Angiotensin-(1-7) and low-dose angiotensin II infusion reverse salt-induced endothelial dysfunction via different mechanisms in rat middle cerebral arteries

Matthew J. Durand,1 Gábor Raffai,1,2 Brian D. Weinberg,1 and Julian H. Lombard1

1Department of Physiology, Medical College of Wisconsin, Milwaukee, Wisconsin; and 2Institute of Human Physiology and Clinical Experimental Research, Semmelweis University, Budapest, Hungary

Submitted 31 March 2010; accepted in final form 20 July 2010

D-ALA. These findings demonstrate that circulating ANG-(1-7), a Mas receptor agonist AVE0991 exerts effects similar to ANG-(1-7) (36), suggesting that drugs that activate the Mas receptor may have a benefit as cardiovascular therapeutic agents, particularly in diseases characterized by endothelial dysfunction.

The effects of ANG-(1-7) under conditions of altered dietary salt intake are presently unclear. Iyer et al. (12) reported that salt-restricted spontaneously hypertensive rats and (mRen-2)27 renin transgenic hypertensive rats exhibit a striking pressor response when the Mas receptor is blocked by the infusion of the inhibitor [D-Ala(7)]-ANG-(1-7) (D-ALA) or when ANG-(1-7) is eliminated by the infusion of neutralizing antibodies for ANG-(1-7). Because salt restriction stimulates the renin-angiotensin system and elevates circulating ANG II levels (10), the findings of that study are consistent with the proposed counterbalancing action of ANG-(1-7) on the vasoconstrictor effects of ANG II (9, 12, 31). On the other hand, Roks et al. (31) reported that ANG-(1-7) significantly reduced maximal ANG II-induced vasoconstriction in Wistar rats fed a normal or HS diet but that this antagonistic effect of ANG-(1-7) on ANG II...

The ingestion of a HS diet causes the suppression of plasma ANG II levels. This physiological mechanism not only plays a crucial role in excreting the increased sodium load but can also lead to increased oxidant stress (19, 39, 52–53) and impaired nitric oxide (NO)-dependent and -independent vasodilation (2, 20, 39, 45). A chronic intravenous infusion of a pressor dose of ANG II to restore normal circulating levels of ANG II in salt-fed rats restores vascular relaxation, NO release, and endothelial Ca2+ signaling in salt-fed rats and also returns elevated vascular superoxide levels to control (normal salt) values (25, 45, 51–53).

There is also growing interest in the role of the small peptide ANG-(1-7) in cardiovascular regulation. When compared with ANG II, plasma levels of ANG-(1-7) have been reported to be 10–100 times lower in normal human subjects (16) or Sprague-Dawley rats (4). Interestingly, angiotensin-converting enzyme inhibition, which has been shown to be beneficial in restoring endothelial function in patients with cardiovascular disease (21, 38), increases plasma ANG-(1-7) levels 10- to 25-fold, whereas ANG II levels decrease only modestly (4, 16). The activation of the Mas receptor for ANG-(1-7) inhibits ANG II-induced vasoconstriction in human internal mammary arteries (32) and forearm resistance vessels (42), supporting the hypothesis that ANG-(1-7) has vascular protective effects that counteract the vasoconstrictor effects of ANG II. Other studies suggest that Mas receptor activation by ANG-(1-7) improves endothelial function by activating endothelial NO synthase (eNOS) (34) and facilitating NO release (8). The orally active Mas receptor agonist AVE0991 exerts effects similar to ANG-(1-7) (36), suggesting that drugs that activate the Mas receptor may have a benefit as cardiovascular therapeutic agents, particularly in diseases characterized by endothelial dysfunction.

LONG-TERM, FOLLOW-UP STUDIES have shown that salt-sensitive humans are not only more prone to develop hypertension than salt-resistant individuals but also have a higher mortality rate, even if they do not develop hypertension (47). The ingestion of a high-salt (HS) diet leads to a significant reduction in endothelium-dependent flow-induced dilation in young healthy human volunteers (41). The latter finding highlights an additional concern in the ongoing debate about dietary salt intake and cardiovascular disease, in light of a compelling review by Widlansky et al. (48) stressing that endothelial dysfunction, similar to that occurring in animal models of elevated dietary salt intake, is a powerful predictor of adverse cardiovascular events, including death, in humans (48).

