Role of superoxide and angiotensin II suppression in salt-induced changes in endothelial Ca\(^{2+}\) signaling and NO production in rat aorta


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Zhu, Jiaxuan, Ines Drenjancevic-Peric, Scott McEwen, Jill Friesema, Danielle Schulta, Ming Yu, Richard J. Roman, and Julian H. Lombard. Role of superoxide and angiotensin II suppression in salt-induced changes in endothelial Ca\(^{2+}\) signaling and NO production in rat aorta. *Am J Physiol Heart Circ Physiol* 291: H929–H938, 2006. First published April 7, 2006; doi:10.1152/ajpheart.00692.2005.—Male Sprague-Dawley rats were maintained on a low-salt (LS) diet (0.4% NaCl) or changed to a high-salt (HS) diet (4% NaCl) for 3 days. Increases in intracellular Ca\(^{2+}\) ([Ca\(^{2+}\)]\(i\)) in response to methacholine (10 μM) and histamine (10 μM) were significantly attenuated in aortic endothelial cells from rats fed a HS diet, whereas thapsigargin (10 μM)-induced increases in [Ca\(^{2+}\)]\(i\) were unaffected. Methacholine-induced nitric oxide (NO) production was eliminated in endothelial cells of aortas from rats fed a HS diet. Low-dose ANG II infusion (5 ng·kg\(^{-1}\)·min\(^{-1}\) iv) for 3 days prevented impaired [Ca\(^{2+}\)]\(i\), signaling response to methacholine and histamine and restored methacholine-induced NO production in aortas from rats on a HS diet. Adding Tempol (500 μM) to the tissue bath to scavenge superoxide anions increased NO release and caused N\(^{6}\)-nitro-l-arginine methyl ester-sensitive vascular relaxation in aortas from rats fed a HS diet but had no effect on methacholine-induced Ca\(^{2+}\) responses. Chronic treatment with Tempol (1 mM) in the drinking water restored NO release, augmented vessel relaxation, and increased methacholine-induced Ca\(^{2+}\) responses significantly in aortas from rats on a HS diet but not in aortas from rats on a LS diet. These findings suggest that J) agonist-induced Ca\(^{2+}\) responses and NO levels are reduced in aortas of rats on a HS diet; 2) increased vascular superoxide levels contribute to NO destruction, and, eventually, to impaired Ca\(^{2+}\) signaling in the vascular endothelial cells; and J) reduced circulating ANG II levels during elevated dietary salt lead to elevated superoxide levels, impaired endothelial Ca\(^{2+}\) signaling, and reduced NO production in the endothelium.

endothelium; sodium; dietary salt intake; vascular relaxation; nitric oxide

A VARIETY OF AGENTS, including muscarinic agonists such as ACh, dilate blood vessels by stimulating the synthesis of nitric oxide (NO) by endothelial NO synthase. Endothelial cell activation in response to agonists and autacoids leads to NO formation via receptor-mediated increases in intracellular calcium ion concentration ([Ca\(^{2+}\)]\(i\)). However, emerging evidence indicates that endothelial NO synthase activity can be affected by a variety of other mechanisms that are independent of [Ca\(^{2+}\)]\(i\). (7, 12, 21, 26, 27). This raises the possibility that changes in NO production under some physiological conditions may be independent of changes in endothelial [Ca\(^{2+}\)]\(i\).

Endothelium-dependent relaxation in response to ACh is impaired in the aorta, resistance arteries, and microvessels of rats fed a high-salt (HS) diet (2–5, 15, 16, 18, 28). Elevated dietary salt intake causes suppression of plasma ANG II levels (8, 9), and previous studies (18, 30, 31) have demonstrated that the impaired vascular relaxation in Sprague-Dawley rats fed a HS diet can be prevented by a continuous intravenous infusion of a low dose of ANG II to maintain normal circulating levels of ANG II.

