Angiotensin-(1–7) Selectively Induces Relaxation and Modulates Endothelium-Dependent Dilation in Mesenteric Arteries of Salt-Fed Rats

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Key Words

Angiotensin-(1–7) · Endothelium · Mas receptor · Renin-angiotensin system · Dietary salt · Vascular dysfunction · Vasodilatation · Angiotensin II

Abstract

This study investigated the acute effects of angiotensin-(1–7) and AVE0991 on active tone and vasodilator responses to bradykinin and acetylcholine in isolated mesenteric arteries from Sprague-Dawley rats fed a high-salt (HS; 4% NaCl) versus a normal salt (NS; 0.4% NaCl) diet. Angiotensin-(1–7) and AVE0991 elicited relaxation, and angiotensin-(1–7) unmasked vasodilator responses to bradykinin in arteries from HS-fed rats. These effects of angiotensin-(1–7) and AVE0991 were inhibited by endothelium removal, A779, PD123319, HOE140 and L-NAME. Angiotensin-(1–7) also restored the acetylcholine-induced relaxation that was suppressed by the HS diet. Vasodilator responses to bradykinin and acetylcholine in the presence of angiotensin-(1–7) were mimicked by captopril and the AT₂ receptor agonist CGP42112 in arteries from HS-fed rats. Thus, in contrast to salt-induced impairment of vascular relaxation in response to vasodilator stimuli, angiotensin-(1–7) induces endothelium-dependent and NO-mediated relaxation, unmasks bradykinin responses via

activation of mas and AT₂ receptors, and restores acetylcholine-induced vasodilation in HS-fed rats. AT₂ receptor activation and angiotensin-converting enzyme (ACE) inhibition shared the ability of angiotensin-(1–7) to enhance bradykinin and acetylcholine responses in HS-fed rats. These findings suggest a therapeutic potential for mas and/or AT₂ receptor activation and ACE inhibition in restoring endothelial function impaired by elevated dietary salt intake or other pathological conditions.

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Introduction

The small peptide angiotensin-(1–7) triggers active regional vasodilatation causing decreases in total peripheral resistance [1] and a dose-dependent reduction of arterial blood pressure [2] when infused intravenously at the whole animal level. Angiotensin-(1–7) also relaxes various arteries and arterioles from different species in vivo [3, 4] and in vitro [5–7]. From a therapeutic perspective, angiotensin-(1–7) is potentially a very important peptide because of its ability to antagonize many of the known deleterious actions of angiotensin II [7, 8].

The major enzymatic route of angiotensin-(1-7) formation is from its precursor angiotensin II by the angiotensin-converting enzyme (ACE) homologue ACE2 [9, 10]. The diverse physiological actions of angiotensin-(1-7) are transduced through its specific G protein-coupled receptor mas [11-13], which can be mimicked by the nonpeptide mas receptor agonist AVE0991 [12, 14], and the effects of angiotensin-(1-7) and AVE0991 can be selectively inhibited by the mas receptor antagonist A779 [3-5, 7, 11-20]. However, a number of studies indicate that AT₂ and BK₂ receptors may also contribute to the physiological effects of angiotensin-(1-7)/mas receptor activation [5-7, 12, 14, 18, 20, 21].

In addition to its direct cardiovascular effects, one of the unique roles of angiotensin-(1-7) is to potentiate the hypotensive [22, 23] and vasodilator [3, 4, 6, 7, 24-26] effects of bradykinin, which are attributed to its ability to inhibit ACE [7, 8, 22, 24, 25]. In a similar fashion, ACE inhibitors not only reduce angiotensin II formation, but can also augment the effects of angiotensin-(1-7) and bradykinin because ACE enzymatically degrades both these peptides [7].

Increased oxidative stress reduces NO bioavailability and causes endothelial dysfunction in the vessel walls of salt-insensitive (for blood pressure) species fed a high-salt (HS) diet [5, 20, 27-31]. The heart and blood vessels are important targets for the formation and the action of angiotensin-(1-7) because ACE2 and mas receptors are present in the vessel wall [32]. Previous studies have shown that chronic infusion of a low dose of angiotensin-(1-7) ameliorates endothelial dysfunction in the arteries of rats fed an HS diet [5, 20]. Because angiotensin-(1-7) receptor activation has been reported to activate eNOS and stimulate endothelial NO release [11, 14, 16, 18], it is possible that even acute mas receptor activation may ameliorate endothelial dysfunction that occurs under pathological conditions [5, 20, 27-31, 33, 34].

The Sprague-Dawley rat is a valuable experimental model with which to study salt-induced vascular dysfunction because these animals exhibit dramatically impaired relaxation to multiple vasodilator stimuli when the rats are fed an HS diet (4.0% NaCl) versus a normal salt (NS) diet (0.4% NaCl) without a salt-induced increase in blood pressure [20, 28, 30]. The goal of the present study was to investigate the mechanisms of: (1) the direct vascular effects of mas receptor activation on vascular tone, and (2) the indirect effects of angiotensin-(1-7) on the responses to endothelium-dependent and independent vasodilators in isolated mesenteric resistance arteries from Sprague-Dawley rats fed NS and HS diets.