Address for reprint requests and other correspondence: J. H. Lombard, Dept. of Physiology, Medical College of Wisconsin, 8701 Watertown Plank Rd., Milwaukee, WI 53226 (e-mail: jlombard@mcw.edu).
induced constriction disappeared when the animals were fed a low-salt diet.

Studies suggesting that Mas receptor activation improves endothelial function (8, 34) naturally raise the question of whether ANG-(1-7) will alleviate the severe endothelial dysfunction that occurs in arteries of normotensive animals fed a HS diet (2, 20, 45) and whether any protective effects of ANG-(1-7) are mediated through the Mas receptor alone or if the ANG II types 1 (AT1) and 2 (AT2) receptors play a role in independent of ANG-(1-7) and the Mas receptor.

In the present study, we also determined the response of isolated middle cerebral arteries (MCAs) from rats fed a normal salt (NS; 0.4% NaCl) diet to an acute addition of ANG-(1-7) and evaluated the contributions of the Mas receptor, AT1 receptor, and AT2 receptor to these responses. The final goal of the study was to determine whether the protective effect of low-dose ANG II infusion to restore endothelium-dependent vasodilation in cerebral arteries of salt-fed rats could be prevented by Mas receptor blockade or whether the ability of subpressor ANG II infusion to reverse endothelial dysfunction in salt-fed rats is independent of ANG-(1-7) and the Mas receptor.

**MATERIALS AND METHODS**

**Experimental groups.** Male Sprague-Dawley rats between 8–10 wk of age were used for all experiments, and all protocols were approved by the Medical College of Wisconsin Institutional Animal Care and Use Committee. The animals were fed either a NS (0.4% NaCl; Dyets; Bethlehem, PA) diet or switched to a HS (4.0% NaCl; Dyets) diet for 3 days before the isolated vessel experiments and were given water to drink ad libitum. The animals receiving chronic intravenous infusions of ANG-(1-7) were pharmacological inhibitors of the AT1 receptor (losartan), the AT2 receptor (PD-123319), or the Mas receptor [D-Ala(7)]-ANG-(1-7) (D-ALA), and animals receiving a chronic intravenous infusion of low-dose ANG II ± losartan or D-ALA were anesthetized with an intramuscular injection containing ketamine, acepromazine, and anadine as described in Experimental groups. Mean arterial pressures were determined via carotid artery catheterization. To test the efficiency of AT1 receptor blockade in losartan-infused animals, a bolus injection of a pressor dose of ANG II (50 ng/kg) was given intravenously (29), which causes an increase in arterial pressure of ∼45 mm Hg in the absence of the inhibitor (29). Losartan-treated animals with an observed blood pressure increase >15 mm Hg in response to the ANG II bolus were excluded from the study. The brain was then removed and immersed in physiological salt solution (PSS) having the following ionic composition: (in mM) 119.0 NaCl, 4.7 KCl, 1.6 CaCl2, 1.18 NaH2PO4, 1.17 MgSO4, 24.0 NaHCO3, 5.5 d-glucose, and 0.03 EDTA. MCAs were carefully dissected under a dissecting microscope (Leica; Buffalo, NY), cannulated at the proximal and distal ends using glass micropipettes (80–120 μm; FHC; Brunswick, ME), and extended to their approximate in situ length. Side branches were ligated to prevent leaks and to allow the vessel to be pressurized. The vessel was continuously perfused with PSS (37°C) and equilibrated with 21% O2-5% CO2-74% N2 gas mixture, and the intraluminal pressure was maintained at 80 mm Hg to approximate in vivo conditions. The internal diameter was measured using television microscopy and a video micrometer (model IV-550, FOR-A; Tokyo, Japan). Vessels lacking intrinsic resting tone were excluded from analysis.