In the present study, we tested the hypothesis that receptor-mediated increases in [Ca\(^{2+}\)]\(i\), and/or entry of Ca\(^{2+}\) via store-operated calcium channels are attenuated in aortic endothelial cells of animals fed a HS diet. The present study also determined whether prevention of ANG II suppression by continuous intravenous infusion of a low dose of the peptide can prevent any decreases in the endothelial [Ca\(^{2+}\)]\(i\), signal and NO production in animals fed a HS diet. Because elevated levels of superoxide can also affect receptor-mediated signaling, either directly (22) or via increased formation of peroxynitrite (20), we also tested the hypothesis that superoxide contributes to the impaired regulation of endothelial cell [Ca\(^{2+}\)]\(i\), during elevated dietary salt intake by determining the acute and long-term effects of the superoxide dismutase mimetic Tempol on methacholine-induced changes in endothelial [Ca\(^{2+}\)]\(i\), NO production, and vascular function in animals fed a HS diet and low-salt (LS) diet.

This study provides the first evidence that ANG II suppression in response to elevated dietary salt intake leads to an attenuation of receptor-mediated increases in endothelial cell [Ca\(^{2+}\)]\(i\), and that prolonged exposure to elevated levels of O\(_2\)\(^{\bullet}\) in animals fed a HS diet attenuates receptor-mediated increases in endothelial cell [Ca\(^{2+}\)]\(i\); (in addition to the ability of O\(_2\)\(^{\bullet}\) to destroy NO via direct inactivation). These findings may have widespread implications for receptor-mediated signal transduction during elevated dietary salt intake.

MATERIALS AND METHODS

Experimental animal groups. Experiments were performed on 10- to 12-week-old male Sprague-Dawley rats maintained in an Association for Assessment and Accreditation of Laboratory Animal Care-approved animal resource center. Seven experimental groups were studied. These included rats fed a HS diet (4% NaCl) for 3 days (group 1) or a LS diet (0.4% NaCl) for 3 days (group 2). Other groups of rats were maintained on a LS diet, switched to a HS diet on the fourth day after surgery, and continuously infused for another 3 days with a low dose of ANG II (5 ng·kg\(^{-1}\)·min\(^{-1}\) iv) (group 3); infused with ANG II (5 ng·kg\(^{-1}\)·min\(^{-1}\) iv) plus the ANG II type 1 (AT\(_1\)) receptor blocker losartan (20 μg·kg\(^{-1}\)·min\(^{-1}\) iv) (group 4); or infused with 0.9% saline vehicle for ANG II (group 5). Groups 6 and 7

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consisted of rats receiving Tempol (1 mM) in their water for 1 wk and then continued on a LS diet or switched to a HS diet for 3 days before the start of the experiment.

For the infusion studies, rats were anesthetized with an intramuscular injection containing ketamine (78.0 mg/kg) and acepromazine (2.2 mg/kg). Chronic indwelling catheters were introduced via the femoral artery and femoral vein to monitor blood pressure and for intravenous infusions, respectively, using procedures identical to those described previously (30, 31). The animals were allowed a 3-day recovery period before the experiment began, and all animals had tap water to drink ad libitum. The Medical College of Wisconsin International Animal Care and Use Committee approved all procedures used in the study.

**Plasma ANG II concentration.** Plasma ANG II levels were measured in the Physiology Department Analytical Core facility (Medical College of Wisconsin, Milwaukee, WI) with the use of previously described techniques (24). Arterial blood samples (1 ml) from undisturbed, chronically cannulated rats were drawn into chilled tubes containing 50 μl/ml of 0.125 mol/l Na2EDTA, 0.025 mol/l phenanthroline, and 0.5 mmol/l neomycin sulfate. Samples were immediately centrifuged, and plasma was separated and frozen at −70°C until extracted from plasma with the use of a C18 Sep-Pak column (Waters). ANG II was separated from ANG I, ANG III, and ANG II metabolites by reverse-phase, high-performance liquid chromatography. Retention times of ANG I, ANG II, and the primary angiotensin metabolites ANG II-(1–7), ANG II-(2–8), ANG II-(3–8), and ANG II-(2–10) were determined by injecting mixtures containing 1–5 ng of each peptide and monitoring with an ultraviolet detector at 210 nm. All peptides were obtained from Peninsula Laboratories (San Carlos, CA).

**Assessment of changes in [Ca2+]i.** On the day of the experiment, rats were anesthetized with pentobarbital sodium (50 mg/ml). The thoracic aorta was removed and rinsed with cold physiological salt solution (PSS) with the following composition (in mM): 119 NaCl, 4.7 KCl, 1.17 MgSO4, 1.6 CaCl2, 1.18 NaH2PO4, 24 NaHCO3, 0.026 EDTA, and 5.5 glucose. Before each experiment, the aorta was cut into several segments. Each segment was opened with fine scissors, affixed with several fine supporting pins onto a 0.9-cm microdissecting dish filled with Sylgard (World Precision Instruments, Sarasota, FL) with the endothelial surface facing up, and immersed in cold-PSS, taking care not to disrupt the endothelium.