Methods

Experimental Animals

The present study used male Sprague-Dawley rats (8-10 weeks old) fed NS (0.4% NaCl) or changed to HS (4% NaCl) diets for 3-6 days, with water to drink ad libitum. On the day of the experiment, the rats were anesthetized with an intraperitoneal injection of ketamine (75 mg/kg), acepromazine (2.5 mg/kg), and Anased (10 mg/kg), and blood pressure was measured by direct cannulation of the carotid artery. All experimental protocols were approved by the Medical College of Wisconsin IACUC.

Evaluation of Mesenteric Vascular Reactivity

Mesenteric resistance arteries feeding the small intestine (~300 µm in diameter) were isolated and cannulated in a tissue culture myograph system (Danish Myo-Technology, Aarhus, Denmark). The arteries were incubated at 37°C and 75 mm Hg intraluminal pressure for 60 min while continually perfusing and superfusing the vessel with PSS (physiological salt solution) equilibrated with a 21% O₂/5% CO₂/74% N₂ gas mixture. The steady state outer diameter of the vessels was measured using video microscopy. The PSS used in these experiments had the following composition (in mM): 119 NaCl, 4.7 KCl, 1.17 MgSO₄, 1.6 CaCl₂, 1.18 NaH₂PO₄, 24 NaHCO₃, and 0.03 Na₂-EDTA.

The arteries were precontracted with an EC₅₀ concentration of norepinephrine (4 µM) and their responses to vasodilator agonists were evaluated by adding increasing concentrations of: (1) angiotensin-(1-7); (2) AVE0991, (3) bradykinin, and (4) acetylcholine (all in 10⁻¹⁰-10⁻⁵ M concentrations), or (5) the NO donor diethylenetriamine NONOate (DETA NONOate; 10⁻⁶-10⁻³ M) to the tissue bath. Bradykinin, acetylcholine and DETA NONOate responses were also measured in the presence of angiotensin-(1-7) (10⁻⁶ M), captopril (10⁻⁵ M), and the AT₂ receptor agonist CGP-42112 (10⁻⁶ M) in the tissue bath. Concentration-dependent responses to the various vasodilators alone or in combination with 10⁻⁶ M angiotensin-(1-7) were also measured after removal of the endothelium by air bolus perfusion, or in the presence of the NOS inhibitor L-NAME (10⁻⁴ M), the AT₂ receptor antagonist PD123319 (10⁻⁵ M), the mas receptor antagonist A779 (10⁻⁵ M), or the BK₂ receptor blocker HOE140 (2 × 10⁻⁷ M).

Real-Time PCR Analysis of the Expression of ACE2 mRNA and mRNA for the AT₁, AT₂ and Mas Receptors

Harvested vessel samples were placed in RNeasySecure™ Reagent (Applied Biosystems, Foster City, Calif., USA) and were homogenized in Lysing Matrix D tubes containing ice-cold Trizol Reagent (Invitrogen, Carlsbad, Calif., USA) at approximately 1 ml/100 mg of tissue by FastPrep-24 homogenizer (MP Biomedicals, Solon, Ohio, USA). Chloroform (200 µl/ml) was added to the homogenates, vortexed and spun at 20,000 rcf (relative centrifugal force) for 15 min at 4°C. The upper layer was decanted and an equal volume of isopropanol was added, vortexed, and stored at room temperature for 10 min to precipitate the RNA. The samples were spun at 20,000 rcf for 10 min at 4°C. The pellet was washed twice with DEPC-treated ice-cold 70% EtOH and dried in the tubes. Isolated RNA was dissolved in 20 µl of DEPC-treated water and stored at -80°C. RNA concentrations were determined using a Biomate spectrophotometer (Thermo Scientific, Waltham, Mass., USA) measuring the 260-nm absorbance (A260) of the diluted samples. The RNA concentration (µg/µl) was calculated as: (A260 × 40 ×

dilution factor)/1,000. The RNA quality and integrity was analyzed using an Agilent 2,100 Bioanalyzer and RNA 6,000 Nano chip kit (Agilent, Foster City, Calif., USA). Only high-quality RNA samples (RNA integrity number >8) were used in the experiments.

An AffinityScript QPCR cDNA Synthesis Kit (Stratagene, La Jolla, Calif., USA) was used to make DNA from the total RNA samples. Reverse transcriptase (RT) reactions were performed with the mixture of 3 µg of RNA sample, 10 µl of Master Mix (2×), 3 µl of random primers (0.1 µg/µl) and 1 µl of AffinityScript RT/RNase Block Enzyme Mixture diluted to 20 µl with RNase-free H₂O. No RT control reactions were performed by substituting the enzyme with H₂O. RT reactions were performed in a thermal cycler (Perkin Elmer, Ramsey, Minn., USA) by incubating the samples at 25°C for 5 min, 42°C for 15 min, and 95°C for 5 min. cDNA samples were stored at -20°C.

