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REDUCED ANGIOTENSIN II LEVELS CAUSE GENERALIZED VASCULAR DYSFUNCTION VIA OXIDANT STRESS IN HAMSTER CHEEK POUCH ARTERIOLES

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Abstract

Objectives—We investigated the effect of suppressing plasma angiotensin II (ANG II) levels on arteriolar relaxation in the hamster cheek pouch.

Methods—Arteriolar diameters were measured via television microscopy during short-term (3–6 days) high salt (HS; 4% NaCl) diet and angiotensin converting enzyme (ACE) inhibition with captopril (100 mg/kg/day).

Results—ACE inhibition and/or HS diet eliminated endothelium-dependent arteriolar dilation to acetylcholine, endothelium-independent dilation to the NO donor sodium nitroprusside, the prostacyclin analogues carbacyclin and iloprost, and the K_{ATP} channel opener cromakalim; and eliminated arteriolar constriction during K_{ATP} channel blockade with glibenclamide. Scavenging of superoxide radicals and low dose ANG II infusion (25 ng/kg/min, subcutaneous) reduced oxidant stress and restored arteriolar dilation in arterioles of HS-fed hamsters. Vasoconstriction to topically-applied ANG II was unaffected by HS diet while arteriolar responses to elevation of superfusion solution PO₂ were unaffected (5% O₂, 10% O₂) or reduced (21% O₂) by HS diet.

Conclusions—These findings indicate that sustained exposure to low levels of circulating ANG II leads to widespread dysfunction in endothelium-dependent and independent vascular relaxation mechanisms in cheek pouch arterioles by increasing vascular oxidant stress, but does not potentiate O₂- or ANG II-induced constriction of arterioles in the distal microcirculation of normotensive hamsters.

Keywords

Salt; angiotensin II; microcirculation; oxidative stress; hypertension

INTRODUCTION

Long term follow up studies in humans have shown that individuals with salt-sensitivity of blood pressure not only have a greater chance of developing hypertension than their salt-

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resistant counterparts, but also have a significantly higher mortality rate than salt-resistant individuals even if they fail to develop hypertension (68). That study also found that individuals with low plasma renin activity (PRA) had a significantly higher mortality from cardiovascular causes than survivors (68). Taken together, those findings indicate that chronic exposure to low PRA and low levels of angiotensin II (ANG II): 1) may have unforeseen effects on vascular function; 2) may contribute to cardiovascular morbidity and mortality in low renin forms of hypertension; and 3) may do so independent of and prior to any elevation of arterial blood pressure.

It is well known and widely accepted that various forms of hypertension are associated with impaired endothelium-dependent vasodilation and enhanced responses to a wide variety of vasoconstrictor stimuli (29, 42, 62, 63). However, much less is known regarding very early changes in vascular reactivity during increases in dietary salt intake in salt-sensitive hypertension and, especially, in experimental models that do not exhibit salt sensitivity of blood pressure. Studies investigating this question are particularly important in light of the growing awareness of the adverse effects of dietary salt intake on a multitude of disease processes independent of elevated blood pressure (45, 47, 68).

One recent study reported that two weeks of low-salt diet (50 mM Na⁺/day vs. 150 mM Na⁺/day) augmented endothelium-dependent flow-mediated vasodilation independent of changes in blood pressure in a population of overweight and obese subjects (9). The rapid onset of deleterious effects of dietary salt on endothelium dependent dilation in humans is further emphasized by another study showing that short-term increases in dietary salt intake (5 days) lead to significant reductions in the endothelium-dependent increase in forearm blood flow during acetylcholine infusion in normotensive, healthy volunteers (61). Those findings suggest that the deleterious effects of elevated salt intake on endothelial function in humans materialize more quickly than previously believed.

While there is increasing evidence that dietary salt can adversely affect endothelial function in major vascular beds, much less is known regarding the blood pressure-independent effects of dietary salt intake on endothelium-independent vascular relaxation and on vascular responses to vasoconstrictor stimuli such as ANG II itself and increased O₂ availability. In the latter case, it is especially important to investigate the effects of dietary salt intake on arteriolar O₂ sensitivity in normotensive animals, as enhanced vasoconstriction in response to elevated PO₂ has been shown to occur in the earliest stages of salt-sensitive hypertension [e.g. reduced renal mass (RRM) hypertension (42) and in Dahl salt-sensitive rats (65)]. However, the relative contributions of dietary salt vs. vascular alterations associated with the hypertensive state to changes in arteriolar O₂ sensitivity are not clearly defined.

Because multiple clinical studies have shown a correlation between endothelial dysfunction and adverse cardiovascular events (including death) in humans (69) and between salt-sensitivity and increased mortality in humans (68), the recent studies showing rapidly developing alterations in vascular reactivity with changes in dietary salt intake (9, 61) emphasize the need to increase our understanding of the mechanisms of early salt-related endothelial dysfunction. The present study tested the hypotheses that tonic activation of vascular AT₁ receptors is necessary to maintain vascular relaxation mechanisms in the hamster microcirculation; and that a sustained reduction of circulating ANG II levels as a result of increased dietary salt intake or inhibition of angiotensin converting enzyme (ACE) leads to increased vascular oxidant stress and impaired endothelium-dependent and – independent vasodilation in cheek pouch arterioles of normotensive hamsters. The final three goals of the study were to evaluate the effect of dietary salt on vascular sensitivity to ANG II itself, to determine whether elevated dietary salt affects K_{ATP} channel function in the hamster microcirculation, and to determine whether arteriolar sensitivity to increased O₂

availability is affected by increased dietary salt intake independent of an increase in arterial blood pressure.