Responses to vasodilator agonists and Ca2+-free solution. To determine the acute effects of ANG-(1-7) on cerebral arteries, MCAs were isolated from rats fed a NS diet and the arterial diameter was measured during an acute addition of ANG-(1-7) (10−10–10−5 M) to the tissue bath. Experiments were conducted before and after the addition of selective antagonists of the AT1 receptor (losartan; 10 μM), the AT2 receptor (PD-123319; 1 μM), and the Mas receptor (D-ALA; 10 μM) to the superfusate for 20 min before the addition of ANG-(1-7) to the tissue bath.

For studies investigating the effect of chronic infusion of ANG-(1-7) on vascular relaxation, diameter changes in response to acetylcholine (ACH; 10−10–10−5 M) and sodium nitroprusside (SNP; 10−12–10−4 M) were assessed in MCAs from each group of rats. In another series of experiments, we determined whether the protective effect of chronic low-dose ANG II infusion to restore vascular relaxation in response to ACh in HS-fed animals was distinct from the effect of ANG-(1-7) to restore vascular relaxation by measuring vascular responses to ACh in HS-fed rats receiving chronic intravenous infusion of ANG-(1-7) ± the AT1 receptor blocker losartan, as described previously (25, 44) or ± the Mas receptor antagonist D-ALA.

ACh was used as the classic indicator of endothelium-dependent dilation mediated via NO (39). Vessel responses to SNP were included as a control to demonstrate that arteries from all the experimental groups dilated in response to the NO donor and that any differences in the experimental groups were not due to a nonspecific loss of NO sensitivity in the arteries. To verify that the restored vasodilator response to ACh was NO dependent in ANG-(1-7)-
infused rats, MCAs from a separate group of HS-fed rats receiving ANG-(1-7) infusion were preincubated with the NO synthase inhibitor N^o-nitro-L-arginine methyl ester (100 μM) for 20 min before measuring the response of the arteries to an addition of ACh to the tissue bath.

At the end of the experiment, the resting tone and maximum diameter of the artery (Table 1) were assessed by superfusing the vessel with Ca^{2+}-free PSS having the following composition: (in mM) 119.0 NaCl, 4.7 KCl, 1.18 NaH_2PO_4, 1.17 MgSO_4, 24.0 NaHCO_3, 5.5 d-glucose, and 0.03 EDTA. Active resting tone (in %) was calculated as [(D_{max} - D_{rest})/D_{max}] × 100, where D_{max} is the maximum diameter in Ca^{2+}-free solution and D_{rest} is the resting control diameter.

Assessment of vascular superoxide levels with dihydroethidium. On the day of the experiment, basilar arteries were removed from the ventral surface of the brain, cleaned of connective tissue, and incubated in warm PSS bubbled with a 21% O_2-5% CO_2-74% N_2 gas mixture for 1 h. Basilar arteries were used to assess vascular superoxide levels because this vessel has a slightly larger diameter than the MCA and can be cleaned of connective tissue more easily, allowing for better cross-sectioning of the vessel. However, similar to other cerebral resistance arteries, the responses of in vivo basilar arteries to ACh are NO dependent (22–24), making this vessel an appropriate surrogate for the assessment of endothelial function in the cerebral circulation.

After incubation in warm PSS, dihydroethidium (5 μM) was applied to the arteries for 15 min in the dark. In the presence of superoxide, membrane permeable dihydroethidium undergoes oxidation to form the membrane impermeable compound 2-hydroxyethidium (50). This positively charged compound binds to DNA and emits a red fluorescence that is proportional to the amount of superoxide that is present. The arteries were washed three times to remove excess dihydroethidium and frozen at −80°C for 1 h in a block of optimum cutting temperature compound (Tissue-Tek; Sakura Finetek USA; Torrance, CA). The frozen blocks were then sliced in 10-μm transverse cross sections at −20°C with a Thermo Scientific HM 550 cryostat (Waltham, MA). The tissue slices were applied to glass microscope slides, mounted with an aqueous mounting medium containing antifading agents (Gel/Mount; Biomeda; Foster City, CA), covered with 1.5-mm coverslips, and allowed to dry for 30 min in the dark. After the tissue slices were dried, bright-field and fluorescent images of multiple cross sections of each individual basilar artery were taken using a Nikon Eclipse TS100 (Tokyo, Japan) microscope equipped with a ×20 objective, a 540-nm excitation filter and 605-nm emission filter (Chroma Technology; Bellows Falls, VT). Digital images were captured using a QImaging Retiga-2000R digital camera (Surrey, BC, Canada) and Metamorph imaging and analysis software (Universal Imaging; Downingtown, PA). Images were quantified by using the ImageJ software to subtract background fluorescence values from free hand-selected cross sections of the basilar artery. The quantified values from all images of a single basilar artery were averaged together to give a single brightness value for that artery.

