Endothelin Activation of Reactive Oxygen Species Mediates Stress-Induced Pressor Response in Dahl Salt-Sensitive Prehypertensive Rats

Gerard D’Angelo, Analia S. Loria, David M. Pollock, Jennifer S. Pollock

Abstract—Experiments were designed to test the hypothesis that endothelin (ET) and/or reactive oxygen species contribute to the pressor response induced by acute air jet stress in normotensive Dahl salt-sensitive rats maintained on a normal salt diet (prehypertensive). Mean arterial pressure was chronically monitored by telemetry before and after 3-day treatment with the free radical scavenger 4-hydroxy-2,2,6,6-tetramethyl piperidinoxyl (Tempol) or ET receptor antagonists ABT-627 (ET A antagonist) or A-182086 (ET A/B antagonist) supplied in the drinking water. Rats were restrained and subjected to pulsatile air jet stress (3 minutes). Plasma samples at baseline and during acute stress were analyzed for 8-isoprostane (measure of reactive oxygen species production) and ET. Neither Tempol nor ET receptor antagonist treatment had an effect on baseline mean arterial pressure or plasma 8-isoprostane. The pressor response to acute stress was accompanied by significant increases in plasma 8-isoprostane and ET. Tempol significantly reduced both the total pressor response (area under the curve) and the stress-mediated increase in plasma 8-isoprostane; conversely, Tempol had no effect on the stress-induced increase in plasma ET. Combined ET_A/B antagonism, but not selective ET_A receptor blockade, similarly suppressed the pressor response to stress and stress-mediated rise in 8-isoprostane. Together these results indicate that reactive oxygen species contribute to the pressor response to acute air jet stress. Furthermore, the increase in reactive oxygen species occurs downstream of ET_B receptor activation. (Hypertension. 2010;56:282-289.)

Key Words: endothelin ▶ reactive oxygen species ▶ air jet stress ▶ Dahl salt-sensitive rat ▶ blood pressure

Reactive oxygen species (ROS) contribute to the pathogenesis of cardiovascular dysfunction associated with several diseases, including hypertension, chronic heart failure, ischemic heart disease, hyperlipidemia, and diabetes mellitus.1-3 In addition, results from several laboratories suggest that ROS are implicated in normal cardiovascular function. Systemic administration of the free radical scavenger 4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl (Tempol) leads to significant decreases in mean arterial pressure (MAP), heart rate (HR), and sympathetic nerve activity in normotensive animals,4-6 suggesting an important role for ROS in the regulation of arterial pressure. Studies performed in vitro7,8 and in vivo9,10 also demonstrate that ROS are involved in the constrictor or pressor response, respectively, to various agonists.

A growing body of evidence suggests that behavioral stress elicits production of ROS; most of these reports, however, focus on chronic stress paradigms.11,12 Numerous studies suggest that acute stress may induce cardiovascular dysfunction,13-20 and additional studies have shown that cardiovascular hyperreactivity is strongly associated with future cardiovascular disease.21-28 Proposed as a mechanistic link between acute stress events and chronic disease later in life, the concept of allostatic load suggests that the cumulative effect of repeated challenges over time may lead to disease.29 Thus, we reasoned that delineation of the mechanism(s) mediating the response to a single stress event would aid in understanding the consequences of cumulative effects.

We have used a model of acute behavioral stress that combines restraint with pulsatile, unavoidable bursts of air to the head (referred to as air jet stress).30-32 We chose the Dahl salt-sensitive (DS) rat because of its use as a model to evaluate genetically defined risk for salt-sensitive hypertension. Tempol significantly attenuates the high salt–mediated increase in arterial pressure in the DS rat.33,34 Very little is known, however, regarding to what extent ROS influence pressor responses in DS animals maintained on a normal salt diet or under prehypertensive conditions.

Endothelin (ET)-1 is an endothelial-derived potent vasoconstrictor peptide. ET-1 is released abuminally, and circulating levels are thought to be the result of spillover from elevations in ET-1 production or reduced clearance. ET-1 is...
also produced in sympathetic nerves and the renal tubular epithelium. Plasma ET-1 levels increase in response to acute mental (mental arithmetic) and physical (cold pressor) stress in human adults and in adolescents. Treiber et al demonstrated that acute stress-induced elevations in plasma ET-1 correlated with stress-induced increases in blood pressure in prehypertensive young adults with verified family histories of cardiovascular disease, suggesting that the stress-induced release of ET-1 may be involved in the acute stress-induced pressor response.