MATERIALS AND METHODS

Experimental Animals

Adult male, age-matched, Golden Syrian hamsters (Charles River Laboratories; Indianapolis, IN) were housed in the AAALAC-accredited animal care facility at the Medical College of Wisconsin (MCW, Milwaukee, WI). All protocols involving animals were approved by the MCW IACUC. The hamsters were maintained on a low salt diet (LS; 0.4% NaCl) or switched to a high-salt diet (HS; 4.0% NaCl) (Dyets; Bethlehem, PA) for 3–6 days immediately prior to the experiment and drank tap water *ad libitum*. Body weights evaluated at the time of the experiment were similar in hamsters fed LS diet (117 ± 2.6 g, $n=23$) and HS diet (115 ± 1.5 g, $n=23$).

Experimental Preparation

On the day of the experiment, the animals were anesthetized with sodium pentobarbital (60 mg/kg ip, Abbott Laboratories; Chicago, IL, USA) and supplemental anesthesia was administered through a femoral or jugular vein cannula as needed. The trachea was cannulated with polyethylene tubing to maintain a patent airway and blood pressure was measured via a femoral artery cannula.

A single-layered cheek-pouch was prepared for viewing via intravital microscopy (43). Under control conditions, the preparation was superfused with physiological salt solution (PSS) warmed to 35°C and equilibrated with 0% O₂-5% CO₂-95% N₂ to insure that all O₂ delivery occurred via the microcirculation, as previously described (43). The PSS used in these experiments had the following constituents (mM): 131.9 NaCl, 4.7 KCl, 2.0 CaCl₂, 1.17 MgSO₄, and 20.0 NaHCO₃. Arterioles (generally one per animal) were selected by branching order and the presence of brisk blood flow velocity. After an initial 30–60 minute equilibration period, active tone in the arterioles was verified by topical application of adenosine (10⁻³ M) from a Pasteur pipette. Any vessel that did not dilate in response to adenosine was not studied. Maximum dilation of the vessel was assessed at the end of the experiment by superfusing the cheek pouch with Ca²⁺-free PSS having the following constituents (mM): 119 NaCl, 4.7 KCl, 1.17 MgSO₄·7H₂O, 20 MgCl₂·6 H₂O, 1.18 NaH₂PO₄, 24 NaH₂CO₃, 0.03 EDTA and 2.0 EGTA.

Experimental Protocols

Arteriolar reactivity to: 1) the endothelium-dependent vasodilator acetylcholine (ACh); 2) endothelium-independent prostacyclin analogues iloprost and carbacyclin, 3) the K_{ATP} channel activator cromakalim, 4) the K_{ATP} channel inhibitor glibenclamide, and 5) the endothelium-independent nitric oxide (NO) donor sodium nitroprusside (SNP) was assessed by measuring vessel diameters during topical addition of the agonist. The role of vascular oxidant stress in affecting arteriolar dilation in different experimental protocols was tested by measuring arteriolar responses the agonist before and after addition of polyethylene glycol-superoxide dismutase (PEG-SOD; 200 U/mL for 30 minutes) (Sigma; St. Louis, MO) or the superoxide dismutase mimetic tempol (10⁻⁴ M) to the superfusion solution for 30 minutes. The role of NO in mediating endothelium-dependent dilation in response to ACh was assessed by comparing arteriolar responses to ACh in the presence and absence of 10⁻⁴ M NG-nitro-L-arginine methyl ester (L-NAME) to inhibit NO synthase. In those experiments, the preparation was superfused with L-NAME for an initial 10 minute equilibration period before arteriolar responses were assessed in the continued presence of the inhibitor.

In other experiments, osmotic minipumps (Durect Corporation, Cupertino, CA) were implanted subcutaneously in the mid-scapular region of additional groups of LS- and HS-fed hamsters anesthetized with an intramuscular injection of ketamine (150 mg/kg) and acepromazine maleate (5 mg/kg). After implantation, the minipumps delivered a continuous infusion of ANG II (25 ng/kg/min, subcutaneous) or isotonic saline vehicle until arteriolar responses to the vasodilator agonists were evaluated in the *in situ* microcirculation 3 days later. In a separate series of experiments, arteriolar responses to ACh and SNP were evaluated in HS-fed hamsters receiving AT₁ receptor blockade with losartan (20 mg/kg/day) in the drinking water for 3 days concurrent with HS diet and ANG II infusion.

We also evaluated the effect of ACE inhibition with captopril (100 mg/kg in the drinking water for 3 days) on arteriolar reactivity to ACh and SNP in hamsters fed standard Purina rodent chow. The goal of those experiments was to determine whether ACE inhibition would impair arteriolar dilation under normal physiological conditions, i.e., in the absence of dietary influences.

In additional experiments, we evaluated arteriolar responses to topical application of ANG II and arteriolar responses to increased O₂ availability produced by equilibrating the superfusion solution with gas mixtures containing 5% O₂, 10% O₂, or 21% O₂, with 5% CO₂ and the balance N₂ (43, 65).

Dihydroethidium (DHE) Staining Protocol

Age-matched animals were maintained on LS diet or switched to HS diet for 3 days and infused with isotonic saline vehicle or a low dose of ANG II (25 ng/kg/min, subcutaneous) from an osmotic minipump, as described above. An additional group of ANG II-infused hamsters on HS diet was treated with losartan (20 mg/kg/day) in the drinking water for 3 days to block the AT₁ receptor.