For the Ca2+ measurements, the microdissecting dish was put into a recording chamber filled with PSS (kept at 37°C) with the lumen side of the vessel on the bottom, facing the objective of an inverted microscope (Nikon TS-100). A 1.5-mm space was maintained between the endothelium and the glass bottom of the recording chamber to prevent the endothelium from contacting the bottom of the perfusion chamber and to allow the PSS to pass over the endothelial side of the vessel.

[Ca2+]i in endothelial cells of the isolated aortas was determined by using an Incyt Im2 imaging system (Intracellular Imaging, Cincinnati, OH) mounted on an inverted microscope (Nikon TS-100). A 1.5-mm space was maintained between the endothelium and the glass bottom of the recording chamber to prevent the endothelium from contacting the bottom of the perfusion chamber and to allow the PSS to pass over the endothelial side of the vessel.

[Ca2+]i in endothelial cells of the isolated aortas was determined by using a fluorescent dye loading and that methacholine treatment did not cause saturation of the dye. Aortic ring studies. The role of superoxide and NO in modulating contractile force in the different groups was evaluated by measuring changes in contractile force in norepinephrine-contracted aortic rings. In these experiments, rats were anesthetized with pentobarbital sodium (50 mg/kg), and the thoracic aorta was removed and cleaned of connective tissue in ice-cold PSS. Aortic rings were mounted in a tissue chamber containing PSS maintained at 37°C and equilibrated with a 95% O2-5% CO2 gas mixture. The vessel rings were attached other investigators (19, 32, 34). The system was calibrated weekly at 37°C in the absence of a vessel, using standard calcium solutions purchased from Molecular Probes.

The changes in endothelial [Ca2+]i, in response to methacholine (10 μM) and histamine (10 μM) were recorded in aortas from rats fed a HS or LS diet and in aortas from rats fed a HS diet and infused with low-dose ANG II (with and without a simultaneous infusion of losartan to evaluate the role of the AT1 receptor in mediating the effects of ANG II on [Ca2+]i, responses in the vessels). The influx of Ca2+ via store-operated Ca2+ channels was evaluated by measuring thapsigargin (10 μM)-induced changes in endothelial [Ca2+]i, in aortas from rats fed a LS diet or HS diet in both normal PSS or Ca2+-free PSS containing (in mM) 119 NaCl, 4.7 KCl, 1.17 MgSO4, 1.17 MgCl2, 20 NaH2PO4, 24 NaHCO3, 0.026 EDTA, and 5.5 glucose. In other experiments, the superoxide dismutase mimetic Tempol (4-hydroxy-2,2,6,6-tetramethyl piperidinoxyloxyl) (500 μM) was added to the tissue bath to evaluate the direct contribution of superoxide to any changes in [Ca2+]i regulation. In those experiments, the vessels were preincubated with Tempol (500 μM) for 20 min, after which the response of 20 cells to methacholine was determined in each aortic segment.

**Measurement of NO production.** To evaluate NO production by the vessels, thoracic aortas were isolated, opened along their longitudinal axis, and mounted with the endothelial side facing upward, as previously described (35). Aortic segments were loaded with the indicator by incubating them for 30 min at 37°C in HEPES-buffered PSS (pH 7.4) containing 5 μM 4,5-diaminofluorescein (DAF-2DA), a membrane-permeable probe that is trapped inside the cells when the ester bonds are hydrolyzed, generating the relatively nonfluorescent and membrane-impermeable DAF-2. DAF-2 rapidly and irreversibly interacts with NO to produce the highly fluorescent product triazolofluorescein (DAF-2T), which accumulates inside the cells over time (11). DAF-2T fluorescence exhibits a linear increase that can be used as an indicator of the rate of NO production, as demonstrated by Yi et al. (33). Once loading was finished, the vessels were rinsed three times and placed in a chamber containing HEPES-buffered PSS maintained at 37°C with a water bath. L-Arginine (100 μM) was added to the chamber during measurements to ensure adequate substrate availability for NO synthase.