We reasoned that the normotensive DS rat is an appropriate model of prehypertensive young adults with a family history of cardiovascular disease. Our focus in this study was to test the hypothesis that ROS contribute to the pressor response to acute air jet stress in prehypertensive DS rats. Prehypertensive DS rats are more sensitive to the pressor effects of ET-1 than Dahl salt-resistant (DR) counterparts, and ET-1 plays a prominent role in the salt-dependent hypertension. Furthermore, previous studies have shown that ROS stimulate sympathetic nerve activity and promote ET-1 production. Therefore, we also tested the hypothesis that the stress-induced increase in ROS in DS rats is downstream of ET-1 receptor activation.

### Methods

#### Animal Model

All of the experiments used 9- to 12-week–old male DS rats (Harlan Laboratories, Indianapolis, IN) fed standard rat chow containing 0.4% NaCl and tap water, ad libitum. One experimental protocol also used 9- to 12-week–old male DR rats. Rats were housed in the animal care facility at the Medical College of Georgia, which is approved by the American Association for the Accreditation of Laboratory Animal Care. All of the protocols have been approved by the Institutional Animal Care and Use Committee.

#### Telemetry

Telemetry transmitters (Data Sciences, Inc) were implanted according to the manufacturer’s specifications, as published previously.

#### Air Jet Stress

DS rats were subjected to 2 sessions of acute air jet stress as described previously, spaced 1 week apart, during which the animals were left untreated (week 1) or put on a 3-day regimen of either Tempol (1 mmol/L in the drinking water; n=10) or the dual ETA/B receptor antagonist A-182086 (30 mg/kg per day in the food; n=4; week 2). All of the animals to air jet stress. Statistical analyses of plasma determinations were made by 2-way ANOVA, followed by Newman-Keuls test for multiple comparisons. Differences are considered significant at P<0.05.

#### Determination of Plasma Concentrations of 8-Isoprostane, ET-1, and Catecholamines

Rats were anesthetized with ketamine/xylazine (50/10 mg/kg IP) and catheters (Braintree Scientific Inc) were inserted into the jugular vein. Catheters were routed subcutaneously and exteriorized at the back of the neck; catheters were filled with heparin (1000 U/mL). Blood (1 mL) was drawn from restrained animals on 2 successive days before air jet stress to determine baseline (unstressed) plasma levels in the absence or presence of pharmacological treatments and on the day of stress over the 30- to 60-second interval of air jet stress. Blood samples were centrifuged at 10 000g for 10 minutes at 4°C, and plasma was removed, aliquoted, and stored at −80°C until analyses could be performed. Please see the online Data Supplement at http://hyper.ahajournals.org for detailed methods.

### Results

Table. Baseline (24-hour) Cardiovascular Hemodynamics

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<tr>
<td>HR, bpm</td>
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*P<0.05 vs untreated.

Whole Animal Pressor Responses

Separate groups of DS animals were either left untreated (tap water alone) or given Tempol (1 mmol/L in the drinking water for 3 days; n=5 for each). Animals were anesthetized with thiobutabarbital (Inactin; 65 mg/kg IP), and the right femoral artery and vein were isolated and cannulated with polyethylene 50 for monitoring MAP and drug infusion, respectively. Peak and steady-state (1 minute before introduction of the next dose) responses to ET peptides and phenylephrine were determined. All of the measurements were recorded using a PowerLab data acquisition system (ADInstruments, Inc). Please see the online Data Supplement for detailed methods and graphic results.

Statistical Analysis

Data are expressed as mean±SE. All of the baseline MAP and HR values are reported as 24-hour means. Total pressor response refers to the change in MAP during the 3 minutes of air jet stress and is expressed as the area under the curve (AUC; in millimeters of mercury×minutes). Statistical analyses of baseline MAP and HR and of the total pressor response were made by paired t test. Baseline plasma values of 8-isoprostane, ET-1, and catecholamines represent the average values obtained for the 2 days before subjecting the animals to air jet stress. Statistical analyses of plasma determinations were made by 2-way ANOVA, followed by Newman-Keuls test for multiple comparisons. Differences are considered significant at P<0.05.