The animals were anesthetized with sodium pentobarbital (60 mg/kg ip). The aorta was quickly removed, placed in PSS, and equilibrated for 30 minutes at 37°C. Arteries were treated with DHE (5 μ M) for 30 minutes at 37°C, embedded in Tissue-Tek O.C.T Compound (Sakura Finetek USA, Inc., Torrance, CA), frozen, cut to 10 μ m sections, and mounted on slides (73). Tissue levels of reactive oxygen species (ROS) were assessed via DHE fluorescence viewed on a Nikon E 600 microscope (Nikon; Tokyo, Japan) (73). The average fluorescence intensity (excitation wavelength = 490 nm; emission wavelength = 605 nm) was captured using a Micromax Cooled CCD camera (Princeton Instruments; Trenton, NJ) and quantified using MetaMorph 4.6 software (Molecular Devices, Sunnyvale, CA).

Data Analysis

Data were expressed as mean $\pm$ SEM for all experiments. Homogeneity of variance was evaluated with a Cochran's test. Data sets failing the Cochran's test were transformed and subsequent analysis performed on the transformed data. Differences between multiple means were evaluated 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 Arteriolar Diameters

Mean arterial pressure (MAP) was not significantly different in animals fed LS diet (99 ± 4.9 mmHg, $n=20$) or HS diet (100 ± 4.4 mmHg, $n=27$). Resting diameter was evaluated under control conditions (0% O₂ superfusion) in an exceptionally large sample of arterioles from multiple experimental series including those reported below and control diameters

from other experiments not reported below, e.g., preliminary experiments for other potential protocols. In that sample, resting diameter ($20.1 \pm 0.5 \mu\text{m}$, $n=86$ in LS and $19.7.1 \pm 0.5 \mu\text{m}$, $n=81$ in HS) and the change in diameter during maximum dilation with Ca^{2+} -free solution (from $19.8 \pm 0.5 \mu\text{m}$ to $29.1 \pm 0.6 \mu\text{m}$ $n=68$ in LS and from $19.9 \pm 0.6 \mu\text{m}$ to $28.2 \pm 0.9 \mu\text{m}$ $n=47$ in HS) were unaffected by high salt diet. There were also no significant differences in pretreatment control diameter vs. post-treatment diameter within a specific diet group for any of the experimental perturbations (Table 1). These findings conclusively demonstrate that short term elevations in dietary salt intake do not affect resting tone or cause structural remodeling of the vessel and/or a generalized reduction in the ability of the vessel to dilate.

Effect of High Salt Diet, Angiotensin II Infusion, and Superoxide Scavenging on Arteriolar Responses to Acetylcholine and Sodium Nitroprusside

HS diet eliminated endothelium-dependent arteriolar dilation in response to ACh and endothelium-independent vasodilation to the NO donor SNP (Figure 1A, B). Loss of the vasodilator response to SNP was due to mechanisms proximal to cGMP, as the maximum dilation in response to the cGMP analogue 8-bromo-cGMP (10^{-5} M) was similar in hamsters fed LS ($15 \pm 2.1\%$, $n=15$) vs. HS ($15 \pm 0.8\%$, $n=14$) diet. Low-dose ANG II infusion restored arteriolar dilation to ACh and SNP in HS-fed hamsters (C, D), but had no effect on these responses in LS-fed hamsters (not shown). MAP was similar in hamsters receiving LS +saline ($82 \pm 1.5 \text{ mmHg}$, $n=10$), HS+saline ($81 \pm 1.3 \text{ mmHg}$, $n=10$), and HS+ANG II ($83 \pm 1.4 \text{ mmHg}$, $n=10$).

Addition of PEG-SOD to the superfusion solution to scavenge superoxide radicals (Figure 2) restored arteriolar dilation to ACh (A) and SNP (B) in hamsters fed HS diet, while arteriolar responses to these agonists were unaffected by PEG-SOD in hamsters maintained on LS diet (C, D). The endothelium-dependent dilation to ACh that was restored by PEG-SOD in animals fed HS diet was NO dependent, as it was eliminated by blocking NO synthase with L-NAME (E). In separate experiments (not shown), arteriolar responses to acetylcholine and SNP were also restored by acute addition of the SOD mimetic tempol (10^{-4} M) to the superfusate or by chronic treatment with tempol (1–3 mM) in the drinking water.

Effect of Angiotensin Converting Enzyme Inhibition on Arteriolar Responses to Acetylcholine and Sodium Nitroprusside

ACE inhibition with captopril had no significant effect on blood pressure (MAP = $89 \pm 2.8 \text{ mmHg}$, $n=6$ in control vs. $91 \pm 2.1 \text{ mmHg}$, $n=8$ in captopril-treated), but eliminated arteriolar dilation to ACh and SNP in animals maintained on standard rodent chow (Figure 3A, B). Acute addition of PEG-SOD to the superfusate to scavenge superoxide radicals ameliorated the impaired dilation of arterioles to ACh and SNP in captopril-treated hamsters (C, D).

Effect of AT₁ Receptor Blockade on Restoration of Arteriolar Dilation to ACh and SNP in HS-Fed Hamsters Infused with ANG II

In a separate series of experiments summarized in Figure 4, AT₁ receptor blockade with losartan (20 mg/kg/day in the drinking water) inhibited the ability of ANG II infusion to restore arteriolar dilation in response to ACh and SNP in hamsters fed a high salt diet (A, B). Losartan treatment alone failed to restore arteriolar dilation in response to ACh or SNP in HS-fed hamsters that did not receive ANG II infusion ($n=5$, not shown). Scavenging of superoxide radicals with PEG-SOD restored arteriolar dilation to acetylcholine in ANG II-infused hamsters fed high salt diet and treated with losartan to block the AT₁ receptor (C).

