Role of Angiotensin-(1–7) in Gastroprotection against Stress-Induced Ulcerogenesis. The Involvement of Mas Receptor, Nitric Oxide, Prostaglandins, and Sensory Neuropeptides

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ABSTRACT

Angiotensin-(1–7) [Ang-(1–7)] is a major vasoactive metabolite of the renin-angiotensin system (RAS). Ang-(1–7) activity via the Mas receptor was documented in kidneys, heart, brain, and gastrointestinal (GI) tract. We studied the gastroprotective activity of exogenous Ang-(1–7) in stress-induced water immersion and restraint stress (WRS) ulcers in rats, and the effects of COX-2 inhibitors or L-NNA (5-[imino(nitroamino)methyl]-L-ornithine) on gastric blood flow (GBF) and luminal content of NO. COX-1 and COX-2 inhibitors reduced the WRS ulcerogenesis, whereas L-NNA reversed the reduction in lesion number and the rise in GBF evoked by Ang-(1–7). Ang II augmented the WRS lesions, decreased GBF and increased the plasma IL-1β and TNF-α levels. Capsaicin denervation and withdrawal of the reduction in Ang-(1–7)–induced gastric lesions and rise in GBF; these effects were restored by supplementation with calcitonin gene–related peptide (CGRP). The cNOS mRNA was upregulated while iNOS, IL-1β, and TNF-α mRNAs were downregulated in Ang-(1–7)–pretreated rats. We conclude that Ang-(1–7), in contrast to Ang II, which worsens WRS ulcerogenesis, affords potent gastroprotection against WRS ulcerogenesis via an increase in GBF, suppression of COX-2 mRNAs, downregulation of iNOS, proinflammatory markers iNOS, IL-1β, and TNF-α, and anti-inflammatory action involving the inhibition of proinflammatory markers iNOS, IL-1β, and TNF-α.

Introduction

The renin-angiotensin system (RAS) is a critical endocrine system involved in physiologic regulation of blood pressure and water, glucose, gastrointestinal motility, mucosal secretion, gastric inflammation, and carcinogenesis (Pandriks 2011; Garg et al., 2012). Recently, the essential Ang I and Ang II metabolites have been identified throughout the GI tract, including stomach, colon, pancreatic islets, and liver (Carl-McGrath et al., 2009; Olszanecki et al., 2009; Hasegawa et al., 2009). Ang II is the central product of RAS and potent constrictor of vascular smooth muscles (Heinemann et al., 1999). Ang II acts via angiotensin receptor type 1 (AT1) and contributes to vasoconstriction, inflammation, vascular and cardiac hypertrophy, and extracellular tissue remodeling by inhibition of cell growth and stimulation of apoptosis (Lemarie et al., 2009). Stimulation of the AT1 receptors activates NADPH oxidase in vascular smooth muscle cells (VSMCs), enhances the production of reactive oxygen species such as nitric oxide (NO), and inhibits the production of prostaglandins. Therefore, the disruption of NO production via AT1 receptors induces increases in blood pressure, hypertrophy, and fibrosis, whereas NO production via AT2 receptors induces vasodilation, increased glomerular filtration, and vascular smooth muscle apoptosis and growth, and inhibition of extracellular matrix synthesis.

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ABBREVIATIONS: A-779, d-Ala7-ANG-(1–7); ACE, angiotensin-converting enzyme; Ang II, angiotensin II; Ang-(1–7), angiotensin-(1–7); AT1, angiotensin receptor type 1; AVE 0991, 5-formyl-4-methoxy-2-phenyl-1-[4-[2-(ethylaminocarbonylsulfonamido)-5-isobutyl-3-thienyl]-phenyl]-methyl-imidazole; CGRP, calcitonin gene–related peptide; cNOS, constitutively expressed nitric-oxide synthase; COX, cyclo-oxygenase; GBF, gastric blood flow; GI, gastrointestinal; iG, intragastric; iNOS, inducible nitric-oxide synthase; IL, interleukin; i-OMe-L-NNA, 5-[imino(nitroamino)methyl]–L-ornithine; NOS, nitric-oxide synthase; PG, prostaglandin; RAS, renin-angiotensin system; SC-560, 5-[4-chloro-phenyl]-1-(4-methoxyphenyl)-3-trifluoromethyl-pyrazole; TNF, tumor necrosis factor; WRS, water immersion and restraint stress.
superoxide and hydrogen peroxide (H₂O₂), and inactivates NO pathway (Mehta and Griendling, 2007). Ang II-activating phospholipase C (PLC) and protein kinase C (PKC) or phospholipase A₂ enhanced synthesis of vasoconstrictive leukotrienes and smooth muscle cell contraction (Mehta and Griendling, 2007; Lemarie et al., 2009). Increased reactive oxygen species (ROS) and decreased blood flow play fundamental roles in the pathogenesis of GI mucosal injury (Bregenzio et al., 2003; Nakagiri et al., 2010).