Measurements were performed on a Nikon E 600 microscope (Nikon, Tokyo, Japan) equipped with a ×10 Plan Fluo phase objective. The signals were acquired by a Princeton Instruments Micromax Cooled CCD camera (RS Princeton Instruments, Trenton, NJ). To evaluate NO production, DAF-2T was excited at 490 nm, and the fluorescence was collected through a 530-nm band-pass emission filter. Fluorescence intensity was analyzed using MetaMorph version 4.6 software (Universal Imaging, Downingtown, PA). NO fluorescence was measured every 5 min for 20 min in the same area of the endothelial surface. The ratio of the slopes of the fluorescence intensity changes during the control period and after addition of 10 μM methacholine or 10 μM histamine to the bath was determined as an indicator of the relative rates of NO production under the two conditions, as previously described (35). At the end of the experiment, the response to the NO donor (Z)-1-[N-(2-aminoethoxy)-N-(2-ammonioethoxy)amino]-diazen-1-ium-1,2-diolate (Cayman Chemical, Ann Arbor, MI) was determined as a positive control to verify that a HS diet did not affect dye loading and that methacholine treatment did not cause saturation of the dye.
to a model FT-03 isometric force transducer (Grass Astro-Med, West Warwick, RI) for continuous measurement of force. Data were acquired and analyzed using WinDaq software (DataQ Instruments, Akron, OH).

Passive force was set at 2–3 g, and the aortic rings were equilibrated and preconditioned as previously described (35). At the end of the equilibration period, the rings were precontracted with norepinephrine (0.1 μM). When the contraction reached a stable level, N-nitro-L-arginine methyl ester (L-NAME; 100 μM) or Tempol (500 μM) was added to the tissue bath to evaluate the contribution of resting levels of NO or superoxide, respectively, to the modulation of vascular tone. After a steady level of tone was reached in the Tempol experiments, L-NAME (100 μM) was added to the tissue bath to assess the effect of NO synthase inhibition on Tempol-induced relaxation of the vessels.

In another series of experiments, responses to methacholine were compared in aortic rings from rats fed a LS or HS diet. In those experiments, norepinephrine (0.1 μM) was added to the chamber to produce a submaximal contraction of the vessel. When the contraction reached a stable value, increasing concentrations of methacholine were added to the chamber to obtain a cumulative concentration-response curve over the range of 10^{-M} to 10^{-4} M. The relaxation response to methacholine at each concentration was expressed as a percentage of the norepinephrine-induced contraction. To assess the role of superoxide in modulating the response of the vessels to methacholine, changes in contractile force in response to increasing concentrations of methacholine were compared with and without Tempol in the drinking water or before and after acute addition of Tempol to the tissue bath. In the latter studies, Tempol-treated aortic rings were paired with untreated rings from the same animal.

Statistics. Data were summarized as means ± SE. One-way or two-way ANOVA was used to identify differences between various experimental groups that were being compared, and a Student-Newman-Keuls test was used to identify differences between individual means, post hoc. A probability level of P < 0.05 was considered to be statistically significant.

RESULTS

Arterial blood pressure and plasma ANG II levels. Mean arterial pressure measurements in rats in the various experimental groups are summarized in Table 1, which includes values obtained from shared rats used in previous studies (36). In these experiments, arterial pressure was significantly elevated in ANG II-infused rats on a HS diet compared with controls on a LS diet. The latter results are in contrast to previous studies by our laboratory (30, 31), which has generally found that low-dose ANG II infusion does not cause an elevation in arterial pressure. The reasons for this elevation of arterial pressure are not readily apparent but may be related to differing environmental conditions in the recording room.

Figure 1 compares plasma ANG II levels in rats on a LS and HS diet (with and without Tempol in the drinking water) and in rats fed a HS diet and receiving a continuous intravenous infusion of a low dose of ANG II to prevent ANG II suppression with a HS diet. In untreated animals, elevated dietary salt intake led to a significant reduction in plasma ANG II levels that could be prevented by continuous intravenous infusion of a low dose of the peptide. Plasma ANG II levels were significantly elevated in Tempol-treated rats on a LS diet compared with untreated controls. However, a HS diet still led to ANG II suppression in Tempol-treated rats; plasma ANG II levels in the latter group were not significantly different from those in animals that were fed a HS diet without Tempol in the drinking water.