Vascular Reactive Oxygen Species

DHE fluorescence was significantly higher in aortas of hamsters fed HS diet compared to LS controls, and chronic infusion of ANG II reduced DHE fluorescence significantly in aortas of HS fed hamsters (Figure 5). AT₁ receptor blockade with losartan attenuated the ability of ANG II infusion to reduce oxidant stress in vessels of HS-fed hamsters receiving ANG II infusion (Figure 6). In a final series of experiments (Figure 7), we found that low dose ANG II infusion had no effect on vascular ROS levels in hamsters maintained on LS diet.

Effect of High Salt Diet on Responses to Prostacyclin Analogues

HS diet also eliminated endothelium-independent arteriolar dilation in response to the stable prostacyclin analogues carbacyclin and iloprost (Figure 8A, B). Arteriolar dilation to iloprost in HS-fed hamsters was restored by ANG II infusion and by addition of the superoxide scavenger tempol to the superfusate (C).

Effect of High Salt Diet on Responses to Cromakalim and Glibenclamide

In other experiments, HS diet significantly reduced arteriolar dilation in response to the K_{ATP} channel activator cromakalim (Figure 9A). Similar to its effect on arteriolar responses to iloprost, low dose ANG II infusion restored arteriolar dilation to cromakalim in HS-fed hamsters (Figure 9A). Addition of the K_{ATP} channel antagonist glibenclamide to the superfusion solution caused a progressive constriction of arterioles in LS-fed hamsters that was absent in hamsters fed HS diet (B). Collectively, these findings indicate that tonic activation of K_{ATP} channels is significantly impaired in hamsters fed HS diet, and that impaired function of K_{ATP} channels in HS-fed hamsters is related to salt-induced ANG II suppression.

Effect of High Salt Diet on Responses to Angiotensin II and Elevated PO₂: Figure 10

summarizes the effect of HS diet on arteriolar constriction to ANG II and elevation of superfusion solution PO₂ in hamsters fed LS vs. HS diet. ANG II-induced constriction was unaffected by elevated dietary salt intake (A). O₂-induced constriction of arterioles (B) was similar during superfusion with PSS equilibrated with 5% O₂ and 10% O₂, but was significantly reduced during 21% O₂ superfusion in hamsters fed HS diet.

DISCUSSION

Two persuasive reviews (2, 47) have noted that current arguments regarding salt and blood pressure are often based on “defensive authoritarianism” in support of “entrenched positions.” Regrettably, this has diverted attention from the possibility that elevated dietary salt intake may exert harmful effects on the cardiovascular system, independent of blood pressure. As a result, the pressure-independent effects of dietary salt on cardiovascular morbidity and mortality have not received their due attention (2).

There is increasing evidence that elevated dietary salt intake leads to a surprisingly rapid development of endothelial dysfunction in humans (61); and several recent studies in Sprague-Dawley rats (40, 44, 66, 67, 72, 73) and Dahl salt-sensitive (SS) rats (10–12) (a genetic model of human salt-sensitive hypertension characterized by chronically low PRA) (1, 7, 31) have unexpectedly suggested that chronic exposure to low levels of ANG II in the blood may contribute to endothelial dysfunction, oxidant stress, and impaired vascular relaxation in the peripheral circulation without an increase in blood pressure.

Vascular oxidant stress often plays a major role in endothelial dysfunction (4) and is more frequently observed in salt-sensitive than in salt-resistant patients (6). Apart from its

association with hypertension, endothelial dysfunction has been found to be an independent predictor of myocardial infarction (18, 28, 52, 55, 60), ischemic stroke (18, 25, 28, 55, 56), unstable angina (19, 25, 52, 56), and cardiovascular death (18, 19, 25, 28, 52, 56, 60). Elevated vascular superoxide ($O_2^{\bullet-}$) levels are also deleterious insofar as they oppose the anti-inflammatory and anti-proliferative effects of NO, and can contribute to oxidant-related damage to multiple transduction mechanisms involved in both endothelium-dependent and endothelium-independent vascular relaxation. In this regard, it is important to note that, in contrast to widespread identification of endothelial dysfunction as a contributor to pathophysiological conditions in humans, much less is known regarding the occurrence and role of generalized defects in vascular relaxation mechanisms in human disease.

Despite increasing evidence that chronic exposure to reduced levels of circulating ANG II can contribute to endothelial dysfunction and vascular oxidant stress in rats (64, 72, 73), there is continuing skepticism regarding the deleterious effects of low ANG II levels on oxidant stress and vascular function. As a result, the potential contribution of chronically low levels of ANG II to vascular dysfunction with high salt diet and during other conditions associated with low levels of circulating ANG II, e.g. low PRA associated with low renin hypertension and increased cardiovascular mortality in humans (68), often goes unnoticed. In the present study, the role of ANG II in maintaining vascular function and preventing oxidant stress in the terminal microcirculation was directly tested for the first time in a novel experimental model that is also suitable for investigation of microvascular function with implanted chambers and in allografts of multiple parenchymal tissues (8, 21, 32, 33).

The finding that ANG II suppression may lead to increased oxidant stress and impaired vasodilatation during elevated dietary salt intake in normotensive animals is a departure from conventional and often dogmatic thinking regarding the role of ANG II in vascular regulation. Specifically, because higher doses of ANG II clearly stimulate $O_2^{\bullet-}$ production in blood vessels (22, 71) it is often assumed that any decrease in ANG II levels is beneficial for vascular function regardless of the context.