Forward (FWD) and reverse (REV) primers for the housekeeping gene (18S) and the target genes had the following sequence: 18S, FWD 5-atacgcagctaggaataatgaata-3, REV 5-ctctagcggcgcaatacgaa-3; ACE2, FWD 5-aaatgagatggcaagacaaaca-3, REV 5-ctcgccaataatccccatagtct-3; AT1, FWD 5-gccagcgtcttcttctcaatct-3, REV 5-gggccagcggtactccatag-3; AT2, FWD 5-ctgtgtgttggaattcatcatt-3, REV 5-cccatccaggtcagagcatc-3; Mas, FWD 5-cggtgac-ttttctattggctaca-3, REV 5-cacaggagggcacagacgaa-3. For each set of reactions, 2.5-µl cDNA (10 ng/µl) samples were mixed with 8 µl of RNase-free H₂O, 12.5 µl SYBR Green Master Mix (2×), 1 µl of FWD and 1 µl of REV primers. RT PCR reactions were performed with the following thermal profile: (1) 95°C for 10 min initial denaturation, (2) 95°C for 30 s denaturation, (3) 55°C for 1 min annealing, (4) 72°C for 30 s extension, (5) 95°C for 1 min and 55°C for 1 min final extension, 55–95°C for 30 s of melting curve analysis with a Stratagene Mx3000P qPCR machine (Agilent Technologies, Santa Clara, Calif., USA), where steps 2–4 were repeated 40 times. MxPro v3.20 QPCR software (Agilent Technologies) was used to determine Ct values, and the target gene expression levels were calculated as $10^6 \times 2^{-(\Delta Ct)}$, where $\Delta Ct = Ct_{\text{target}} - Ct_{18s}$.

Western Blots for ACE2 and AT₁, AT₂, and Mas Receptors

The expression of ACE2 and AT₁, AT₂, and mas receptor protein was evaluated by Western blot analysis in mesenteric arteries from NS- and HS-fed rats. Whole mesenteric vascular beds were isolated, cleaned, and homogenized in a solution containing 250 mM of sucrose, 1 mM of EDTA, 1.5 µl of protease and phosphatase inhibitor cocktail, 1 mM of KH₂PO₄, and 10 mM of K₂HPO₄ (pH 7.7). Tissue debris and nuclear fragments were removed by centrifugation (12,000 g for 20 min at 4°C) and the protein concentration of the supernatant was determined using a Bradford protein assay (Thermo Scientific, Rockford, Ill., USA). Western blotting was performed by loading 10 µg of mesenteric artery tissue homogenate onto a 10–20% SDS-polyacrylamide gel and separated by electrophoresis. The samples were transferred to a nitrocellulose membrane (0.45 µm), and blocked for 1 h in TBST solution (10 mM Tris, 150 mM NaCl, 0.1% Tween-20) containing 10% non-fat dried milk at room temperature.

The membranes were then incubated with a 1:800 dilution of rabbit anti-AT₁ (sc-1173) antibody, a 1:1,000 dilution of rabbit anti-AT₂ (sc-9040) antibody, a 1:400 dilution of goat anti-mas (sc-54682) antibody and a 1:200 dilution of rabbit anti-ACE2 (sc-20998; all from Santa Cruz Biotechnology) antibody overnight. The next day, the membranes were washed with TBST and incubated for 2–3 h with the following secondary antibodies (Santa

Cruz Biotechnology): horseradish peroxidase-conjugated goat anti-rabbit (sc-2004) for AT₁, AT₂ (1:4,000 dilution) and ACE2 (1:5,000 dilution), and donkey anti-goat (sc-2020) for mas (1:8,000 dilution). The protein expression of β-actin was measured by incubation of the membranes with mouse primary antibody for β-actin (A-5441; Sigma, St. Louis, Mo., USA) and goat anti-mouse secondary antibody (sc-2005) in dilutions of 1:25,000 for 1 h each.

After incubation with the secondary antibodies, the membranes were washed and protein bands were visualized using chemiluminescence (SuperSignal; Pierce, Rockford Ill., USA). The membranes were exposed to Blue Lite Autorad film (F-9024-8X10, GeneMate; BioExpress, Kaysville, Utah, USA), and developed in a Kodak X-OMAT 2000A developer (Eastman Kodak, Rochester, N.Y., USA). Densitometry values (pixels) were obtained using Un-ScanIT 6.1 software (Silk Scientific, Orem, Utah, USA). The expression of bands of the target protein for each animal was expressed as the percentage of β-actin or total protein using Ponceau S (P7170; Sigma) to stain the protein bands. The results for each antibody were normalized as 100% for the NS control group.

Drugs and Reagents

All chemicals used in this study were purchased from Sigma except A779 (Bachem, Torrance, Calif., USA), PD123319, CGP-42112 and HOE140 (Tocris, Ellisville, Mo., USA), and DETA NONOate (Cayman, Ann Arbor, Mich., USA). AVE0991 was a generous gift from Sanofi-Aventis (Frankfurt, Germany).

Statistical Analysis

Data are presented as mean values ± SEM. Resting and EC₅₀ diameters were measured at the end of the incubation period and following precontraction with an EC₅₀ concentration of norepinephrine in the tissue bath, respectively. Precontraction levels (%) were calculated as [(EC₅₀ diameter/resting diameter) × 100%]. Diameter changes were calculated as the difference from the EC₅₀ control diameter established before the addition of any drugs or agonists.

Differences between individual groups were evaluated using Student's t test for the comparison of two groups or ANOVA with post hoc Dunnett's test for comparisons of more than two experimental groups. In the case of concentration-dependent responses, one-way repeated-measures ANOVA was used and Dunnett's pair-wise multiple comparison procedure was applied to test for significant differences from the precontracted diameter. Statistical significance was taken as $p < 0.05$.