Statistical methods. Data are presented as means ± SE. For comparisons of two groups, an unpaired Student’s t-test was used. For all concentration-response curves, the differences between multiple groups at each concentration were determined using a one-way ANOVA. The differences between individual means following ANOVA were evaluated using a post hoc Student-Newman-Keuls or Holm-Sidak test. A probability level of P < 0.05 was considered to be statistically significant.

RESULTS

Arterial blood pressure, vessel diameter, and active tone. Table 1 summarizes mean arterial pressure, resting diameter, maximum diameter, and active resting tone in MCAs for the various experimental groups. Consistent with previous studies by our laboratory and others (2, 25), arterial blood pressure and active resting tone in the MCAs were unaffected by a HS diet. ANG-(1-7) infusion did not cause a significant change in mean arterial pressure and had no effect on active resting tone of isolated MCAs in any of the experimental groups. ANG II infusion or ANG II infusion with d-ALA had no effect on mean arterial pressure, whereas a coinfusion of losartan with ANG II led to the expected decrease in blood pressure in the infused animals. An infusion of ANG II, either alone or in the presence of losartan or d-ALA, did not have a significant effect on the resting tone of isolated MCAs in the present experiments. The failure of any of the treatments to affect active tone in these experiments is important because it shows that any changes in the magnitude of vascular relaxation in the different experimental groups are independent of differences in resting tone, i.e., that they do not reflect a preexisting constriction of the artery.

Responses of MCAs to acute addition of ANG-(1-7). Isolated MCAs from rats fed a NS diet dilated in response to an acute addition of ANG-(1-7) (10^{-10}–10^{-5} M) to the tissue bath (Fig. 1). The relaxation of the isolated MCAs in response to ANG-(1-7) was blocked by incubating the vessel with the Mas receptor.

Table 1. Mean arterial blood pressures and middle cerebral artery diameters from all animals used in this study