Effect of methacholine, histamine, and thapsigargin on [Ca^{2+}]i. Aortic endothelial cells become fluorescent after incubation with fura-2 AM and have a characteristic round-shaped appearance, enabling them to be observed individually (Fig. 2, A and B). Endothelial denudation after dye loading eliminated the fluorescence signal (Fig. 2, C and D), demonstrating that the signal was coming from the endothelial cells. Resting [Ca^{2+}]i values were unaffected by any of the treatments (data not shown).

Methacholine and histamine both elicited a biphasic increase in [Ca^{2+}]i in the endothelial cells that consisted of an initial peak increase, followed by a sustained plateau phase in which [Ca^{2+}]i remained relatively stable (Fig. 3). The increases in endothelial [Ca^{2+}]i during the peak and plateau phases of activation were significantly lower in aortas from rats fed a HS

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### Table 1. Mean arterial pressures

<table>
<thead>
<tr>
<th>Group</th>
<th>Mean Arterial Pressure, mmHg</th>
<th>n</th>
<th>Source</th>
</tr>
</thead>
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<tr>
<td>Low-salt diet</td>
<td>116±2</td>
<td>12</td>
<td>Ref. 36</td>
</tr>
<tr>
<td>High-salt diet</td>
<td>117±5</td>
<td>12</td>
<td>Ref. 36</td>
</tr>
<tr>
<td>High salt + ANG II</td>
<td>135±4†</td>
<td>9</td>
<td>Ref. 36 and current study*</td>
</tr>
<tr>
<td>High salt + ANG II + losartan water</td>
<td>106±4</td>
<td>6</td>
<td>Ref. 36</td>
</tr>
<tr>
<td>Low salt + Tempol in drinking water</td>
<td>127±2</td>
<td>13</td>
<td>Current study</td>
</tr>
<tr>
<td>High salt + Tempol in drinking water</td>
<td>126±3</td>
<td>12</td>
<td>Current study</td>
</tr>
</tbody>
</table>

Values are means ± SE for n rats. *Mean value recalculated from all animals used in present experiments. †Significantly different from low-salt diet (P < 0.05); ‡significantly different from noninfused animals on high-salt diet (P < 0.05).
diet compared with those from rats fed a LS diet. The changes in endothelial $\left[Ca^{2+}\right]_{i}$ in response to both methacholine and histamine in aortas from ANG II-infused rats fed a HS diet were similar to those occurring in vessels from animals on a LS diet (Fig. 3, B and D). Simultaneous infusion of losartan to block the AT1 receptor for ANG II eliminated the ability of ANG II to preserve methacholine-induced $\left[Ca^{2+}\right]_{i}$ responses in aortas from rats fed a HS diet (Fig. 4).

In another series of experiments, thapsigargin ($10 \mu M$) was added to the tissue bath to evaluate the effect of a HS diet on intracellular $\left[Ca^{2+}\right]_{i}$ stores and on the function of store-operated $\left[Ca^{2+}\right]_{i}$ channels. In those experiments, thapsigargin-induced increases in $\left[Ca^{2+}\right]_{i}$ in aortas from rats fed a LS diet and HS diet were virtually identical to each other, either in $\left[Ca^{2+}\right]_{i}$-free or $\left[Ca^{2+}\right]_{i}$-containing solutions (Fig. 5).

**Effect of Tempol on methacholine-induced $Ca^{2+}$ release.** Figure 6 compares the effect of Tempol on endothelial $\left[Ca^{2+}\right]_{i}$ responses in aortas from rats fed a HS and LS diet. Acute addition of Tempol ($500 \mu M$) to the tissue bath to determine whether superoxide directly inhibits endothelial $\left[Ca^{2+}\right]_{i}$ signaling did not restore the attenuated increase in $\left[Ca^{2+}\right]_{i}$ in response to methacholine stimulation in aortas from rats fed a HS diet. However, chronic administration of Tempol ($1 \mathrm{nM}$) in the drinking water before and during exposure to a HS diet significantly augmented the methacholine-induced increase in cytosolic $\left[Ca^{2+}\right]_{i}$ in aortas from rats on a HS diet. In contrast, neither acute nor chronic Tempol treatment had a significant effect on $Ca^{2+}$ responses in vessels from animals fed a LS diet.