Results

Experimental Groups and Vessel Diameters

Table 1 summarizes the age, body weight, arterial blood pressure, resting control diameters, and the magnitude of the precontraction, expressed as the percentage of the control diameter in the various experimental groups of rats fed an NS or HS diet. As shown previously [20], blood pressure was not affected by dietary salt intake. Control diameters were not influenced by salt diet,

Table 1. Age, body weight, and blood pressure of Sprague-Dawley rats fed NS or HS diets

	NS diet	HS diet
Age, weeks	9.3±0.1 (n = 99)	9.6±0.1 (n = 172)
Body weight, g	276.5±1.9 (n = 99)	282.7±1.9 ^a (n = 172)
Blood pressure, mm Hg	113.6±1.4 (n = 88)	114.0±1.6 (n = 154)
Diameter, μm [EC ₅₀ diameter, % of resting]		
Control	305.0±4.3 [82.5±0.8] (n = 45)	305.7±3.4 [82.5±0.5] (n = 54)
+A779	284.2±7.3 [83.5±2.2] (n = 9)	283.6±6.4 [81.2±1.6] (n = 16)
+PD123319		299.7±8.7 [80.2±1.2] (n = 16)
+L-NAME	315.5±10.1 [77.6±1.1] (n = 8)	302.9±4.3 [80.3±1.1] (n = 26)
– endothelium	301.3±6.9 [81.0±1.4] (n = 16)	284.8±6.4 [82.2±1.7] (n = 16)
+ HOE140		313.4±5.2 [78.6±0.6] (n = 24)
+ angiotensin-(1–7)	328.8±5.1 [77.9±0.7] (n = 8)	315.9±14.6 [78.1±1.1] (n = 8)
+ angiotensin-(1–7) + A779		333.4±13.7 [79.0±1.6] (n = 8)
+ angiotensin-(1–7) + PD123319		306.1±6.6 [78.8±1.0] (n = 8)
+ angiotensin-(1–7) + L-NAME		310.0±6.8 [79.2±2.0] (n = 8)
+ angiotensin-(1–7) – endothelium		316.8±7.8 [75.6±2.3] ^b (n = 8)
+ angiotensin-(1–7) + HOE140		326.8±8.8 [74.7±1.4] ^b (n = 8)
+ captopril	301.3±6.5 [80.5±1.7] (n = 8)	319.0±9.9 [78.6±1.4] (n = 8)
+ CGP42112	307.5±5.1 [79.0±1.5] (n = 8)	301.8±7.8 [79.0±1.6] (n = 8)

Square brackets contain the percentage of control diameters and precontraction (4 μM norepinephrine) levels of mesenteric arteries for each experimental group. Data are expressed as mean $\pm$ SEM. ^a and ^b indicate statistically significant differences ($p < 0.05$) from NS and control groups, respectively.

endothelium removal, or by the presence of different drugs in the tissue bath before application of the various vasodilators.

Direct Effect of Angiotensin-(1–7) and AVE0991 on Vessel Diameter

Norepinephrine-constricted arteries from NS-fed animals showed no response to angiotensin-(1–7) or only a small dilation to the mas receptor agonist AVE0991 (fig. 1a, 2a, respectively). By contrast, mesenteric arteries from HS-fed rats unexpectedly dilated in a concentration-dependent manner in response to both angiotensin-(1–7) (fig. 1b) and AVE0991 (fig. 2b).

Effect of Endothelium Removal and NOS Inhibition on Vessel Responses to Angiotensin-(1–7) and AVE0991

Endothelium removal abolished the vasodilator responses to angiotensin-(1–7) and AVE0991 in the arteries of HS-fed animals (fig. 1b, 2b). NOS inhibition with L-NAME also eliminated vasodilation in response to angiotensin-(1–7) and AVE0991 in the arteries of HS-fed rats (fig. 1c, 2c). As expected, arteries from rats fed the NS

diet failed to dilate in response to either of the agonists following endothelium removal (fig. 1a, 2a) or during NOS inhibition (not shown).

Effect of Mas Receptor, AT₂ Receptor, and BK₂ Receptor Blockade on Vessel Responses to Angiotensin-(1–7) and AVE0991

The addition of A779 eliminated the vasodilator responses to angiotensin-(1–7) (fig. 1d) and AVE0991 (fig. 2d) in the arteries of HS-fed rats, thus confirming that those responses are mediated by the mas receptor. Vasodilator responses to angiotensin-(1–7) and AVE0991 were also inhibited by the AT₂ receptor antagonist, PD123319 (fig. 1d, 2d), and by the BK₂ receptor antagonist, HOE140 (fig. 1e, 2e).

Effect of Angiotensin-(1–7) on Vessel Responses to Bradykinin

Bradykinin alone induced only a slight increase in the diameter of arteries from HS-fed rats and, in contrast to acetylcholine (see below) and previous reports in mesenteric arterioles [3, 4], had no effect on the diameter of arteries from NS-fed rats (fig. 3a). The addition of 10^{–6} M of

Fig. 1. Vascular responses to direct application of angiotensin-(1-7) in mesenteric arteries of Sprague-Dawley rats fed NS (**a**) or HS (**b-e**) diets. Responses were measured in intact and endothelium-denuded preparations (**a, b**), or after the addition of L-NAME (**c**), A779 or PD123319 (**d**), or HOE140 (**e**) to the tissue

bath. Data are presented as the mean $\pm$ SEM ($n = 8-11$); * and + show statistically significant differences ($p < 0.05$) from the pre-constricted diameters in the HS and HS + PD123312 groups, respectively.

angiotensin-(1-7) to the tissue bath selectively enhanced the vasodilator responses to bradykinin in the arteries of HS-fed animals (fig. 3b), and the maximal response to bradykinin was comparable to that occurring in response to the direct application of angiotensin-(1-7) and AVE0991 (fig. 1b, 2b). In the arteries of rats fed the NS diet, responses to bradykinin were unaffected by angiotensin-(1-7) (fig. 3a, b).