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### Results

Three days of pretreatment with the free radical scavenger Tempol had no effect on baseline (24-hour) MAP but caused a small yet significant decrease in baseline HR (Table). DS animals were restrained and subjected to acute air jet stress. Comparison of the integrated pressor response, calculated as the AUC, indicated that the stress response was significantly lower in Tempol-treated rats (15.9±2.5 versus 27.5±3.8 mm Hg×3 minutes, Tempol versus untreated; P<0.05; Figure 1). MAP was monitored for 20 minutes after the stress period to assess the extent of blood pressure recovery. Poststress AUC was significantly more negative in the rats given Tempol (109.4±29.2 versus 4.8±19.6, Tempol versus untreated; P<0.05; Figure 1).

To examine whether acute air jet stress caused an increase in ROS and whether this was affected by Tempol, plasma levels of 8-isoprostane were measured as an index of ROS production. DS rats were fitted with venous catheters, and blood was drawn at baseline and then again during air jet stress. In untreated animals, air jet stress caused a near doubling in plasma 8-isoprostane; Tempol had no effect on the baseline plasma level of 8-isoprostane but abolished the stress-mediated rise in 8-isoprostane (Figure 2A).
sympathetic nerve activity,5,6,43 thus we measured plasma (Figure 2B).

nor on the plasma ET-1 levels during stress had no effect on the baseline plasma concentrations of ET-1 pressed the stress-induced rise in plasma ET-1 levels. Tempol treatment. Tempol did not alter the baseline epinephrine (Epi; pathetic activity in the presence and absence of Tempol levels of catecholamines as an indirect determinate of sym-
pol (1 mmol/L in the drinking water; week 2) for 3 days. *P<0.05.

Animals were either untreated (week 1) or given the free radical scavenger, Tempol (1 mmol/L in the drinking water; week 2; n=10) for 3 days. AUC was calculated as the sum of the MAP data points during or after air jet stress minus the average MAP obtained over the 3 minutes before the start of air jet stress. *P<0.05.

We were interested in assessing whether the stress-induced rise in arterial pressure and plasma 8-isoprostane in DS rats was related to the hypertensive genetic predisposition of DS rats or was a general phenomenon. Therefore, we measured the effect of Tempol on the stress-mediated pressor response and the stress-mediated plasma 8-isoprostane levels in DR rats. Opposite to what was observed in DS rats, Tempol enhanced the integrated pressor response in DR rats (27.6±2.0 versus 17.6±2.3 mm Hg×3 minutes, Tempol versus untreated; P<0.05). Plasma levels of 8-isoprostane in DR rats were similar before and during air jet stress (18±1 versus 17±2 pg/mL, baseline versus stress).

Previous studies have shown that ROS promote ET-1 production.44 Therefore, we evaluated whether Tempol suppressed the stress-induced rise in plasma ET-1 levels. Tempol had no effect on the baseline plasma concentrations of ET-1 (Figure 2B) nor on the plasma ET-1 levels during stress (Figure 2B).

Other laboratories have demonstrated that ROS stimulate sympathetic nerve activity,5,6,43 thus we measured plasma levels of catecholamines as an indirect determinate of sympathetic activity in the presence and absence of Tempol treatment. Tempol did not alter the baseline epinephrine (Epi; Figure 3A) or norepinephrine (NE; Figure 3B) levels. Although Tempol significantly reduced the stress-induced increase in plasma Epi (368±33 versus 522±36 pg/mL, Tempol versus untreated; P<0.05; Figure 3A), Tempol augmented the stress-mediated rise in plasma NE concentration (523±96 versus 303±33 pg/mL, Tempol versus untreated; P<0.05; Figure 3B).

Because ET-1 has been demonstrated to mediate an increase in ROS,46,47 we tested the effects of ET receptor blockade on plasma 8-isoprostane levels to determine whether ROS production was downstream of ET-1 receptor activation. We demonstrated previously that selective ETA receptor blockade does not affect the pressor response to air jet stress or the stress-mediated increase in catecholamines in prehypertensive DS rats.45 Nevertheless, we examined whether ETA receptor blockade altered the production of ROS. Treatment of DS rats with the ETA receptor antagonist ABT-627 had no effect on plasma 8-isoprostane either at baseline or during stress (Figure 4A). We next examined the effect of dual ETA/B receptor inhibition with A-182086. Pretreatment with A-182086 had no effect on baseline (24-hour) MAP or HR (Table). A-182086 also had no effect on baseline plasma 8-isoprostane but blocked the stress-induced increase in plasma 8-isoprostane (Figure 4B).