**Effect of Tempol and ANG II infusion on NO production.** Methacholine significantly increased the slope of DAF-2T fluorescence in aortas obtained from rats fed a LS diet (with and without Tempol in the drinking water; Fig. 7, A and C) but not in aortas from rats fed a HS diet (Fig. 7B). Chronic administration of Tempol in the drinking water (Fig. 7D) and low-dose ANG II infusion (Fig. 7E) restored methacholine-induced NO release in aortas from rats fed a HS diet. Addition of l-NAME ($100 \mu M$) to the tissue bath prevented methacholine-induced increases in DAF-2T fluorescence (Fig. 7, A, C, D, and E), demonstrating that these are due to NO release. The protective effect of ANG II infusion to restore NO production in aortas from rats on a HS diet was prevented by simultaneously infusing the AT1 receptor blocker losartan with the ANG II (Fig. 7F).

**Effect of l-NAME and Tempol on contractile force in aortic rings.** Addition of l-NAME ($100 \mu M$) to the tissue bath caused an increase in contractile force in norepinephrine-contracted aortic rings that was significantly larger in aortas from rats fed a LS diet or maintained on a HS diet with ANG II infusion than in aortas from animals fed a HS diet alone (Fig. 8A). Addition of Tempol ($500 \mu M$) to the tissue bath caused a relaxation of norepinephrine-contracted aortic rings that was significantly...
larger in aortas from rats fed a HS diet than in vessels from rats fed a LS diet or rats fed a HS diet and receiving ANG II infusion (Fig. 8B). Tempol-induced relaxation was completely eliminated by l-NAME (100 μM) in all the groups.

Methacholine-induced vascular relaxation in aortic rings from rats on a HS diet was augmented by both acute and chronic Tempol administration (Fig. 9). Methacholine-induced vascular relaxation of aortas from animals on a LS diet was increased by acute addition of Tempol to the tissue bath and tended to be augmented by chronic administration of Tempol, although the latter effect was not significant (Fig. 9).

DISCUSSION

We have previously demonstrated that NO production is reduced in aortas from rats on a HS diet, leading to impaired relaxation in response to methacholine (35). The results of those studies suggested that elevated levels of superoxide contribute to the reduced NO production in aortas from rats on a HS diet. However, it is unknown whether mechanisms other than the destruction of NO by elevated levels of superoxide may contribute to impaired muscarinic relaxation in aortas of animals on a HS diet.

The present study presents new evidence that short-term increases in dietary salt intake cause a significant reduction in the amplitude of the [Ca^{2+}]_{i} increase that occurs in response to muscarinic receptor activation with methacholine in aortic endothelial cells of normal Sprague-Dawley rats, compared with responses in vessels from rats fed a LS diet or vessels from rats fed a HS diet and receiving low-dose angiotensin infusion to maintain normal circulating levels of ANG II. We also found that a HS diet led to a reduction in the amplitude of the histamine-induced increase in endothelial [Ca^{2+}]_{i} in the aorta, demonstrating that the reduced endothelial [Ca^{2+}]_{i} response in rats on a HS salt diet is not specific to M3 muscarinic receptor activation. In contrast, thapsigargin-induced increases in endothelial [Ca^{2+}]_{i} were virtually identical in the aortas from rats on a HS diet and a LS diet, indicating that filling of inositol 1,4,5-trisphosphate-sensitive Ca^{2+} stores (22) and...
Ca\textsuperscript{2+} entry through store-operated channels are not affected by a HS diet.

Previous studies (18) demonstrated that cerebral arteries from rats on a HS diet fail to relax in response to either direct activation of I prostanoind (IP) receptors with iloprost or direct activation of the \( \alpha \)-subunit of the G\textsubscript{i} protein with cholera toxin, suggesting that the impaired relaxation of resistance arteries of animals on a HS diet may be localized to the receptor-G\textsubscript{i} protein complex. The present study provides new findings indicating that a HS diet also compromises transduction events related to G\textsubscript{q} protein activation or coupling of G\textsubscript{q} proteins to the M\textsubscript{3} or H\textsubscript{1} receptor in the vascular endothelium.