The present experiments demonstrate that high salt diet, a known suppressor of plasma ANG II levels, eliminates arteriolar dilation to the endothelium-dependent vasodilator ACh, endothelium-independent prostacyclin analogues carbacyclin and iloprost, the K_{ATP} channel opener cromakalim and the endothelium-independent NO donor SNP in the hamster cheek pouch. High salt diet also appears to compromise the tonic role of K_{ATP} channels in regulating arteriolar tone in the cheek pouch, as arteriolar constriction in response to the K_{ATP} channel antagonist glibenclamide that occurred in arterioles of hamsters fed low salt diet was eliminated when the animals were fed a high salt diet.

The original goal of this study was to evaluate early-onset endothelial dysfunction in arterioles with ANG II suppression due to high salt diet and ACE inhibition. However, these experiments revealed impaired vascular relaxations that are much more extensive than those found in conventional studies of endothelial dysfunction, and which are indicative of widespread vascular dysfunction associated with reduced activation of the AT_1 receptor. For example, the observation that high salt diet affects K_{ATP} channel function in HS-fed hamsters was unexpected, because it contrasts with earlier studies showing that responses to the K_{ATP} channel opener aprikalim are intact in middle cerebral arteries of rats fed HS diet (4% NaCl) (44). However, other investigators have reported that the relaxation of aortas from Sprague-Dawley rats fed 8% NaCl diet (48) and rats with DOCA-salt hypertension (17) exhibit a reduced relaxation to cromakalim, suggesting that K_{ATP} channels may be deactivated during the development of salt loading hypertension. In the latter study (17), cromakalim also induced a dramatic increase in whole cell K_{ATP} channel currents in smooth muscle cells from sham operated controls while cromakalim-induced K_{ATP} channel currents

were weak or absent in cells from DOCA- salt hypertensive animals. The authors of those studies (17, 48) concluded that K_{ATP} channels were deactivated or K_{ATP} channel function was impaired in vascular smooth muscle cells from animals with these forms of salt-induced hypertension. However, the results of the present study suggest that salt-induced impairment of K_{ATP} channel function may precede the increase in blood pressure in salt-dependent forms of hypertension. Based on studies of Liu and coworkers in experimental models of hyperglycemia and diabetes (5, 41), the reduced response to glibenclamide and cromakalim in HS-fed hamsters may reflect a reduced open probability of K_{ATP} channels related to oxidant stress. The latter observations are consistent with increased vascular oxidant stress (73) and down regulation of antioxidant defenses reported in rats exposed to chronically low levels of plasma ANG II due to salt-induced ANG II suppression (14, 46) or an inability to regulate plasma renin activity normally (Dahl salt-sensitive rats) (13).

The finding that arteriolar responses to the NO donor SNP were lost in hamsters fed high salt diet was totally unexpected. This dramatic reduction in arteriolar sensitivity to SNP and suggests either that salt-induced oxidant stress is especially prominent in the cheek pouch microcirculation, or that NO-mediated vascular relaxation mechanisms are especially vulnerable to oxidant stress in this tissue. While the PEG-SOD and DHE fluorescence experiments (Figures 2–6) would suggest that elevated levels of $O_2^{\bullet-}$ compromise ACh-induced dilation by reducing NO availability in HS-fed hamsters, the loss of arteriolar dilation in response to SNP in hamsters fed high salt diet and in animals treated with captopril clearly indicate that suppression of ANG II-dependent mechanisms that maintain NO sensitivity could also play an important role in the loss of arteriolar dilation to ACh. At the present time, the mechanism responsible for the loss of arteriolar dilation to SNP is unknown. This question is especially intriguing in light of multiple studies reporting that NO sensitivity (assessed with NO donors) is not depressed with hypertension (39, 50, 51) or with HS diet (38, 40, 44, 67).

Because the restoration of acetylcholine-induced relaxation in HS animals by PEG-SOD was prevented by L-NAME, it is possible that loss of arteriolar responses to ACh and SNP in HS fed hamsters and in hamsters treated with ACE inhibitors may both be due to NO quenching by $O_2^{\bullet-}$. However, this does not seem to be a likely mechanism to explain the impaired SNP induced relaxation, particularly at higher doses of SNP. The similar responses of arterioles to 8-Br-cGMP in hamsters fed LS vs. HS diet indicate that vessel sensitivity to the vasodilator actions of cGMP is similar in the two groups on animals. Therefore, one possible mechanism for the loss of NO sensitivity in the HS fed hamsters is that oxidant stress due to high salt diet or ACE inhibition compromises the ability of the guanylyl cyclase to form cGMP in response to NO. The latter interpretation is consistent with reports that increased levels of ROS can lower guanylyl cyclase expression and activity, (16, 34, 35) which would impair arteriolar dilation in response to the NO donor. Relevant to this interpretation, Kagota et al. (34) showed that reduced expression and activity of soluble guanylyl cyclase in salt-fed SHR was a function of dietary salt intake, rather than elevated blood pressure itself.

It is important to stress that the profound effects of HS diet on vascular relaxation mechanisms in this study occurred with no effect on blood pressure, resting tone, or maximum diameter in an exceptionally large sample of arterioles, demonstrating that even short term high salt diet leads to dramatic and widespread defects in vascular relaxation mechanisms in the distal microcirculation of the hamster. Taken together, these observations support the value of this experimental model for investigating early-onset, salt-induced vascular dysfunction in microvessels independent of elevated blood pressure or structural remodeling. The present study also provides additional evidence supporting the universal nature of salt-induced impairment of vascular relaxation, including the distal microcirculation, in a novel species with great potential value for chronic studies of

developmental changes and trophic influences of the renin-angiotensin system on vascular reactivity using implanted chambers (8, 21, 32, 33) and allografts from diverse parenchymal tissues including heart (21), lung (8), kidney (32), and brain (33).