Effect of Endothelium Removal and NOS Inhibition on Vessel Responses to Bradykinin

Inhibition of NOS eliminated the small direct effect of bradykinin to dilate the arteries of rats fed the HS diet (not shown). Endothelium removal and NOS inhibition also eliminated the enhanced responses to bradykinin induced by exogenous addition of 10^{-6} M of angioten-

sin-(1-7) to the tissue bath in vessels from HS-fed animals (fig. 3b, c).

Effect of Mas Receptor, AT₂ Receptor, and BK₂ Receptor Blockade on Vasodilator Responses to Bradykinin

Similar to their effect on the direct response of the vessels to angiotensin-(1-7) and AVE0991, A779 and PD123319 abolished the potentiated response to bradykinin in the presence of angiotensin-(1-7) (fig. 3d) in the vessels of HS-fed rats. The initial small dilation that occurred in response to bradykinin alone (not shown) and the enhanced response to bradykinin in the presence of angiotensin-(1-7) (fig. 3e) were both inhibited by the BK₂ receptor antagonist HOE140 in the arteries of HS-fed rats.

Fig. 2. Vascular responses to direct application of the mas receptor agonist AVE0991 in mesenteric arteries of Sprague-Dawley rats fed NS (a) or HS (b–e) diets. Responses were measured in intact and endothelium-denuded preparations (a, b), or after the addition of L-NAME (c), A779 or PD123319 (d), or HOE140 (e) to the

tissue bath. Data are presented as the mean $\pm$ SEM ($n = 8-10$); * and + show statistically significant differences ($p < 0.05$) from the precontracted diameters in the NS or HS, and HS + A779 groups, respectively.

Effect of ACE Inhibition and AT_2 Receptor Activation on Bradykinin Responses

As angiotensin-(1–7) can act as an ACE inhibitor and vasodilation to angiotensin-(1–7) in mesenteric arteries involves AT_2 receptors, we tested the effects of the ACE inhibitor captopril and the AT_2 receptor agonist CGP42112 on vasodilator responses to bradykinin in arteries from NS- and HS-fed rats. Those experiments showed that both captopril and CGP42112 were able to mimic the ability of angiotensin-(1–7) to enhance the response to bradykinin in the arteries of HS-fed rats (fig. 4, right), while they had no effect on bradykinin responses in arteries from rats fed an NS diet (fig. 4, left).

Effect of Angiotensin-(1–7), ACE Inhibition and AT_2 Receptor Activation on Responses to Acetylcholine

To further assess the effects of angiotensin-(1–7) on endothelium-dependent vasodilation, we also tested whether administration of angiotensin-(1–7) improves the vasodilator responses to muscarinic receptor activation that are suppressed by an HS diet in rat small mesenteric arteries [20, 31], and whether captopril and CGP42112 can mimic the effect of angiotensin-(1–7). In these experiments, angiotensin-(1–7), ACE inhibition and AT_2 receptor activation were all able to ameliorate the impaired vasodilator responses to acetylcholine in arteries from HS-fed animals (fig. 5, right), while responses to acetylcholine were unaffected in the arteries of NS-fed rats (fig. 5, left).

Fig. 3. Vascular responses to bradykinin alone (control; **a**) or in the presence of 10^{-6} M angiotensin-(1-7) (**b-e**) in mesenteric arteries of Sprague-Dawley rats fed NS or HS diets. Responses were measured in intact and endothelium-denuded preparations (**a, b**), or

after the addition of L-NAME (**c**), A779 or PD123319 (**d**), or HOE140 (**e**) to the tissue bath. Data are presented as the mean $\pm$ SEM (n = 8). Statistically significant differences (* p < 0.05) from the precontracted diameters in HS are indicated.

Effect of Angiotensin-(1-7), ACE Inhibition, and AT_2 Receptor Activation on Responses to DETA-NONOate

Figure 6 compares vascular NO sensitivity in the various groups as assessed by measuring the concentration-dependent relaxations to the endothelium-independent NO donor DETA NONOate. Arterial responses to DETA NONOate were unaffected by an HS diet or by treatment with angiotensin-(1-7), captopril, or CGP42112, demonstrating that changes in vessel sensitivity to NO itself are not responsible for any differences in the response of the vessels to endothelium- and NO-dependent vasodilator stimuli in any of the groups.

ACE2, AT_1 Receptor, AT_2 Receptor, and Mas Receptor Expression

The expression of mRNA and protein for ACE2, and for the AT_1 , AT_2 , and mas receptors was evaluated by RT-

PCR and Western blot, respectively. The expression of ACE2 and all of the receptors were unaffected by dietary salt intake at both the mRNA level (fig. 7a) and the protein level (fig. 7b).