Similar to the results obtained with Tempol, combined ET_A/B receptor antagonism significantly reduced the integrated pressor response to acute stress (6.9±6.7 versus 43.8±12.5 mm Hg×3 minutes, A-182086 versus untreated; P<0.05; Figure 5, left); poststress recovery of MAP appeared to be greater, but this difference was not statistically significant (P=0.11; Figure 5, right). Dual ETA/B receptor antagonism did not affect the baseline plasma catecholamines (Epi: 147±14 versus 145±15 pg/mL, A-182086 versus untreated; NE: 406±37 versus 357±43 pg/mL, A-182086 versus untreated) or stress-mediated elevation in catecholamines (Epi: 231±34 versus 282±26 pg/mL, A-182086 versus untreated; NE: 562±123 versus 482±55 pg/mL, A-182086 versus untreated). Treatment with A-182086 increased baseline plasma ET-1 levels (22.65±1.42 pg/mL versus 0.73±0.14 pg/mL, A-182086 versus untreated; P<0.0001). DS rats treated with A-182086 during stress did not demonstrate a stress-induced increase in plasma ET-1 (22.65±1.42 pg/mL versus 20.36±2.74 pg/mL, baseline versus stress).

ROS have been shown to partially mediate the constrictor response to various agonists, including ET-1.9 The whole
animal pressor response to ET-1 and S6c, a selective ETB receptor agonist, in anesthetized DS rats with and without Tempol treatment was determined. Peak and steady-state pressor responses to exogenous ET-1 (Figure S3) or S6c (Figure S4) were unaffected by treatment with Tempol. We examined the whole animal pressor response to exogenous phenylephrine in anesthetized animals to determine whether there is reduced responsiveness of the vascular smooth muscle to \(\alpha_1\)-adrenergic stimulation. Experiments were performed in both the absence and presence of autonomic ganglion blockade with chlorisondamine. Chlorisondamine produced comparable decreases in MAP in untreated and Tempol-treated animals (Figure S5). Tempol had no effect on the phenylephrine-mediated pressor response in the absence and presence of chlorisondamine (Figure S6).

**Discussion**

The principal finding of this study is that the blood pressure responsiveness during acute air jet stress in normotensive DS animals depends on ET-mediated increases in ROS. Specifically, the free radical scavenger Tempol significantly lowered the pressor response to air jet stress and abolished the stress-mediated rise in ROS. Similar responses were obtained with combined ETA/B but not selective ETA receptor blockade. Neither Tempol nor combined ETAB receptor blockade had any effect on 24-hour baseline MAP.

Several studies have shown that ROS can increase ET-1 production in cultured endothelial and vascular smooth muscle cells.\(^{48-50}\) Moreover, under various experimental or pathological conditions, Tempol reduces ET-1 generation in vivo.\(^{44,51,52}\) Tempol had no effect on basal or stress-mediated increases in circulating ET-1, suggesting that ROS, per se, is not the stimulus for ET-1 release during acute stress. Conversely, ET-1 can stimulate superoxide generation in aortic rings,\(^*\) setting up a potential feed-forward mechanism for further production of ET-1 and ROS. In the present study, ET receptor antagonism prevented the stress-mediated increases in plasma 8-isoprostane and arterial pressure without changes in baseline values. These data indicate a causal relationship between ROS and the pressor response to acute stress and that the increase in ROS occurs downstream of ET receptor activation. Specifically, dual ETAB receptor antagonism prevented the stress-mediated rise in ROS, whereas selective ETA receptor blockade had no effect. These data indicate that the increase in ROS most likely occurs in response to ETB receptor stimulation. Our experimental approach used the comparison of a dual ETAB antagonist and a selective ETA antagonist to discern the effects of ET receptors in response to stress. We used this approach because treatment with an ETB selective antagonist will produce large increases in arterial pressure, vascular resistance, and increase ETA receptor activity, which would make interpretation of results especially difficult. We recognize that the inability to directly
nerve activity in both normotensive and hypertensive rats. Possibly, these isoprostane-specific biological effects may as well have direct biological effects in the vasculature.

oxidative stress and lipid peroxidation. In the majority of studies, changes in plasma isoprostane are examined under the context of a chronic disease state or after a more prolonged pathological insult. In this regard, our results are indeed novel in that we reproducibly detect changes after the start of air jet stress in DS rats. We found that DR rats did not display a similar stress-induced increase in plasma isoprostane, thus we reasoned that the increased isoprostane levels in DS rats are relevant. Isoprostanes can mediate increases in DNA synthesis, cellular proliferation, and collagen synthesis. Thus, isoprostanes are biomarkers of ROS production as well as having direct biological effects in the vasculature. Possibly, these isoprostane-specific biological effects may play a role in the vascular pathologies observed in chronic repetitive stress paradigms.