The reduced responses to histamine and methacholine in rats fed a HS diet appear to be due to a fall in circulating levels of ANG II, because they can be fully restored by continuous intravenous infusion of a low dose of the peptide to prevent the ANG II suppression that occurs during exposure of rats to a HS diet (8, 9) (Figs. 1 and 3). These observations are consistent with earlier reports that low-dose ANG II infusion restores impaired vascular relaxation in response to ACh and other vasodilator stimuli in cerebral and skeletal muscle resistance arteries of rats on a HS diet (18, 30, 31). The protective effect of ANG II infusion to restore endothelial Ca\textsubscript{2+} signaling in animals on a HS diet provides additional evidence in support of the hypothesis that ANG II plays a role in maintaining normal vascular relaxation mechanisms, even in the absence of salt-induced elevations in blood pressure.

Previous studies indicate that the resting levels of oxidative stress are elevated in aortas (35) from rats fed a HS diet. Superoxide scavenging with Tempol restores methacholine-induced NO release and prevents methacholine-induced increases in \( \Delta [\text{Ca}\textsubscript{2+}] \) levels in aortas from rats on a HS diet (35), suggesting that elevated levels of superoxide in animals on a HS diet impair endothelium-dependent vasodilator responses by combining with NO to form peroxynitrite, thereby reducing NO bioavailability. However, studies by other investigators (22) suggest that elevated superoxide levels could contribute directly to impaired \([\text{Ca}\textsubscript{2+}]\), signaling in endothelial cells by depleting releasable Ca\textsubscript{2+} pools. Therefore, the present study also explored whether elevated levels of superoxide contribute to the salt-induced attenuation of receptor-mediated \([\text{Ca}\textsubscript{2+}]\), signaling in the endothelium that was demonstrated for the first time in the present experiments.

**Fig. 4.** A: methacholine-induced changes in \([\text{Ca}\textsubscript{2+}]\) in endothelial cells of aortas from rats maintained on a HS diet with saline infusion (open circles) or HS diet with ANG II plus losartan infusion (solid circles). Data are plotted as means for 100–120 cells in 5–6 animals with SE omitted for clarity. B: mean change in endothelial \([\text{Ca}\textsubscript{2+}]\), (in nM) (±SE) during peak and plateau phases of methacholine response in aortas from rats fed HS diet with saline infusion vs. rats fed HS diet and receiving a simultaneous infusion of ANG II and losartan. n/n, Total number of cells per number of rats (20 cells/aorta).

**Fig. 5.** A: changes in endothelial \([\text{Ca}\textsubscript{2+}]\), (in nM) vs. time in thapsigargin-treated aortas in Ca\textsubscript{2+}-containing physiological salt solution (PSS) and in Ca\textsuperscript{2+}-free PSS. Data are plotted as means for 120–160 cells in 6–8 animals with SE omitted for clarity. B: mean change in endothelial \([\text{Ca}\textsubscript{2+}]\), (±SE) in response to thapsigargin in aortas from rats fed LS or HS diet. n/n, Total number of cells per number of rats (20 cells/aorta). There was no significant difference in the response of HS and LS vessels to thapsigargin in either condition.
In this study, acute scavenging of superoxide by addition of Tempol to the tissue bath did not ameliorate the attenuated Ca\textsuperscript{2+} response in aortic endothelial cells from rats on a HS diet, suggesting that elevated levels of superoxide do not affect agonist-induced Ca\textsuperscript{2+} signaling directly in those animals. However, chronic administration of Tempol in the drinking water before and during exposure to a HS diet caused a significant increase in the amplitude of the methacholine-induced [Ca\textsuperscript{2+}]i response in aortic endothelial cells compared with aortas from untreated rats on a HS diet. The reason why chronic, but not acute, Tempol treatment restores Ca\textsuperscript{2+} signaling remains to be determined. One possibility is that continued exposure to elevated levels of superoxide directly damages G protein/receptor-mediated signal transduction mechanisms in aortas from rats on a HS diet, independent of effects on NO availability. However, it is conceivable that destruction of NO by elevated levels of superoxide could also affect [Ca\textsuperscript{2+}]i signaling mechanisms, e.g., by nitration of the receptor via the actions of peroxynitrite formed by combination of NO with superoxide radicals (20).

The ability of chronic Tempol treatment to restore [Ca\textsuperscript{2+}]i signaling does not appear to be due to any ability of Tempol to prevent ANG II suppression with HS diet. In these experiments, chronic Tempol treatment caused a significant increase in plasma ANG II in animals fed a LS diet. The reasons and mechanisms for this increase in plasma ANG II in Tempol-treated rats on a LS diet need to be determined. However, despite this elevation in ANG II levels, methacholine-induced Ca\textsuperscript{2+} release and NO production were not augmented compared with values in untreated animals on a LS diet. More importantly, Tempol treatment did not prevent ANG II suppression by HS diet, and plasma ANG II levels in Tempol-treated rats on a HS diet were not significantly different from those in untreated rats on a HS diet.