Discussion

In the present study, we tested whether the acute application of angiotensin-(1-7) or AVE0991 relaxes the small mesenteric arteries of Sprague-Dawley rats fed an NS diet, and whether these vasodilator responses are suppressed by an HS diet. Contrary to our hypothesis, mesenteric arteries from rats fed an HS diet exhibited a significant endothelium- and NOS-dependent dilation in response to angiotensin-(1-7) and AVE0991. We also determined the indirect effect of mas receptor stimulation

Fig. 4. Vascular responses to bradykinin in the absence (control) and in the presence of the ACE inhibitor captopril (10^{-5} M) or the AT_2 receptor agonist CGP42112 (10^{-6} M) in mesenteric arteries of Sprague-Dawley rats fed NS (a) or HS (b) diets. Data are presented as the mean $\pm$ SEM ($n = 8$); *, +, and $\ddagger$ show statistically significant differences ($p < 0.05$) from the preconstricted diameters in the HS control, HS + captopril, and HS + CGP42112 groups, respectively.

Fig. 5. Vascular responses to acetylcholine alone (control) or in the presence of angiotensin-(1-7) (10^{-6} M), the ACE inhibitor captopril (10^{-5} M), or the AT_2 receptor agonist CGP42112 (10^{-6} M) in small mesenteric arteries of Sprague-Dawley rats fed NS (a) or HS (b) diets. Data are presented as the mean $\pm$ SEM ($n = 8$); *, +, $\ddagger$, and $^{\circ}$ indicate statistically significant differences ($p < 0.05$) from the preconstricted diameters in the control, angiotensin-(1-7), captopril, and CGP42112 groups, respectively.

on endothelium-dependent relaxations to bradykinin and acetylcholine. These experiments showed that angiotensin-(1-7) unexpectedly potentiated the small dilation occurring in response to bradykinin and restored vascular relaxation in response to acetylcholine that was lost in the arteries of HS-fed rats. Finally, we compared the vascular effects of mas receptor activation with those of ACE inhibition and activation of the AT_2 receptor, finding that

the effects of angiotensin-(1-7) on the vasodilator responses to bradykinin and acetylcholine were mimicked by captopril and CGP42112.

Vasorelaxant and Vasoprotective Effects of Mas Receptor Activation in HS-Fed Rats

The ACE2-angiotensin-(1-7)-mas receptor axis represents the depressor arm of the renin-angiotensin system

Fig. 6. Vascular responses to NO donor DETA NONOate alone (control) or in the presence of angiotensin-(1-7) (10^{-6} M), the ACE inhibitor captopril (10^{-5} M) or the AT_2 receptor agonist CGP42112 (10^{-6} M) in mesenteric arteries of Sprague-Dawley rats fed NS (**a**) or HS (**b**) diets. Data are presented as the mean $\pm$ SEM ($n = 8$); *, +, ‡, and ° indicate statistically significant differences ($p < 0.05$) from the preconstricted diameters in the control, angiotensin-(1-7), captopril, and CGP42112 groups, respectively.

that opposes the effects of the ACE-angiotensin II- AT_1 receptor axis [35, 36]. Although elevated dietary salt intake suppresses plasma angiotensin II levels [37, 38], an HS diet generally leads to widespread impairment of endothelium-dependent and endothelium-independent vasodilator responses in different vessel types and in multiple regions of the vasculature [5, 20, 27–31, 34]. These deleterious effects of an HS diet on vascular relaxation are mediated by elevated oxidative stress that compromises endothelial Ca^{2+} signaling and agonist-induced NO release and reduces NO bioavailability [5, 20, 27, 29, 31, 38, 39].

The effects of an HS diet in the cerebral vasculature are reversed by returning to a low-salt diet for 2 weeks and by continuous intravenous infusion of a low dose of angiotensin II for 3 days [40] to restore normal plasma angiotensin II levels [38]. In a similar fashion, chronic intravenous infusion of angiotensin-(1-7) and oral treatment with AVE0991 also ameliorate endothelial dysfunction and restore NO-mediated vasodilatation to acetylcholine in middle cerebral arteries [5] and mesenteric resistance arteries [20] of salt-fed Sprague-Dawley rats. Consequently, it appears that mas receptor activation has protective effects during elevated dietary salt intake that are distinct from its well-known ability to directly antagonize the deleterious effects of elevated plasma angiotensin II levels.

Mechanisms of the Vasorelaxant and Vasoprotective Effects of Mas Receptor Activation

In the present study, angiotensin-(1-7) and AVE0991 caused vascular relaxation and angiotensin-(1-7) im-

proved bradykinin- and acetylcholine-induced relaxation in the small mesenteric arteries of HS-fed rats. These direct vasodilator and indirect protective effects of acute mas receptor activation are remarkable in light of the widely reported effects of HS diet to produce vascular oxidant stress, endothelial dysfunction, and impaired vascular relaxation in response to a variety of vasodilator stimuli in multiple vascular beds and in several different species [5, 20, 27–31, 34, 38, 41]. These unexpected effects of angiotensin-(1-7) raise intriguing questions regarding the mechanisms by which mas receptor activation exerts these vasodilator and vasoprotective effects during elevated dietary salt intake.

As an HS diet increases oxidative activity in the vessel wall, which reduces NO bioavailability due to the destruction of NO by superoxide anions, the vasorelaxation and preservation of endothelium-dependent and NO-mediated vascular relaxations by mas receptor activation in HS-fed animals could reflect either an activation of antioxidant defense mechanisms or a reduction in superoxide production (or both).