It stands to reason that reduced sympathetic nerve activity resulting from a decrease in neuronal ROS may contribute to the effect seen with Tempol. Bolus intravenous administration of Tempol has been shown to lower renal sympathetic nerve activity in both normotensive and hypertensive rats. Dai et al demonstrated that ET-1 activation of ROS in celiac ganglia isolated from deoxycorticosterone acetate-salt hypertensive rats was sensitive to ETB but not ETA receptor blockade. ETB receptor stimulation caused a similar increase in ganglionic superoxide levels in normotensive rats. From these studies, one would predict that reducing ROS with Tempol or with ETB receptor blockade would lower the stress-mediated rise in plasma catecholamines. Although Tempol did reduce the stress-mediated increase in Epi, we found that there was a paradoxical increase in plasma NE. Moreover, dual ET receptor blockade had no effect on the stress-mediated rise of either catecholamine. Li et al reported that chronic ETB receptor activation by S6c, a selective ETB agonist, induced hypertension that was ameliorated by Tempol and decreased superoxide levels in ganglia. Furthermore, these investigators found that, although plasma NE levels were not increased in S6c hypertension, surgical ablation of the celiac ganglion plexus, which provides most of the sympathetic innervation to the splanchnic organs, significantly attenuated the development of S6c-induced hypertension. These results are relevant to our study by demonstrating that ETB receptor activation involves sympathetic pathways, without changes in plasma catecholamines. Plasma levels of catecholamines are an indirect surrogate measurement of sympathetic activation and should be interpreted accordingly. Further investigation with direct sympathetic nerve recording is necessary to fully elucidate the roles of ROS and ET receptor activation in the stress-induced pressor response.

Considerable evidence links ROS to cardiovascular disease in various animal models, particularly hypertension, yet ROS also contribute to normal cardiovascular function. Bolus intravenous administration of Tempol has been shown to acutely lower blood pressure in normotensive rats. Using an oral dosing paradigm, however, we did not observe any effect on baseline blood pressure over 3 days in prehypertensive DS rats; a similar lack of effect on pressure was obtained in studies by Schnackenberg and colleagues, with chronic dosing of Wistar-Kyoto rats. A possible explanation of the acute versus long-term effects of Tempol in normotensive rats may be explained by the dose and route of administration. Consistent with no change in baseline pressure, we found no effect of Tempol on basal plasma 8-isoprostane levels. Finally, Tempol can cross the blood-brain barrier, and so we cannot rule out the possibility that reductions in ROS within the central nervous system contribute to the Tempol-mediated attenuation of the pressor response to acute air jet stress.

ROS also partially mediate the response to contractile agonists. We reasoned that, in the absence of an effect of Tempol on the stress-mediated increase in plasma ET-1, Tempol may blunt the constrictor response to ET-1. We found, however, that Tempol had no effect on the anesthetized whole animal pressor responses to exogenous ET-1 or S6c. Given the role of the sympathetic nervous system in the response to an acute stress, we also examined the effect of Tempol on the pressor response to α-adrenergic stimulation in anesthetized DS rats. Tempol similarly had no effect on the phenylephrine-mediated pressor response. This can be explained by previous findings showing that α-adrenergic stimulation does not elicit an increase in ROS. It is plausible that a bolus injection of phenylephrine in anesthetized preparations is not an accurate model for acute stress-mediated adrenergic activation in conscious rats. Therefore, future studies are necessary to fully examine the interaction of the
individuals at increased risk of cardiovascular disease later in life.\textsuperscript{21–28} The concept of allostatic load suggests, however, that disease results from the cumulative effects of multiple exaggerated responses to stress over time.\textsuperscript{29} Because the vascular dysfunction that occurs with aging is associated with increased ROS,\textsuperscript{71} it is, therefore, plausible that repeated responses to stress that invoke a rise in ROS may further contribute to the pathogenesis of cardiovascular disease.