In these experiments, both chronic infusion of a subpressor dose of ANG II and acute scavenging of superoxide radicals restored arteriolar dilation to ACh, iloprost, and SNP in HS-fed animals. ROS levels were elevated in aortas of HS-fed hamsters, and were significantly reduced by chronic infusion of a low dose of ANG II. However, low dose ANG II infusion had no effect on vascular ROS levels in hamsters fed LS diet, where salt-induced ANG II suppression would not occur. The ability of ANG II infusion to restore arteriolar dilation in HS-fed hamsters and to reduce vascular oxidant stress in aortas of hamsters fed high salt diet were both inhibited by AT₁ receptor blockade with losartan (Figure 6), indicating that the protective effect of ANG II infusion to restore arteriolar dilation is mediated via antioxidant effects subsequent to AT₁ receptor activation. Consistent with this interpretation, scavenging of O₂^{•-} radicals with PEG-SOD restored arteriolar dilation to ACh and SNP that was lost in losartan-treated hamsters fed HS diet and infused with ANG II (Figure 4).

Our finding that vascular O₂^{•-} levels in HS-fed animals are significantly reduced by continuous infusion of a low dose ANG II may be related to the ability of ANG II to prevent the down-regulation of microvascular antioxidant defenses with HS diet. The latter hypothesis is supported the studies of Lenda and Boegehold (37) showing that HS diet decreases Cu/Zn SOD activity in rat skeletal muscle arterioles. There is also evidence that ANG II infusion increases Cu/Zn SOD activity significantly in mice lacking extracellular-superoxide dismutase (ecSOD) (20, 70) and increases the expression of ecSOD through an AT₁ receptor-mediated mediated mechanism in mouse aorta and human aortic smooth muscle cells (15). Finally, chronic low dose ANG II infusion also prevents the down-regulation of Cu/Zn SOD in cerebral arteries of HS-fed Sprague-Dawley rats (46) and congenic rats carrying a normally functioning renin allele in the Dahl salt sensitive background (13).

The finding that low dose ANG II infusion reduces vascular oxidant stress and preserves arteriolar dilation in HS-fed hamsters is novel because it contrasts with the more widely investigated action of ANG II to increase vascular oxidant stress (22, 49), leading to impaired vasodilatation (30). While increased oxidant stress and impaired vascular relaxation in the face of salt-induced ANG II suppression appears to be paradoxical, Zhu et al.(73) found that chronic i.v. infusion of a low dose of ANG II (5 ng/kg/min) to restore normal circulating ANG II levels in HS-fed rats (23, 72) restores vascular O₂^{•-} levels to low salt control values while higher doses of ANG II lead to the expected increase in oxidant stress in small arteries of the rat mesentery.

One important limitation of the present study is that the source of elevated vascular superoxide levels in HS-fed fed hamsters remains to be determined. While it is well known that supraphysiological levels of ANG II lead to increased superoxide production by NADPH oxidase, the potential source of the elevated superoxide levels under conditions of suppressed ANG II is more perplexing. Recent studies have shown that chronic exposure to low levels of ANG II in the plasma lead to reduced expression of antioxidant enzymes such as Cu/Zn SOD, (13, 14, 46) and that ANG II increases the expression of ecSOD in mouse aorta and in human vascular smooth muscle cells (15), and ANG II infusion increases Cu/Zn SOD activity in ecSOD knockout mice (20). In our estimation, the most likely explanation for the increased oxidant stress in blood vessels of the salt fed hamsters is that multiple enzymatic sources that produce superoxide during their normal catalytic activity contribute to oxidant stress because antioxidant defenses are down regulated in HS-fed animals.

Potential enzymatic sources of superoxide under these conditions include NADPH oxidase, xanthine oxidase, cyclooxygenase, uncoupled nitric oxide synthase, the leukotriene pathway, and cytochrome P450 enzymes forming EETs and 20-HETE, among others. The interpretation that inhibition of enzymes that produce superoxide during normal catalytic activity lead to a nonspecific elevation of vascular oxidant stress when antioxidant mechanisms are suppressed is supported by earlier studies of small mesenteric arteries and aortas of HS-fed rats, where inhibition of NADPH oxidase (73), xanthine oxidase (73), and NO synthase (74) all led to a reduction in vascular superoxide levels in an experimental model that exhibits down regulation of antioxidant enzymes due to salt-induced ANG II suppression (14, 46).

A role for tonic AT₁ receptor activation in maintaining normal vascular relaxation is also supported by our finding that ACE inhibition with captopril eliminated arteriolar dilation in hamsters fed standard rodent chow. Similar to our findings in hamsters fed high salt diet, scavenging of O₂^{•-}radicals restored arteriolar dilation to ACh and SNP in captopril-treated animals, indicating that ACE inhibition increases vascular oxidant stress by reducing plasma ANG II levels.