<table>
<thead>
<tr>
<th>Experimental Group</th>
<th>MAP, mmHg</th>
<th>Resting Diameter, μm</th>
<th>Maximum Diameter, μm</th>
<th>Active Tone, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal salt</td>
<td>NA</td>
<td>154 ± 4.9 (8)</td>
<td>263 ± 3.7 (8)</td>
<td>42 ± 1.9 (8)</td>
</tr>
<tr>
<td>Normal salt-d-ALA acute</td>
<td>NA</td>
<td>170 ± 8.4 (6)</td>
<td>253 ± 3.0 (6)</td>
<td>33 ± 3.2 (6)</td>
</tr>
<tr>
<td>Normal salt-losartan acute</td>
<td>NA</td>
<td>161 ± 5.9 (6)</td>
<td>254 ± 3.9 (6)</td>
<td>37 ± 1.7 (6)</td>
</tr>
<tr>
<td>Normal salt-PD-123319 acute</td>
<td>NA</td>
<td>163 ± 8.5 (6)</td>
<td>254 ± 6.6 (8)</td>
<td>36 ± 3.1 (6)</td>
</tr>
<tr>
<td>Normal salt-saline infused</td>
<td>93 ± 4.9 (7)</td>
<td>156 ± 9.6 (8)</td>
<td>256 ± 3.3 (7)</td>
<td>40 ± 4.3 (7)</td>
</tr>
<tr>
<td>Normal salt-ANG-(1-7) infused</td>
<td>96 ± 3.5 (6)</td>
<td>147 ± 9.3 (7)</td>
<td>254 ± 8.6 (7)</td>
<td>43 ± 1.9 (7)</td>
</tr>
<tr>
<td>High salt-ANG-(1-7) infused</td>
<td>110 ± 2.3 (9)</td>
<td>152 ± 5.0 (13)</td>
<td>245 ± 4.9 (13)</td>
<td>37 ± 2.5 (13)</td>
</tr>
<tr>
<td>High salt-ANG-(1-7) + d-ALA infused</td>
<td>105 ± 3.8 (13)</td>
<td>148 ± 7.1 (9)</td>
<td>251 ± 6.5 (7)</td>
<td>39 ± 1.9 (7)</td>
</tr>
<tr>
<td>High salt-ANG-(1-7) + losartan infused</td>
<td>113 ± 3.8 (6)</td>
<td>146 ± 5.9 (6)</td>
<td>247 ± 4.1 (6)</td>
<td>41 ± 2.4 (6)</td>
</tr>
<tr>
<td>High salt-ANG-(1-7) + PD-123319 infused</td>
<td>91 ± 5.4 (8)</td>
<td>151 ± 6.1 (8)</td>
<td>250 ± 3.6 (8)</td>
<td>40 ± 2.2 (8)</td>
</tr>
<tr>
<td>High salt-ANG-(1-7) + d-ALA infused</td>
<td>100 ± 3.2 (10)</td>
<td>148 ± 7.2 (10)</td>
<td>254 ± 6.5 (9)</td>
<td>42 ± 2.0 (9)</td>
</tr>
<tr>
<td>High salt-ANG-(1-7) + losartan infused</td>
<td>96 ± 6.2 (5)</td>
<td>143 ± 4.3 (6)</td>
<td>234 ± 12.7 (6)</td>
<td>38 ± 2.1 (6)</td>
</tr>
<tr>
<td>High salt-ANG II + d-ALA infused</td>
<td>90 ± 3.5 (4)</td>
<td>149 ± 5.4 (6)</td>
<td>229 ± 6.4 (6)</td>
<td>35 ± 2.6 (6)</td>
</tr>
<tr>
<td>High salt-ANG II + losartan infused</td>
<td>76 ± 2.7 (4)*</td>
<td>125 ± 11.7 (6)</td>
<td>238 ± 4.3 (6)</td>
<td>47 ± 5.0 (6)</td>
</tr>
</tbody>
</table>

Values are means ± SE; n, number (shown in parentheses) of animals for MAP and arteries (1 per animal) for resting diameter, maximum diameter, and active tone. MAP, mean arterial pressure; NA, not applicable. *P < 0.05, high salt-ANG II + losartan infused vs. high salt-saline infused, high salt-ANG-(1-7) infused, high salt-ANG-(1-7) + d-ALA infused, high salt-ANG-(1-7) + PD-123319 infused.
Fig. 1. Response of isolated middle cerebral arteries from rats fed a normal salt diet to ANG-(1-7) (10⁻¹⁰–10⁻⁵ M; n = 8) before and after acute addition of the Mas receptor antagonist d-ALA (10 µM; n = 6), the AT₁ receptor antagonist losartan (10 µM; n = 6), or the AT₂ receptor antagonist PD-123319 (1 µM; n = 6) to the tissue bath. *P < 0.05, inhibitor vs. control.

