerbation of increased cerebral vascular ROS production by aldosterone in aged animals compared with adults, despite the presence of hypertension.

Increased ROS production and endothelial dysfunction caused by aldosterone

Chronic treatment with aldosterone causes oxidative stress and endothelial dysfunction in systemic vessels [9, 11, 13–15, 17], but this is the first study to test for such effects in the cerebral circulation. Increased ROS production involves elevated levels of superoxide anion, which can impair endothelium-dependent relaxation by decreasing bioavailability of nitric oxide [20, 33, 34]. NADPH oxidases are known to be a major source of vascular superoxide [35], and we found here that NOX2-deficient mice are indeed resistant to aldosterone-induced increases in ROS production and endothelial dysfunction. Our results also suggest that the increase in ROS production and endothelial dysfunction that occur in the cerebral circulation in response to aldosterone are likely to be independent of increases in blood pressure [13, 15, 16], analogous to previous reports of subpressor doses of angiotensin II (a major stimulus of aldosterone production) causing cerebrovascular oxidative stress and vascular dysfunction [36, 37]. The prevalence of primary aldosteronism in patients with hypertension ranges between 3% and 20%, constituting an important cause of unrecognized secondary hypertension [38]. The lower limit of aldosterone levels in such patients is more than 0.4 nM [39], with concentrations increasing to as high as 8 nM in patients with congestive heart failure [40]. Treatment of rodents with aldosterone for 2–4 weeks at doses equal or similar to that used in this study is reported to produce a serum aldosterone concentration of >4 nM [14, 41, 42]. Thus, the level produced in rodents with this protocol is in the range seen in patients.

Role of NOX2 oxidase

NOX2 is expressed in the endothelium of cerebral blood vessels [18, 19, 43]. Under control conditions, we found NOX2 deficiency to have no effect on endothelial function, consistent with earlier reports [21, 44]. One study has reported that chronic aldosterone treatment increased NOX2 expression in the aorta [8]. Here, we found that aldosterone treatment increased ROS levels and caused endothelial dysfunction in cerebral arteries of WT mice but not NOX2-deficient mice, indicating that superoxide generated by vascular NOX2 oxidase activity is a key mediator of cerebrovascular dysfunction [4]. Moreover, we used PdB to compare the effect of vehicle and aldosterone treatments on the capacity of NOX2 oxidase to acutely generate superoxide. PdB promotes formation of the NOX2 oxidase complex through protein kinase C-dependent phosphorylation of the p47 phox subunit [45] resulting in enhanced Nox2 oxidase activity. Using this approach, the PdB-stimulated increase in superoxide is entirely NOX2-dependent (Figure 3 and [21]), making it a useful tool to study the role of NOX2 oxidase in the vasculature. Increased superoxide levels following stimulation with PdB in arteries from aldosterone-treated mice thus reflects increased expression/activity of NOX2 following aldosterone treatment.

Aged mice

The frequency of stroke and Alzheimer's disease increases with age [23], but mechanisms of ageing-related pathological changes in the cerebral circulation are poorly understood. As there is evidence that expression of vascular MR is higher in aged (30 months) versus adult (8 months) rats [24], and that this receptor mediates an augmented vascular contractile response in mice aged >9 months versus 3–4 months [46], we tested whether the

pathological effects of chronic aldosterone that we observed in young adult mice (average age: 5–6 months old) might be further exacerbated in aged mice (average age: 16–17 months old). Such a finding would be suggestive of a role for aldosterone signaling through the MR as a contributing mechanism to worsening cerebral vascular pathology during aging. However, we found no evidence for exacerbated oxidative stress in the cerebral circulation in response to aldosterone in aged mice, although there was a greater hypertensive effect in these animals. Our findings are compatible with the conclusion that the cerebrovascular and pressor actions of aldosterone may be independent of one another. Indeed, other studies have reported vascular actions of aldosterone in peripheral blood vessels occur in the absence of an increase in blood pressure [13, 15, 16].