The results of this study naturally raise the question of how our findings relate to the indisputable beneficial effects of ACE inhibitors and angiotensin receptor blockers (ARBs) in treating hypertension and other pathophysiological conditions. The most likely explanation for these disparate findings is that ACE inhibitors and ARBs prevent high levels of ANG II from increasing O₂^{•-}levels under pathological conditions, while other mechanisms determine the responses to these agents when plasma ANG II levels and oxidant stress are normal. For example, studies by Reed et al. (54) showed that the transduction mechanisms and physiological effects of AT₁ receptor blockade on coronary collateral development following repeated transient ischemia differ substantially depending on the levels of oxidant stress in the tissue. In that study, AT₁ receptor blockade was beneficial with elevated oxidant stress in the JCR rat model of metabolic syndrome, but deleterious in WKY rats having normal levels of reactive oxygen species in the tissue. Importantly, those investigators also showed that low dose ANG II infusion augmented coronary collateral development while high dose ANG II infusion abrogated coronary collateral development in WKY rats. Those differences in coronary collateral development in the presence of AT₁ receptor blockade or different ANG II doses in the various experimental groups were associated with fundamental differences in pattern of activation of the Akt and p38 kinase pathways (54).

In the present study, ANG II infusion did not fully restore vascular ROS levels to LS control levels, in contrast to the findings of Zhu et al. (73). This is most likely due to a failure of the subcutaneous infusion to restore plasma ANG II to normal physiological values. While salt-induced suppression of PRA and ANG II is indisputable in larger animals (26, 27, 36), rats (23, 53, 72), and humans (24, 57–59), plasma ANG II levels are notoriously affected by blood loss, stress, and anesthesia. Because the small size of the hamster, obtaining an arterial blood sample of adequate size for ANG II measurements is impossible in conscious animals, and anesthesia and sampling of trunk blood cause dramatic increases in plasma ANG II that obscure physiological differences. Thus, the precise level of plasma ANG II in the subcutaneously-infused hamsters remains an open question.

Nonetheless, the most important observation in this series of experiments is that ANG II infusion did cause a significant reduction in ROS levels concomitant with restoration of vascular relaxation; and that both these actions of ANG II were inhibited by blocking the AT₁ receptor with losartan. We believe that restoration of vasodilator responses by ANG II infusion despite some level of persisting oxidant stress occurs because ANG II infusion is

still able to reduce ROS levels by an amount that is adequate to ameliorate oxidant-dependent defects in vascular relaxation mechanisms that exist in the absence of ANG II infusion.

In a final series of experiments, we also determined whether short term HS diet affects arteriolar responses to exogenously-applied ANG II or elevated PO₂. This is important because much less is known regarding the effects of HS diet alone on arteriolar reactivity to vasoconstrictor stimuli in normotensive animals. Based on findings in hypertensive models, we expected that HS diet would potentiate arteriolar constriction to these stimuli. Contrary to our hypothesis, HS diet had no effect on ANG II-induced constriction; and O₂ induced constriction of arterioles was either unaffected (5% O₂, 10% O₂) or attenuated (21% O₂). The lack of a potentiating effect of HS diet on arteriolar O₂ responses is especially important because it shows that the salt-induced potentiation of arteriolar constriction to elevated PO₂ in Dahl salt-sensitive rats (65) and in rats with reduced renal mass-salt loading hypertension (42) is likely due to intrinsic alterations of microvascular function associated with the hypertensive model, rather than a nonspecific effect of elevated dietary salt intake itself.

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List of Abbreviations

ACE	Angiotensin converting enzyme
ACh	Acetylcholine
ANG II	Angiotensin II
ARB	Angiotensin receptor blocker
AT₁	Angiotensin II type 1 receptor
cGMP	Cyclic guanosine monophosphate
Cu/Zn SOD	Copper zinc (intracellular) superoxide dismutase
DHE	Dihydroethidium
ecSOD	Extracellular superoxide dismutase
HS	High salt (4.0% NaCl)
K_{ATP}	ATP-sensitive potassium channel
L-NAME	NG-nitro-L-arginine methyl ester
LS	Low salt (0.4% NaCl)
MAP	Mean arterial pressure
NO	Nitric oxide
PEG-SOD	Polyethylene glycol-superoxide dismutase
PRA	Plasma renin activity
PSS	Physiological salt solution
ROS	Reactive oxygen species
RRM	Reduced renal mass
SHR	Spontaneously hypertensive rat

SNP	Sodium nitroprusside
SOD	Superoxide dismutase
WYK	Wistar Kyoto rat

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Perspectives

It has been estimated that a population-wide reduction in dietary salt intake would produce cardiovascular benefits on a par with those of population-wide reductions in tobacco use, obesity, and cholesterol levels (3). From a translational standpoint, our finding that salt-induced ANG II suppression or interference with the tonic activation of AT₁ receptors leads to vascular oxidant stress and generalized vascular dysfunction has important implications regarding the development of cardiovascular disease in the presence of low PRA during elevated dietary salt intake and in low renin forms of hypertension. These findings also suggest that reduced dietary salt intake may be beneficial in preventing early vascular dysfunction and, ultimately, cardiovascular morbidity and mortality in humans, especially those exhibiting salt-sensitivity of blood pressure prior to the onset of hypertension (68).

Highlights

High salt diet eliminated vascular relaxation mechanisms in hamster arterioles.

Low dose ANG II infusion with HS diet restored dilation and reduced oxidant stress.

HS diet and low dose ANG II infusion had no effect on arterial blood pressure.

ACE inhibition and AT₁ receptor blockade abrogated the protective effects of ANG II.

Salt-induced ANG II suppression led to impaired K_{ATP} channel function.

Figure 1.

Responses of cheek pouch arterioles to acetylcholine (**A**, **C**), and sodium nitroprusside (**B**, **D**) in hamsters fed low salt (LS) diet (solid lines) or high salt (HS) diet (dashed lines).