Based on our current observations (fig. 1, 2) and established findings in the literature [11, 14, 18, 25], we assume that the acute addition of angiotensin-(1-7) and AVE0991 activates endothelial NOS and stimulates NO release, which acts as a vasodilator mediator. In the present study, the direct vasodilator responses to angiotensin-(1-7) (fig. 1) and AVE0991 (fig. 2) were both eliminated by endothelium removal and by inhibiting NOS with L-NAME, demonstrating an endothelium-dependent and NO-me-

Fig. 7. Expression of ACE2 and AT₁, AT₂ and mas receptor mRNA (**a**) and protein (**b**) evaluated by RT-PCR and Western blot (representative blots are shown) in mesenteric arteries of Sprague-Dawley rats fed NS or HS diets. Messenger RNA and protein expressions are shown relative to 18S (n = 9) and percentage of NS (n = 6–8), respectively, and summarized as the mean ± SEM.

diated mechanism. In a similar fashion, angiotensin-(1–7) may have the indirect effect of rescuing or enhancing acetylcholine- or bradykinin-induced vasodilator responses by increasing NO availability.

Our earlier finding that preincubation with angiotensin-(1–7) reduces vascular oxidant stress in the mesenteric arteries of HS-fed rats [20] is consistent with both the direct and indirect effects of angiotensin-(1–7) described above. One particularly attractive explanation for the ability of mas receptor activation to reduce oxidative stress in the arteries of HS-fed rats is via activation of antioxidant defense mechanisms in the vasculature. For example: (1) angiotensin-(1–7) increases superoxide dismutase (SOD) activity in the brain of spontaneously hypertensive rats [42]; (2), SOD and catalase activities are suppressed in the aorta of mas-deficient mice [43], and (3), angiotensin-(1–7) restores the activity of SOD and catalase in the kidneys of diabetic rats [44].

In addition, studies in human aortic endothelial cells [45] and rat aorta [46] indicate that angiotensin-(1–7) can prevent angiotensin II-induced activation of NAD(P)H oxidase [45] and reduce NAD(P)H oxidase-derived superoxide production [46]. In line with our hypothesis, a reduction of oxidative stress via antioxidant treatment with tempol and the pharmacological inhibition of different superoxide-producing enzymes both cause vasodilation and rescue vascular relaxation in the small mesenteric arteries from HS-fed rats [31]. The latter findings provide an additional mechanism by which acute administration of angiotensin-(1–7) may reduce oxidative stress in the arterial wall, thereby eliciting and enhancing vascular relaxation by preserving NO availability in the vasculature.

Effect of an HS Diet on the Expression of ACE2, Mas Receptors, and Angiotensin AT₁ and AT₂ Receptors

In the present study, we found that the expression of ACE2, AT₁ receptors, AT₂ receptors and mas receptors was unaffected by an HS diet. These observations support the hypothesis that the direct and indirect effects of mas receptor activation in the arteries of salt-fed animals are not due to salt-induced upregulation of ACE2 or mas receptors or changes in the expression of AT₁ or AT₂ receptors for angiotensin II. However, our finding of an unchanged expression of these proteins does not eliminate the possibility that the catalytic activity of ACE2 or receptor activation mechanisms may be altered by an HS diet, suggesting a valuable area for future investigation.

Role of Bradykinin in Angiotensin-(1-7)-Induced Dilation and Indirect Effect of Angiotensin-(1-7) to Enhance Bradykinin-Induced Vascular Relaxation

The direct vasodilator effect of angiotensin-(1-7) and AVE0991 in the arteries of HS-fed rats is complex, because vasodilator responses to angiotensin-(1-7) and AVE0991 in the arteries of HS-fed rats were eliminated not only by NO synthase inhibition, but also by the BK₂ receptor antagonist HOE140. Participation of NO and bradykinin in those vasodilator responses is consistent with known mechanisms mediating the direct effects of mas receptor activation by angiotensin-(1-7) [6, 7, 18] or AVE0991 [14]. In this regard, the present study indicates that vascular relaxation in response to direct mas receptor activation is coupled to endogenous bradykinin formation, and/or to direct or indirect activation of the BK₂ receptor, as reported in many other vascular preparations [3, 6, 7, 14, 18, 21, 47].

In addition to its direct vasodilator effect, we also found that angiotensin-(1-7) potentiated vasodilator responses to exogenously applied bradykinin in HS-fed rats. As previously reported in the rat mesenteric and coronary circulations [3, 15], the enhanced dilation to bradykinin in the presence of angiotensin-(1-7) was eliminated by L-NAME (fig. 3). The latter finding suggests that, similar to the acute vasodilator responses to angiotensin-(1-7) and AVE0991, preservation of vascular NO levels, most likely due to the vasodilator and antioxidant properties of angiotensin-(1-7), is crucial to the ability of this peptide to enhance bradykinin-induced vasodilation in mesenteric arteries of HS-fed rats. One surprising observation in the present study was that, in contrast to other studies [3, 4] (and vessel responses to acetylcholine), bradykinin failed to dilate small mesenteric arteries from rats fed an NS diet. This may be related to the operation of different vascular relaxation mechanisms activated by bradykinin versus acetylcholine at the level of precontraction of the artery used in the present study or to the use of different experimental preparations, i.e. isolated arteries in the present study versus whole mesenteric vascular beds [3, 4].