Our group demonstrated previously that acute stress-induced elevations in plasma ET-1 correlated with stress-induced increases in blood pressure in prehypertensive adolescents and young adults with verified family histories of cardiovascular disease.\textsuperscript{38–40} Our current study in an animal model, the prehypertensive DS rat, determined a mechanism of the stress-induced pressor response is via ET-1 activation of the ROS pathway. The prehypertensive DS rat is a model of prehypertensive young adults with family histories of cardiovascular disease; thus, we predict that behavioral stress in the young adults activates an ET-dependent ROS pathway. Future translational studies will explore these hypotheses.

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Disclosures
None.

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References

Perspectives
There is a growing body of evidence suggesting that exaggerated cardiovascular responses to acute stress can identify adrenergic, ET, and ROS pathways in acute stress-induced pressor responses in normotensive DS rats.

The results in the present study, in conjunction with various reports in the literature, have led us to propose a causal chain of events that, in part, mediate the acute stress-induced pressor response in DS rats. However, the cellular mechanisms by which this occurs remain to be elucidated. Mayorov et al\textsuperscript{68} have shown that bilateral injection of Tempol into the rostral ventrolateral medulla significantly attenuates the pressor response to air jet stress, suggesting that ROS mediate at least in part the cardiovascular response to acute stress. Also, acute increases in blood pressure or increased vascular pressure lead to increased ROS production.\textsuperscript{69,70} Because both ET receptor blockade and Tempol reduced the stress-induced pressor response, we concluded that the rise in blood pressure is most likely not the stimulus for the increased ET-1 or ROS but that ROS activate the pressor response. A link between the increase in ROS and increased blood pressure was not revealed in our study. Tempol is a redox-cycling nitroxide that promotes the metabolism of ROS and improves NO bioavailability in vivo\textsuperscript{55}; thus, we speculate that increased ROS may lead to a loss of NO bioavailability mediating the increase in blood pressure. Vascular NO synthase activity in DS rats compared with DR rats is very low (J.S. Pollock et al, unpublished observations, 2002), so it is possible that the NO buffering capacity is greatly reduced in this animal model. Future experiments are necessary to elucidate the mechanism(s) of the ROS-mediated increase in blood pressure in prehypertensive DS rats. Figure 6 shows our hypothetical scheme that air jet stress stimulates the sympathetic nervous system followed by increased ET-1 and ET receptor activation leading to the production of ROS and finally increased blood pressure. These data indicate a causal relationship between ROS and the pressor response to acute stress and that the increase in ROS occurs downstream of ET receptor activation.

Figure 6. Scheme depicting the causal relationship of the ET pathway and ROS production in the acute stress-mediated rise in blood pressure in prehypertensive DS rats.


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Supplemental Data Section

Endothelin activation of reactive oxygen species mediates acute stress-induced pressor response in Dahl salt-sensitive pre-hypertensive rats

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Running head: Mechanism of acute behavioral stress induced pressor response

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Methods

Telemetry: Telemetry transmitters (Data Sciences, Inc.) were implanted according to the manufacturer’s specifications. Rats were anesthetized with ketamine/xylazine (50 mg/kg / 10 mg/kg, i.p.). The abdominal aorta was then exposed by a midline incision, and briefly occluded. The transmitter catheter was inserted into a hole made by a 21-gauge needle just proximal to the iliac bifurcation, and secured in place with tissue glue (Vetbond). The transmitter body was attached to the abdominal wall along the incision line with 4-O proline suture as the incision was closed. The skin was closed with staples that were removed 7 days after the incision had healed. Rats were returned to individual housing for data collection and allowed 8-10 days to recover from surgery before being subjected to the stress protocol. Animals were housed in a room separate from that used for studying the stress response. The individual rat cages were placed on top of the telemetry receivers, and mean arterial pressure (MAP) and heart rate (HR) were continuously (i.e. 10-sec sampling periods at regularly scheduled 10-min intervals) recorded throughout the study using the Dataquest A.R.T. Acquisition program.

Air jet stress: DS rats were subjected to 2 sessions of acute air jet stress spaced one week apart without treatment. All animals were previously subjected to at least two 15-minute restraint sessions on days prior to an experiment to reduce the stress associated with the restraint itself. On the day they were subjected to air jet stress, rats were quietly brought to a sound-proofed room. Immediately after starting the telemetry recording software, the room was vacated. The animals were then allowed to acclimate to their surroundings for 15-30 minutes in their cages, that is, until which time they ceased exploring the new environment and their pressures stabilized. Animals were then placed in tubular Plexiglas restrainers with sufficient aeration, and MAP and HR were continuously monitored by telemetry for at least 15 minutes before initiating air jet stress. When necessary, animals were monitored for up to an additional 10 minutes to allow the animals to adapt to being restrained, such that 3-5 minutes of stable MAP and HR recordings were obtained prior to exposure to air jet stress. Air jet stress consisted of pulses (2 sec duration delivered every 10 sec for 3 min) of compressed air (15 lb/in^2) aimed at the forehead from a 1/8" opening at the front of the tube. After the 3-minute stress period, MAP and HR were monitored for 20 additional minutes while the animals were still in the restrainer, and post-air jet values obtained. At the end of this post-stress period, animals were returned to their cages and brought back to their holding room.