Conclusions

Our findings support the concept that chronically increased levels of plasma aldosterone may be an important contributor to cerebrovascular disease, and that such effects are likely to be mediated by increased ROS production due to increased NOX2 oxidase activity. Further studies are needed to examine the role of other sources of superoxide, including Nox isoforms, in the cerebrovascular actions of aldosterone, and whether the cerebrovascular actions of aldosterone are MR-dependent with a view to potential use of MR antagonists in the prevention and treatment of cerebrovascular disease and stroke [5, 47–50].

Acknowledgments

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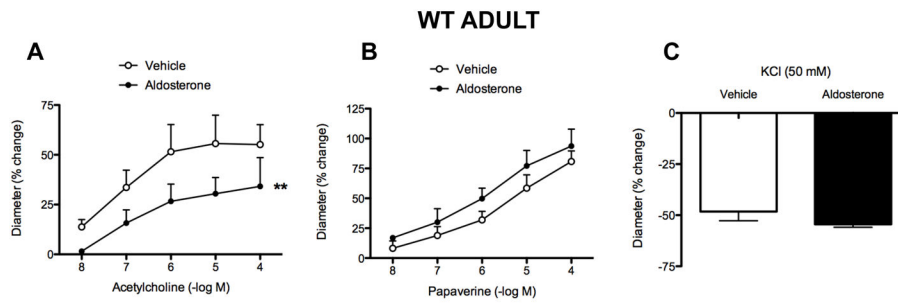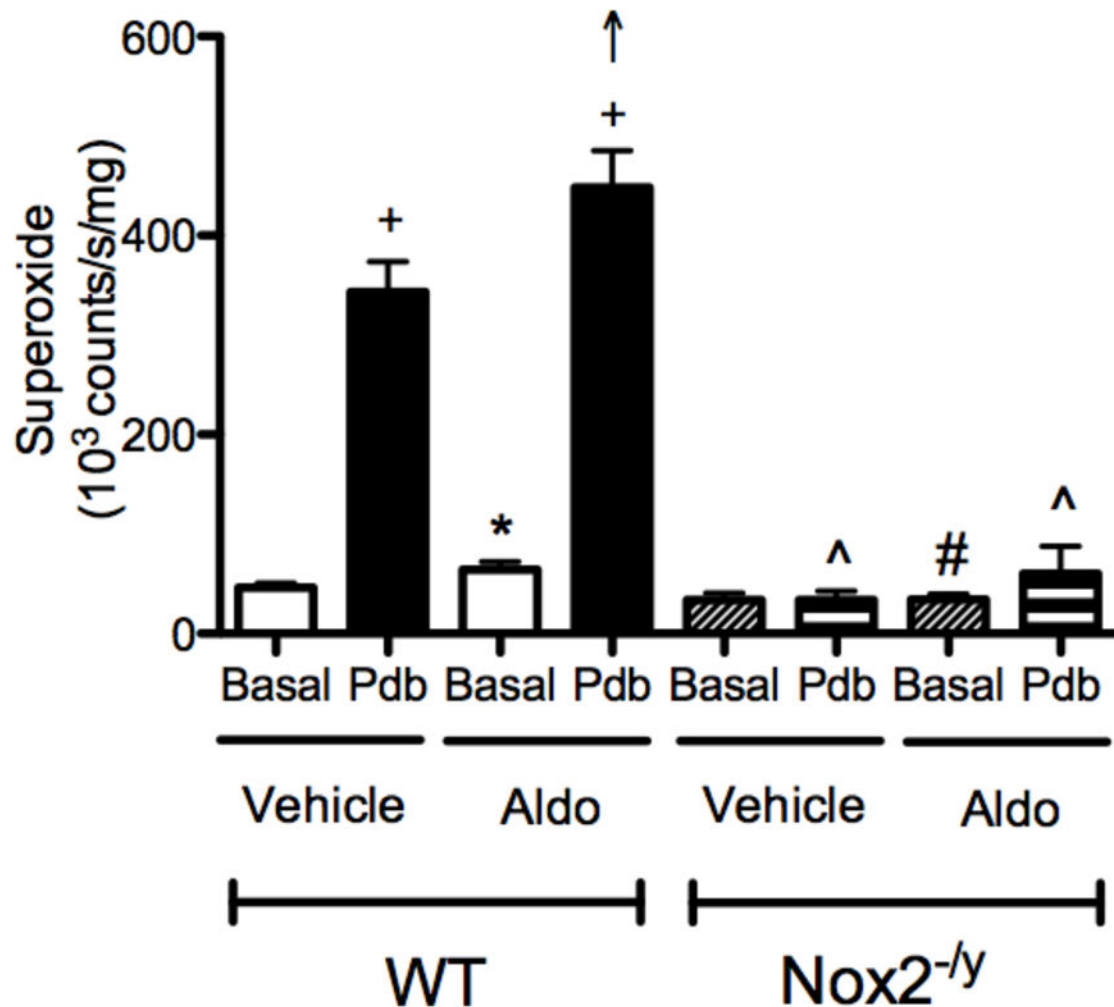
Figure 1.
Vascular responses to acetylcholine (A: vehicle, n=5; aldosterone, n=5), papaverine (B: vehicle, n=4–9; aldosterone, n=4–9), and KCl (C: vehicle, n=5; aldosterone, n=3) in WT adult mice. All data are mean±SE. **P<0.01 vs vehicle.
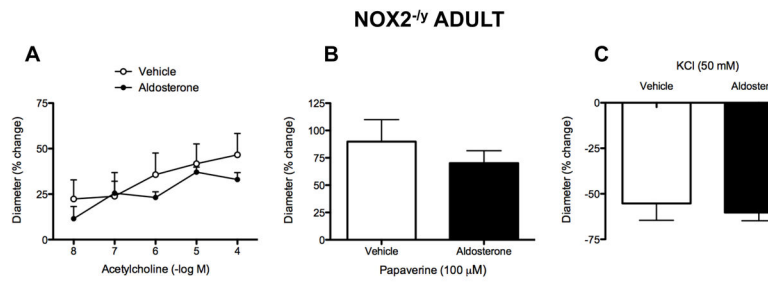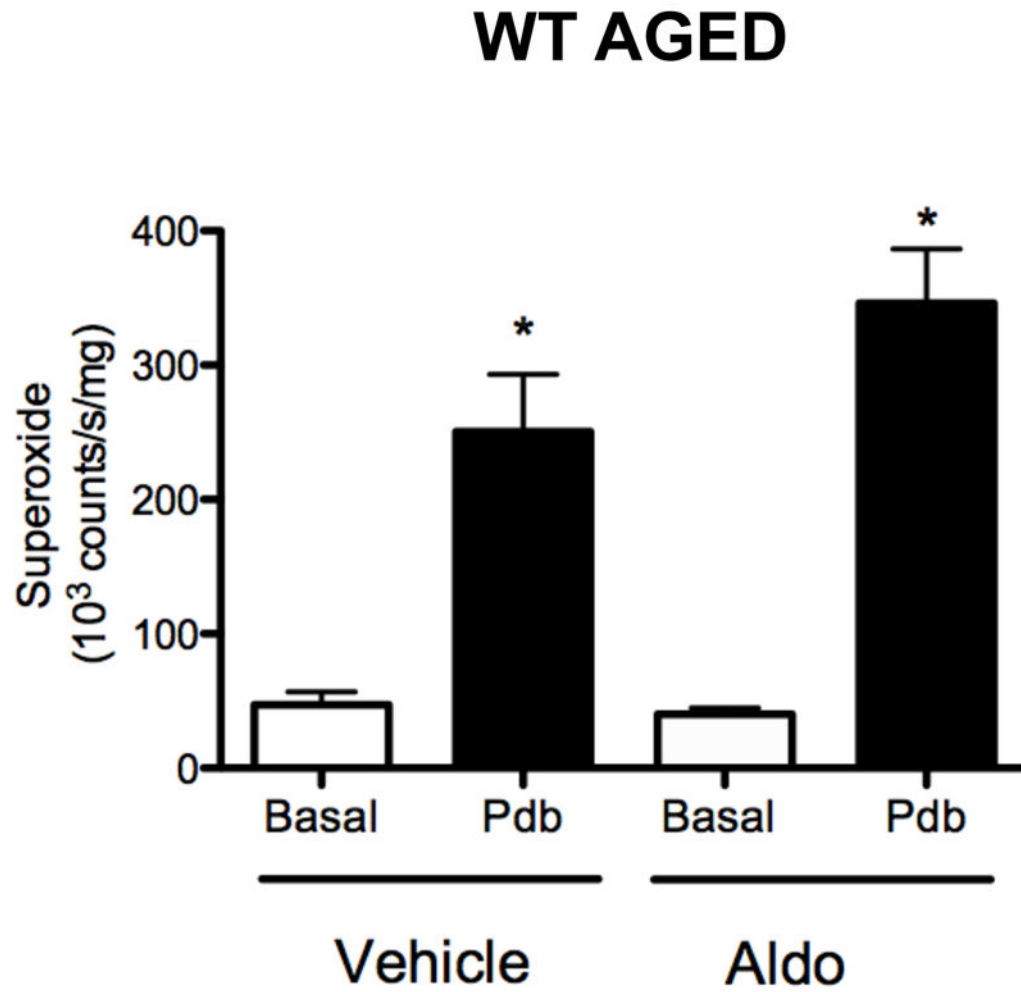
Figure 2.
Vascular responses to acetylcholine (A: vehicle, n=6; aldosterone, n=4), papaverine (B: vehicle, n=6; aldosterone, n=4), and KCl (C: vehicle, n=4; aldosterone, n=3) in Nox2$^{-/y}$ adult mice. All data are mean±SE.