Panels **C** and **D** represent LS or HS receiving chronic infusion of isotonic saline or HS

+ANG II infusion (dotted lines). Data are expressed as mean $\pm$ SEM for 5–9 animals/group.

* $P < 0.05$ vs. LS.

Figure 2.

Effect of acute addition of PEG-SOD (200 U/mL) to the superfusate on arteriolar responses to acetylcholine (**A**, **C**) and sodium nitroprusside (**B**, **D**) in hamsters fed LS (**C**, **D**) or HS (**A**, **B**) diet. **Panel E** shows the effect of NOS inhibition with L-NAME on arteriolar responses to ACh in HS-fed hamsters during superfusion with PEG-SOD to scavenge superoxide radicals. Data are expressed as mean $\pm$ SEM for 6–18 animals/group; * $P < 0.05$ vs. same concentration of the agonist in absence of PEG-SOD (**A**, **B**) or L-NAME (**E**).

Figure 3.

Effect of angiotensin converting enzyme inhibition with captopril on arteriolar responses to acetylcholine and sodium nitroprusside in animals fed standard rodent chow before (**A, B**) and after (**C, D**) superoxide scavenging with PEG-SOD. Data are expressed as mean change in diameter ($\pm$ SEM) from control prior to addition of the agonist for $n = 4-15$ animals/group. * $P < 0.05$ in captopril-treated animals vs. nontreated controls; † $P < 0.05$ captopril vs. captopril + PEG-SOD.

Figure 4.

Effect of AT_1 receptor blockade with losartan (20 mg/kg) on arteriolar responses to acetylcholine (**A**) and sodium nitroprusside (**B**) in HS-fed hamsters receiving chronic infusion of a low dose of ANG II. **Panel C** shows restoration of ACh-induced dilation by PEG-SOD in HS-fed hamsters receiving ANG II infusion with losartan to block the AT_1 receptor. Data are expressed as mean $\pm$ SEM for $n=5-6$ /group. * $P<0.05$ for response in ANG II-infused animals $\pm$ losartan (**A, B**) or $\pm$ PEG-SOD (**C**).

Figure 5. Levels of reactive oxygen species (assessed via DHE fluorescence) in aortas of hamsters fed LS diet (**A**) or HS diet (**B**) and HS diet with low dose ANG II infusion (**C**). **Panel D** summarizes data normalized as percent of average LS DHE fluorescence. Data are expressed as mean $\pm$ SEM for n=8/group; * P<0.05 vs. LS control; † P<0.05 vs. HS.

Figure 6.

Levels of reactive oxygen species (assessed via DHE fluorescence) in aortas of hamsters fed HS diet and infused with saline (**A**) or ANG II $\pm$ losartan (**B, C**). **Panel D** summarizes the data normalized as percent of average HS+saline DHE fluorescence. Data are expressed as mean $\pm$ SEM for n=6/group; * P<0.05 vs. HS+saline control; †--P<0.05 vs. HS+ANG II.

Figure 7. Levels of reactive oxygen species (assessed via DHE fluorescence) in aortas of hamsters fed LS diet and receiving continuous infusion of saline vehicle (**A**) or ANG II (**B**). **Panel C** summarizes the data normalized as percent of average LS+saline DHE fluorescence. Data are expressed as mean $\pm$ SEM for n=6/group.

Figure 8.

Effect HS diet on arteriolar responses to carbacynin (**A**) and iloprost (**B**) and iloprost $\pm$ low dose ANG II infusion or superoxide scavenging with tempol (**C**). Data are expressed as mean $\pm$ SEM for $n=6-8$ /group. * $P<0.05$ vs. LS at same agonist concentration (**A**, **B**) or HS + ANG II (**C**). $\dagger$ $P<0.05$ vs. HS+saline+tempol.

Figure 9.

Arteriolar responses to the K_{ATP} channel activator cromakalim (**A**) and the K_{ATP} channel inhibitor glibenclamide (**B**) in hamsters fed low salt (LS; n=8) diet; high salt (HS; n=5) diet; or HS diet with ANG II infusion (HS + ANG II; n=5) (**A**). Data are expressed as mean change in diameter (μm) $\pm$ SEM. * $P < 0.05$ vs. LS control. † $P < 0.05$ vs. HS+ANG II.

Figure 10.

Arteriolar constriction in response to ANG II (n=11 LS, 15 HS) (**A**) and elevation of superfusion solution O₂ concentration from 0% O₂ to 5% O₂ (n= 8 LS, 5 HS), 10% O₂ (n= 16 LS, 10 HS), and 21% O₂ (n= 16 LS, 16 HS) (**B**) in hamsters fed LS vs. HS diet. Data are expressed as mean change from control diameter ± SEM. * P<0.05 vs. LS diet.

TABLE 1

Control Diameters of Arterioles in Different Experimental Groups

Group	Diameter (μm)
LS	20.1 ± 0.5 (86)
HS	19.7 ± 0.5 (81)
HS + ANG II	21.5 ± 1.2 (8)
HS + Saline	20.5 ± 1.2 (8)
LS + PEG SOD	13.9 ± 0.7 (11)
HS + PEG SOD	15.9 ± 0.8 (13)
HS + PEG SOD + L-NAME	19.2 ± 1.7 (6)
Purina	14.5 ± 0.8 (15)
Purina + Captopril	15.4 ± 0.8 (20)
Purina + Captopril + PEG SOD	15 ± 1.8 (4)
HS + ANG II + Losartan	19.2 ± 1.9 (6)
HS + ANG II + Losartan + PEG SOD	20.7 ± 1.9 (6)