Effect of ACE Inhibition and AT₂ Receptor Activation/Inhibition on Vessel Responses to Bradykinin and Acetylcholine

Based on our initial results and earlier findings [3-6, 8, 15, 20, 22-26, 48-50], the second part of our study tested whether the effect of mas receptor activation to potentiate or restore vasodilator responses to bradykinin and acetylcholine in the arteries of HS-fed rats can

be mimicked by inhibition or activation of different components of the renin-angiotensin system. Vessel responses to bradykinin and acetylcholine were determined before and during ACE inhibition with captopril and AT₂ receptor activation by CGP42112, and compared to the effects of these agents on the corresponding controls [mesenteric arteries from NS- or HS-fed animals in the presence or absence of angiotensin-(1-7)]. Similar to the indirect effects of acute mas receptor activation with angiotensin-(1-7) on bradykinin- and acetylcholine-induced vasodilation, captopril and CGP42112 enhanced vascular relaxation in response to these agonists in vessels from HS-fed animals, with no effect on the responses of mesenteric arteries from NS-fed rats (fig. 4, 5).

The present experiments performed on the arteries of HS-fed rats clearly demonstrate a protective effect of acute ACE inhibition with captopril on salt-induced endothelial dysfunction. The mechanism of the enhanced endothelium-dependent vasodilation during ACE inhibition is still controversial. Beyond the effect of inhibiting the hydrolytic activity of ACE to destroy bradykinin, a variety of alternative mechanisms have been proposed to explain the potentiating effect of both angiotensin-(1-7) [3, 4, 22] and captopril [48-53], and these are important targets for future investigation.

In this regard, one unexpected observation in the present study was the ability of captopril to restore vasodilator responses to acetylcholine in the arteries of the HS-fed rats. However, this effect of ACE inhibition is not unprecedented, as ACE inhibition with captopril or enalapril potentiates acetylcholine-induced dilation in the canine coronary circulation by facilitating the release of NO and prostacyclin via mechanisms coupled to endogenously formed bradykinin [48], and ACE inhibitors have been reported to depress vascular tone, inhibit vasoconstrictor responses, and cause vascular relaxation in response to a variety of vasoactive stimuli [54].

An involvement of angiotensin II receptors in mediating the effects of angiotensin-(1-7) or AVE0991 has been reported in bovine aortic endothelial cells [14, 18], porcine coronary arteries [21], and mouse heart [55]. Previous studies from our laboratory have shown that the protective effects of chronic angiotensin-(1-7) infusion to restore endothelial function in mesenteric and cerebral arteries of HS-fed Sprague-Dawley rats also involves the AT₂ receptor [5, 20]. The mechanisms by which angiotensin II receptors contribute to the physiologic effects of mas receptor activation remain to be determined, but may include different receptor-receptor interactions such

as functional antagonism, cross talk, transactivation, and dimerization [5–7, 12, 14, 18, 20, 21].

Consistent with the results of other studies [5, 14, 18, 20, 21, 55, 56], the AT₂ receptor also plays a role in the acute vascular responses to angiotensin-(1–7), because the vasodilator effects of angiotensin-(1–7) and AVE0991 and the potentiating effect of angiotensin-(1–7) on bradykinin-induced vascular relaxation were all blocked by the AT₂ receptor antagonist PD123319 (fig. 1–3). Consistent with these results, AT₂ receptor activation by CGP42112, mimicking the effects of angiotensin-(1–7), restored the vasodilator responses to bradykinin and acetylcholine in the arteries of HS-fed rats (fig. 4, 5). Taken together, these findings provide further support for the hypothesis that not only chronic [20] but also acute AT₂ receptor activation might play a crucial role in the ability of angiotensin-(1–7) to restore vasodilator responses that are normally suppressed by an HS diet, and also support the hypothesis that the mas and AT₂ receptors play an essential role in the potentiation of bradykinin and acetylcholine responses by angiotensin-(1–7).

Effect of Mas Receptor Activation, ACE Inhibition and AT₂ Receptor Activation on NO Sensitivity

Finally, vessel responses to the NO donor DETA NONOate were assessed in order to test for possible changes in vascular NO sensitivity during the various experimental interventions employed. Neither the suppressed vasodilatation to acetylcholine due to an HS diet nor the enhanced response to vasodilator stimuli in the presence of the different activators or inhibitors of the renin-angiotensin system were due to changes in vascular NO sensitivity, as vessel responses to DETA NONOate were not altered in any of those cases (fig. 6). These findings are in full agreement with previous findings showing that responses to sodium nitroprusside are unaffected by angiotensin-(1–7) [3–5, 16, 23, 25], and indicate that neither the suppression of acetylcholine responses nor the

ability of mas receptor activation, ACE inhibition, or AT₂ receptor activation to improve vessel relaxation in response to bradykinin or acetylcholine in rats fed an HS diet are due to changes in vessel sensitivity to NO.

Perspectives

As reviewed by Nguyen Dinh and Touyz [57], recent findings have shown that essentially all components of the renin-angiotensin system exist in the peripheral organs and vascular tissues, and that these tissue-specific components can work independently of the systemic renin-angiotensin system. In addition to the direct vasorelaxant and indirect vasoprotective effects of angiotensin-(1–7), ACE inhibition and AT₂ receptor activation also ameliorate endothelial dysfunction caused by an HS diet. These findings are especially relevant in light of recent studies showing that an HS diet leads to endothelial dysfunction in healthy normotensive male [58] and female [59] humans. In this regard, the results of the present study suggest novel therapeutic targets including not only the mas receptor, but also other components of the renin-angiotensin system to treat diseases associated with salt-induced endothelial dysfunction, chronically low angiotensin II levels, and vascular oxidant stress [60].

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Disclosure Statement

The authors have no conflicts of interest to disclose.

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