Effect of chronic ANG-(1-7) infusion on vascular responses to ACh and SNP. The effect of a chronic (3 days) intravenous infusion of ANG-(1-7) (4 ng·kg⁻¹·min⁻¹) on vessel responses to ACh (10⁻¹⁰–10⁻⁵ M) and SNP (10⁻¹²–10⁻⁴ M) are summarized in Figs. 2 and 3. In those experiments, a chronic infusion of ANG-(1-7) restored ACh-induced dilation, which was absent in the MCAs of rats fed a HS diet (Fig. 2A). However, ANG-(1-7) infusion had no effect on the vasodilator responses to SNP in rats fed a HS diet (Fig. 2B), demonstrating that any reduction in ACh-induced dilation in the different groups reflected a loss of the endothelium-dependent component of dilation rather than a loss of vessel sensitivity to NO. ANG-(1-7) infusion had no effect on the vasodilator responses to ACh or SNP in rats fed a NS diet (Fig. 3, A and B). The restored dilation in response to ACh in rats fed a HS diet and receiving an intravenous infusion of ANG-(1-7) was eliminated by inhibiting NO synthase with N⁶-nitro-L-arginine methyl ester, showing that the restored dilation is NO dependent (Fig. 4).

Effect of Mas receptor blockade, AT₁ receptor blockade, and AT₂ receptor blockade on the restoration of ACh-induced dilation by chronic low-dose ANG II infusion in rats fed HS diet. The protective effect of ANG-(1-7) infusion to restore vasodilation in response to ACh in rats fed a HS diet was prevented by a simultaneous infusion of the Mas receptor antagonist d-ALA but not by a coinfusion of losartan (Fig. 5, A and B). A chronic infusion of the AT₂ receptor antagonist PD-123319 also inhibited the ability of ANG-(1-7) to restore ACh-induced dilation in rats fed a HS diet (Fig. 5C), indicating that the restoration of ACh-induced vasodilation by ANG-(1-7) infusion in salt-fed rats is mediated via the Mas receptor acting in conjunction with the AT₂ receptor, with no influence of the AT₁ receptor.
In contrast, a chronic infusion of ANG-(1-7) had no effect on vascular superoxide levels in rats fed a NS diet (Fig. 8).

**DISCUSSION**

The major goal of this study was to determine whether the chronic activation of the Mas receptor by ANG-(1-7) infusion would ameliorate the impaired endothelium-dependent vascular relaxation that occurs in the MCAs of normotensive Sprague-Dawley rats fed a HS diet. An additional goal was to use isolated MCAs from rats fed a NS diet to verify that the Mas receptor can play a role in regulating vascular function in cerebral resistance arteries. We also evaluated the role of the AT1 and AT2 receptors in mediating the acute and chronic effects of ANG-(1-7) because existing studies in the literature have reached widely varying conclusions regarding the role of the Mas receptor and the AT1 and AT2 receptors in mediating the response of diverse physiological systems to ANG-(1-7). For example, some studies indicate that the effects of ANG-(1-7) are mediated solely via the Mas receptor and are unaffected by either AT1 or AT2 receptor blockade (3, 40), whereas others indicate that the physiological effects of ANG-(1-7) involve either the AT1 receptor (15, 27), the AT2 receptor (31, 43), or both (6) acting in conjunction with the Mas receptor. The final goal of this study was to determine whether Mas receptor blockade prevents the previously reported protective effect of low-dose ANG II infusion to restore endothelium-dependent vascular relaxation in MCAs of rats fed a HS diet or whether the protective effect of ANG-(1-7) infusion to reverse salt-induced endothelial dysfunction is separate and distinct from that of low-dose ANG II infusion.

In these experiments, we showed that ANG-(1-7) can dilate MCAs from animals fed a NS diet (Fig. 1), supporting a potential role for this peptide in the regulation of vascular function in cerebral resistance arteries. The present study also shows that both the acute actions of ANG-(1-7) to dilate resting MCAs and the ability of chronic ANG-(1-7) infusion to restore ACh-induced dilation in cerebral arteries of salt-fed rats are insensitive to AT1 receptor blockade with losartan but are strongly inhibited by the Mas receptor antagonist D-ALA and by the AT2 receptor antagonist PD-123319. The observation that both of these receptors inhibit the vasodilator response to ANG-(1-7) suggests that ANG-(1-7) acutely acts through both the Mas receptor and the AT2 receptor to produce vascular relaxation and to mediate endothelial protective mechanisms in cerebral arteries of HS-fed rats, whereas the AT1 receptor has no effect on those responses. By contrast, the protective effect of low-dose ANG II infusion to restore endothelium-dependent relaxation in response to ACh was eliminated by a simultaneous coinfusion of losartan as previously reported (44) but was completely unaffected by a coinfusion of the Mas receptor antagonist D-ALA. These results show that the effect of ANG-(1-7) infusion to reverse salt-induced endothelial dysfunction is separate and distinct from that of low-dose ANG II infusion.