Determination of plasma concentrations of 8-isoprostane, ET-1, and catecholamines: Rats were anesthetized with ketamine/xylazine (50 mg/kg / 10 mg/kg, i.p.), and catheters (Braintree Scientific Inc., Braintree, MA) were inserted into the jugular vein. Catheters were routed subcutaneously, and exteriorized at the back of the neck; catheters were filled with heparin (1000 U/ml). For four days after the surgery, animals were placed into the restrainers for at least 15 min, consistent with the duration used for the acute stress protocol, and catheters were flushed to maintain patency. On days 5 and 6 post-surgery, blood (1 ml) was drawn from restrained animals to determine baseline (unstressed) plasma levels. Red blood cells were resuspended in 1 ml sterile saline (0.9% NaCl) containing 6.2% bovine serum albumin and 50 µl of 7.5% EDTA, and replaced in the animal; catheters were subsequently refilled with heparin. On day 7 post-surgery, animals were subjected to the acute stress
protocol, and blood (1 ml) was drawn over the 30-60 sec interval of air jet stress. As before, red blood cells were resuspended in a sterile saline solution, re-infused, and catheters refilled with heparin. Catheter patency was maintained for the next 4 days, after which time the animals were placed on a 3-day regimen of either tempol (1 mM in the drinking water), the selective ET₄ receptor antagonist ABT-627 (5 mg/kg/day in the drinking water), or A-182062 (30 mg/kg/day in the food). Separate groups of animals were used for each of the treatments. Blood (1 ml) was drawn on each of the first 2 days of treatment with tempol, ABT-627, or A-182062, and on the third day of treatment, animals were again subjected to the acute stress protocol. Blood samples were centrifuged at 10000 x g for 10 min at 4 °C, and plasma was removed, aliquoted, and stored at –80 °C until analyses could be performed. The following plasma measurements were made: 8-isoprostane concentration by EIA (Cayman Chemical Company, Ann Arbor, MI), ET-1 by ELISA (QuantiGlo; R&D Systems, Minneapolis, MN), and epinephrine (Epi) and norepinephrine (NE) by RIA (BI-CAT-RIA, ALPCO Diagnostics, Windham, NH) according to manufacturer’s specifications.

Whole animal pressor responses: Separate groups of DS animals were either left untreated (tap water alone) or given tempol (1 mM in the drinking water for 3 days) (n=5 for each). Animals were anesthetized with thiobutabarbital (Inactin; 65 mg/kg, i.p.), and the right femoral artery and vein were isolated and cannulated with PE-50 for monitoring MAP and drug infusion, respectively. After a 30-minute equilibration period, animals were given chlorisondamine (5 mg/kg, i.v) to eliminate endogenous sympathetic vasomotor tone and baroceptor-reflex mediated responses. Effective blockade was confirmed by the absence of reflex bradycardia following constrictor administration. Phenylephrine (0.5, 1, 2, 4, 8, 16, and 24 µg/kg), an α₁ adrenergic specific agonist, was administered in randomized order of doses. MAP was allowed to return to baseline between each dose. Responses to ET-1 and sarafotoxin 6c (S6c; both ET-1 and S6c at 0.1, 0.3, and 1 nmol/kg), a selective ET₆ receptor agonist, were examined in separate sets of animals. ET-1 and S6c was administered in ascending concentration order at 20-minute intervals due to the prolonged nature of pressor responses. Peak and steady-state (one minute before introduction of next dose) responses were reported. ET responses were obtained only in the presence of chlorisondamine. All measurements were recorded using a Power Lab data acquisition system. Responses to PE in anesthetized animals are reported as the peak change in MAP from the baseline MAP; both peak and steady-state responses are reported for S6c. PE and S6c dose-response curves were analyzed two-way analysis of variance with repeated measures.