An important implication of our finding that chronic administration of Tempol ameliorates the salt-induced reduction of Ca\textsuperscript{2+} signaling in response to M\textsubscript{3} receptor activation is that the enhanced oxidative stress accompanying a HS diet may affect a variety of signal transduction pathways, in addition to NO-dependent vascular relaxation. The latter hypothesis is supported by preliminary studies (17) showing that ACh-in-
duced contraction of intestinal smooth muscle is significantly attenuated in rats on a HS diet and that this impairment can be prevented by low-dose ANG II infusion. The possibility that elevated superoxide levels affect multiple receptor-mediated signal transduction mechanisms is further supported by a recent report (1) demonstrating that impaired dopamine receptor-G protein coupling in the kidney of obese Zucker rats can be prevented by treating the animals with Tempol, whereas antioxidant therapy has no effect on lean Zucker controls.

The results of this study suggest that the ANG II suppression in response to elevated dietary salt intake may be the ultimate culprit leading to increased superoxide levels, defects in receptor-G protein coupling, and impaired vascular relaxation. However, the precise mechanisms by which ANG II suppression leads to these changes remain to be determined. The relationship between suppression of ANG II by a HS diet and increased superoxide levels is particularly intriguing, given the well-known ability of ANG II, acting via AT1 receptor activation, to increase vascular O2\textsuperscript{2-} production by NAD(P)H oxidase (23, 25). In this respect, an especially interesting observation is the report (10) that ANG II infusion and excess salt loading (8% NaCl) both increase superoxide levels in the brain of stroke-prone spontaneously hypertensive rats (SHRSP) and

Fig. 7. Effect of methacholine on slope ratio of 4,5-diaminofluorescein (DAF-2T) fluorescence increases in aortas from rats on LS diet (A), HS diet (B), LS diet with Tempol in drinking water (C), HS diet with Tempol in drinking water (D), HS diet receiving ANG II infusion alone (E), or HS diet receiving both ANG II and losartan (F). L-NAME, N\textsuperscript{\text{-}}-nitro-L-arginine methyl ester. Data are means ± SE; n = 6 rats per group. *Significant difference between control and methacholine treatment within a group (P < 0.05).
that both the salt-induced and ANG II-induced increase in superoxide production can be prevented by AT1 receptor blockade with candesartan (which also dramatically improved survival in the salt loaded SHRSP).

One possible mechanism for increased superoxide levels in the face of ANG II suppression may be the downregulation of antioxidant defense mechanisms in the tissue. This hypothesis is supported by the report of Fukai et al. (6), who demonstrated that ANG II increases extracellular SOD expression in mouse aorta, both in vivo and in organoid culture. Additional support for this hypothesis is provided by the findings of other investigators, who reported that a reduction in extracellular SOD activity contributes to elevated superoxide levels and impaired flow-induced dilation in patients with chronic heart failure (13) and that reduced Cu/Zn SOD activity contributes to elevated superoxide levels and loss of arteriolar NO activity in rats on a HS diet (14). The hypothesis that oxidative stress is lower and NO-dependent relaxation is preserved to a greater extent in aortas from ANG II-infused rats fed a HS diet is supported by the results of our functional studies showing that Tempol causes less relaxation and l-NAME causes a larger contraction in aortas from ANG II-infused animals on a HS diet compared with vessels from rats on a HS diet without ANG II infusion (Fig. 8).

In conclusion, the results of this study provide new evidence that [Ca2+]i signaling in response to methacholine and histamine is impaired in vascular endothelial cells from rats fed a short-term HS diet and that normal control of [Ca2+]i and NO production can be restored by low-dose ANG II infusion acting at the level of the AT1 receptor. An important new finding in this study is that prolonged exposure to increased oxidative stress (blocked by chronic Tempol administration) appears to contribute to the impaired [Ca2+]i signaling in endothelial cells from rats on a HS diet. These alterations in vascular function during exposure to a HS diet may be of substantial interest because of their potential contribution to early elevations in vascular resistance before the onset of hypertension in salt-sensitive individuals.

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