ADULT

Figure 3.
Superoxide levels in cerebral arteries from WT (vehicle, n=19; aldosterone, n=18) and Nox2$^{-/y}$ mice (vehicle, n=9; aldosterone, n=8) measured after vehicle or aldosterone treatment, under basal conditions or in the presence of 10 μmol/L PdB. All data are mean ±SE. * P=0.056 vs vehicle-treated WT basal, + P<0.05 vs vehicle-treated WT basal, ↑ P<0.05 vs vehicle-treated WT PdB, # P<0.05 vs aldosterone-treated WT basal, ^ P<0.05 vs vehicle- and aldosterone-treated WT PdB.

WT AGED

Figure 4.
Superoxide levels in cerebral arteries from vehicle- and aldosterone-treated WT aged mice (both n=9) measured under basal conditions and in the presence of 10 μmol/L PdB. All data are mean±SE. *P<0.05 vs vehicle- and aldosterone-treated basal.

Table 1

Adult mice

Initial blood pressure, basilar artery diameter, kidney weight (KW): body weight (BW) ratio, and changes in systolic blood pressure (SBP) following vehicle or aldosterone treatment (number in brackets indicates n value) in adult mice.

	Initial SBP (mmHg)	Treatment Subgroup	SBP (mmHg) Week 1	SBP (mmHg) Week 2	Baseline Diameter (µm)	KW:BW (mg:g)
WT	**115±2**	Vehicle	**4±3 (21)**	**6±3 (21)**	**151±10 (14)**	**12.4±0.2 (30)**
	(41)	Aldo	**6±3 (20)**	**8±4 (20)**	**153±10 (12)**	**16.2±0.2 (31)**[*]
Nox2⁻/ʸ	111±3	Vehicle	0±3 (10)	8±4 (10)	149±15 (6)	12.5±0.3 (15)
	(19)	Aldo	0±6 (9)	2±6 (9)	157±18 (4)	15.3±0.2 (12)[*]

[*] P<0.0001 vs corresponding vehicle

Table 2

Aged mice

Initial blood pressure, kidney weight (KW): body weight (BW) ratio, and changes in systolic blood pressure (SBP) in response to vehicle and aldosterone treatment (number in brackets indicates n value) in aged mice.

	Initial SBP (mmHg)	Treatment subgroup	SBP (mmHg) Week 1	SBP (mmHg) Week 2	KW:BW (mg:g)
WT	103±4 (16) ***	Vehicle	2±4 (6)	3±4 (6)	12.0±0.4 (9)
		Aldo	8±5 (7)	19±4 (7) *	16.9±0.7 (9) **

*
P<0.05 vs corresponding vehicle

**
P<0.0001 vs corresponding vehicle

***
P<0.05 vs adult WT