Statistical Analysis: Data are expressed as means ± SE. All baseline MAP and HR values are reported as 24-hour means. Total pressor response refers to the change in MAP during the 3 minutes of air jet stress, and was determined by the equation: \[ \Sigma((P - P_{\text{pre-stress}}) \times 0.067) \], where \( P \) refers to each MAP data point recorded during the delivery of air jet stress, \( P_{\text{pre-stress}} \) is the average pressure during the 3 minutes just prior to the onset of the air pulses, and 0.067 is the 4 second data collection interval in minutes. Data are expressed as the area under the curve (AUC; mmHg x min). Statistical analyses of baseline MAP and HR and of the total pressor response were made by paired t-test. Baseline plasma values of 8-isoprostane, ET-1, and catecholamines represent the average values obtained for the two days before subjecting the animals to air jet stress. Statistical analyses of plasma determinations were made by two-way analysis of variance, followed by Newman-Keuls test for multiple comparisons. Differences are considered significant at \( p<0.05 \).
Results

Two week successive air jet stress protocol. Changes in arterial pressure in untreated animals during air jet stress are shown in Figure S1. Figure S2 shows that the pressor response is indistinguishable from week one to week two in the same group of DS rats: thus, any effect of the treatments on the stress response during the second week could not be attributed to habituation.

Pressor responses in anesthesized rats. ROS have been shown to partially mediate the constrictor response to various agonists, including ET-1.\(^1\)\(^,\)\(^2\) Peak and steady-state pressor responses to exogenous ET-1 (Fig. S3) or S6c (Fig S4) were unaffected by pretreatment with tempol. The rapid response to stress is mediated by the sympathetic nervous system, and thus in part by \(\alpha\) adrenergic-mediated vasoconstriction. We therefore examined the whole animal pressor response to exogenous phenylephrine in anesthetized animals to determine whether the blunted pressor response to air jet stress in tempol-treated rats was due to reduced responsiveness of the vascular smooth muscle to \(\alpha_1\) adrenergic stimulation. Experiments were performed in both the absence and presence of autonomic ganglion blockade. Chlorisondamine produced comparable decreases in MAP in untreated and tempol-treated animals (Fig S5). Tempol had no effect on the phenylephrine-mediated pressor response in the absence and presence of chlorisondamine (Fig. S6A and B, respectively).
REFERENCES
Figure S1: Effect of air jet stress on mean arterial pressure in pre-hypertensive Dahl salt-sensitive rats. Data represent average absolute mean pressure from 10 untreated animals.
Figure S2: Summary of integrated pressor response (area under the curve; AUC) to acute air jet stress (left panel) and integrated mean arterial pressure during the 20-minute post-stress period (right panel) in pre-hypertensive Dahl salt-sensitive rats. Air jet stress was administered twice in untreated animals ($n=8$) at a weekly interval.
Figure S3: Effect of the free radical scavenger, tempol, on whole animal peak (A) and steady-state (B) pressor response to exogenous endothelin-1 (ET-1) \((n=6)\) in anesthetized, pre-hypertensive Dahl salt-sensitive rats in the presence of autonomic ganglionic blockade using chlorisondamine (5 mg/kg). Animals were either untreated or given tempol (1 mM in the drinking water mg/kg/day) for 3 days.
Figure S4: Effect of the free radical scavenger, tempol, on whole animal peak (A) and steady-state (B) pressor response to the ET<sub>B</sub> receptor selective agonist sarafotoxin 6c (S6c) (n=5-6) in anesthetized, pre-hypertensive Dahl salt-sensitive rats in the presence of autonomic ganglionic blockade using chlorisondamine (5 mg/kg). Animals were either untreated or given tempol (1 mM in the drinking water mg/kg/day) for 3 days.
Figure S5: Effect of autonomic ganglion blockade with chlorisondamine (5 mg/kg) on baseline arterial pressure in pre-hypertensive Dahl salt-sensitive rats. Animals were either untreated ($n=4$) or given tempol ($n=3$) (1 mM in the drinking water mg/kg/day) for 3 days.
Figure S6: Effect of the free radical scavenger, tempol, on whole animal pressor response to exogenous phenylephrine ($n=3-4$) in anesthetized, pre-hypertensive Dahl salt-sensitive rats in the absence (A) or presence (B) of autonomic ganglionic blockade using chlorisondamine (5 mg/kg). Animals were either untreated or given tempol (1 mM in the drinking water mg/kg/day) for 3 days.