Several possible scenarios could explain the permissive role of the AT2 receptors in the ANG-(1-7)-mediated signaling observed in these experiments. It seems unlikely that ANG-(1-7) exerts biological activity by directly binding to the AT2 receptor, because previous studies have shown that ANG-(1-7) binds the AT2 receptor with nearly 6,000-fold less affinity than ANG II (33). It is also unlikely that PD-123319 directly interferes with the Mas receptor block. Instead, the AT2 receptor antagonist PD-123319 may be functioning as an agonist for the AT1 receptor. This explanation is consistent with previous studies showing that ANG-(1-7) can mediate the acute dilation of MCAs from salt-fed rats by activating the AT2 receptor (31, 43). It is also unlikely that the AT1 receptor has a role in mediating the response of MCAs to ANG-(1-7) because the protective effect of ANG-(1-7) infusion to reverse salt-induced endothelial dysfunction is separate and distinct from that of low-dose ANG II infusion.

**Fig. 4.** Response to acetylcholine (10^{-5} to 10^{-10}) M in isolated middle cerebral arteries from rats fed a high-salt diet (n = 7) receiving a 3-day infusion of ANG-(1-7) (4 ng·kg^{-1}·min^{-1}; n = 7) or 3-day isotonic saline infusion (n = 5 to 6).

**Fig. 3.** Response to acetylcholine (10^{-10} to 10^{-5}) M (A) and sodium nitroprusside (10^{-5} to 10^{-4}) M (B) in isolated middle cerebral arteries from rats fed a normal salt diet (n = 6) vs. normal salt-fed rats receiving a chronic 3-day infusion of ANG-(1-7) (4 ng·kg^{-1}·min^{-1}; n = 7) or 3-day isotonic saline infusion (n = 5 to 6).
receptor, because studies in Mas-transfected cells have shown that PD-123319 is unable to significantly displace ANG-(1-7) binding to the Mas receptor (37). Despite the extremely low binding affinity of ANG-(1-7) for the AT2 receptor, numerous studies have shown that many biological actions of ANG-(1-7) are mediated, in part, through the AT2 receptor. For example, a study by Roks et al. (31) showed that ANG-(1-7) decreased the magnitude of ANG II-induced constriction in aortic rings of rats fed a NS diet and that this effect could be blocked by either D-ALA or PD-123319 (31). Other studies have shown that ANG-(1-7) induces both arachidonic acid and prostaglandin release in vascular smooth muscle cells, both of which are strongly inhibited by AT2 receptor antagonism (13, 26). While the exact mechanism for the blockade of ANG-(1-7) responses by PD-123319 is unclear, accumulating evidence suggests that the Mas receptor forms functional dimers with the ANG II receptors (5–6, 14, 30) and that there is likely a functional interaction such as receptor cross talk or a permissive role for the AT2 receptor in the action of the Mas receptor (6). In support of this hypothesis, a study by Hansen et al. (11) showed that ANG-(1-7) specifically promotes the activation of the AT2 receptor-linked G protein, suggesting a possible mechanism for functional cross talk between the Mas receptor and AT2 receptor.

The restoration of ACh-induced dilation by chronic intravenous infusion of ANG-(1-7) in rats fed a HS diet is especially significant since it indicates that the Mas receptor activation has a powerful endothelial protective effect in light of the dramatic reduction or complete abrogation of endothelium-dependent dilation to ACh that occurs in untreated rats fed HS diet (Fig. 2A) (20, 39, 44–45). As such, the findings of the present study are consistent with those of Faria-Silva et al. (8), who showed that Mas receptor stimulation by ANG-(1-7) improves endothelial function in normotensive rats and with other studies showing that Mas receptor activation activates eNOS and stimulates endothelial NO release (34). Taken together, these findings suggest that agents which activate the Mas receptor could have important protective effects to alleviate the endothelial dysfunction that occurs during elevated dietary salt intake (2, 20, 45, 52–53).
A HS diet leads to increased vascular superoxide levels and to reduced NO availability during resting conditions and during agonist-induced activation (19, 51–54). Previous studies from our laboratory and others suggest that superoxide dismutase (SOD) expression and SOD activity are reduced by HS diet, whereas the activity of pro-oxidant enzymes is unchanged (17–18, 25). While it remains to be determined whether increased oxidative stress in arteries of rats fed HS diet is due to an increased production of superoxide, a reduced ability to scavenge superoxide, or a combination of those factors, the enhanced levels of superoxide present in arteries of salt-fed rats lead to reduced NO availability, impaired endothelium-dependent dilation, impaired G protein signaling, and reduced Ca\(^{2+}\) signaling in the endothelial cells (51–54). These alterations undoubtedly play a crucial role in the endothelial dysfunction that occurs in aortas (53) and resistance arteries (52) of salt-fed rats.

Mas\(^{-/-}\) mice suffer from endothelial dysfunction caused by increased gp91\(^{phox}\) expression, reduced NO levels, decreased eNOS expression, decreased SOD activity, and reduced catalase activity (49). In regard to the physiological role of ANG-(1-7) in preserving endothelial function and regulating oxidative stress in the vasculature, studies in cultured endothelial cells have suggested that ANG-(1-7) treatment can improve endothelial function by increasing both eNOS activity and NO production (34). Other studies suggest that ANG-(1-7) treatment reduces NADPH oxidase activity in the kidney (1).

The present study shows that chronic stimulation of the Mas receptor by ANG-(1-7) restores NO-dependent vasodilation to ACh in rats fed a HS diet (Fig. 4) and that this restored dilation...
is accompanied by a reduction in vascular superoxide levels (Fig. 7). By contrast, vascular superoxide levels were unaffected by ANG-(1-7) infusion arteries from rats maintained on a low-salt diet (Fig. 8). Taken together, these observations indicate that ANG-(1-7) restores endothelium-dependent dilation to ACh in salt-fed rats by reducing the elevated vascular superoxide levels that are present with a HS diet relative to the controls maintained on a low-salt diet (52–53). While the exact cellular mechanisms by which chronic ANG-(1-7) treatment lowers vascular superoxide levels and preserves NO-dependent dilation in animals fed HS diet remain to be determined, future studies addressing this question will be vitally important to further understand the role of ANG-(1-7) in preserving endothelial function.

**Perspectives.** The present study shows that Mas receptor activation by ANG-(1-7) has a powerful protective effect to maintain endothelium-dependent vasodilation during conditions of elevated dietary salt intake, which eliminates or drastically impairs the relaxation of resistance arteries and other vessels to multiple vasodilator stimuli, even in the absence of an elevation in arterial blood pressure (2, 20, 52–53). As such, the results of this study suggest that pharmacological agonists targeting the Mas receptor may be effective in alleviating the endothelial dysfunction that occurs during elevated dietary salt intake and other pathophysiological conditions associated with endothelial dysfunction. This action has potential therapeutic value in light of clinical studies showing 1) higher mortality rates in salt-sensitive individuals versus salt-resistant counter-
part (46), 2) impaired endothelial function in healthy normotensive humans ingesting a HS diet (41), 3) the value of endothelial dysfunction as a predictor of adverse cardiovascular events including death (48), and 4) the potential value of assessing endothelial function to guide therapeutic approaches to cardiovascular disease (48).

ACKNOWLEDGMENTS

We thank Sarah Balus and Lynn Dondlinger for chronically instrumenting the animals used in this study.

GRANTS

This work was supported by National Heart, Lung, and Blood Institute Grants HL-65289, HL-72920, and HL-090206 and American Heart Association Midwest Affiliate Predoctoral Fellowship 0815532G and Postdoctoral Fellowship 0920116G.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

REFERENCES