Exposure to stress is commonly recognized as a risk factor of microbleeding and gastric mucosal injury. Reaction to stress is mediated via two distinct but unrelated systems: the hypothalamic-pituitary-adrenocortical (HPA) system and the sympathoadrenal system (Goldstein and McEwen, 2002; Saavedra et al., 2006). Ang II receptor subtypes AT₁ and AT₂ were detected in the human esophageal, gastric, small intestinal, and colonic mucosa (Hirasawa et al., 2002; Casselbrant et al., 2009; Hallersund et al., 2011). The antagonists of Ang II AT₁ receptors attenuated gastric injury induced by ischemia-reperfusion, cold stress, and indomethacin-induced damage in rodents due to an inhibition of sympathetic-adrenal axis and the attenuation of vasoconstrictor and proinflammatory activity of Ang II (Pavel et al., 2007; Morsy et al., 2009; Gemici et al., 2010; Saavedra et al., 2010; Santos et al., 2011).

Antagonists of AT₁ receptor candeisol and candesartan prevented stress-induced gastric lesions (Bregenzio et al., 2003,2004; Merai et al., 2009).

Angiotensin-(1–7) (Ang-(1–7)) known as a vasodilator generated from angiotensin I (Ang I) is a physiological counterpart of the ACE homolog ACE2 or neutral endopeptidase (NEP) from the angiotensin-converting enzyme (ACE) that is extremely susceptible to degradation from angiotensin I through angiotensin-converting enzyme (ACE) homolog ACE2 or neutral endopeptidase (NEP), respectively. The involvement of endogenous PG in the gastroprotective effects of Ang-(1-7) was examined. Capsaicin was injected subcutaneously 1, 2, and 3 weeks before the experiment to induce the functional denervation. We also assessed the effect of Ang-(1–7) on the expression of mRNA for constitutively expressed nitric-oxide synthase (nNOS), inducible nitric-oxide synthase (iNOS), proinflammatory cytokines interleukin (IL)-1β and tumor necrosis factor (TNF)-α, and plasma levels of these cytokines during stress ulcerogenesis.

Materials and Methods

Animals. Male Sprague-Dawley rats total 25–30 g with weight averaging about 250 g were used in this study. Rats were fasted for 24 hours with free access to water before exposure to WRS. The study was approved by the Institutional Animal Care and Use Committee of Jagiellonian University Medical College in Cracow and run in accordance with the statement of the Helsinki Declaration regarding animal experimental and care.

Application. To induce gastric lesions, rats were immobilized in individual Bolman cages and immersed in a water bath (23°C) for 3.5 hours. The rat xyphoid level as reported by our group previously (Brogowzko et al., 2000, Kwiecien et al., 2012). In separate major series of groups of rats rats (F-G) were selected. Thirty minutes before exposure to water immersion and restraint stress (WRS), rats in series A–C received pretreatment with (1) exogenous Ang-(1–7) (6.25–50 μg/kg i.g.), (2) Ang-(1–7) Mas receptor antagonist (Bayorh et al., 1999; Santos et al., 2013) (5 mg/kg i.p.), or (3) perindopril (8 mg/kg i.p.), the ACE-lowering agent (Pierron et al., 2004, Santos et al., 2012). The involvement of endogenous PG in the gastroprotective effects of Ang-(1–7) or vehicle (control) was investigated in rats (G) with or without pretreatment with (1) L-NNA (20 mg/kg i.p.), (2) perindopril (8 mg/kg i.p.), or (3) perindopril, Ang-(1–7) Mas receptor antagonist (Bayorh et al., 1999; Santos et al., 2013) with or without the combination with Ang-(1–7) (5 mg/kg i.p.) in rats exposed 30 minutes later to 3.5 hours of WRS. AVE 0991 (50 mg/kg i.p.), the nonpeptide Ang-(1–7) receptor agonist (Pinheiro SV et al., 2004; Santos and Fereira, 2006), respectively.

In separate major series of groups of rats rats (F-G) were selected. Thirty minutes before exposure to water immersion and restraint stress (WRS), rats in series A–C received pretreatment with (1) exogenous Ang-(1–7) (6.25–50 μg/kg i.g.), (2) Ang-(1–7) Mas receptor antagonist (Bayorh et al., 1999; Santos et al., 2013) (5 mg/kg i.p.), or (3) perindopril (8 mg/kg i.p.), the ACE-lowering agent (Pierron et al., 2004, Santos et al., 2012). In another subgroup with COX-1 and COX-2 inhibitors, rats of series F were coadministered with exogenous prostaglandin E₂ (PGE₂; 5 μg/kg i.g.) in the presence of Ang-(1–7).

In series G, the effect of blockade of sensory nerves induced by large dose of capsaicin (total 125 mg/kg s.c.) on the protective and hyperemic activity of Ang-(1–7) was examined. Capsaicin was injected for 3 consecutive days at a respective dose of 25, 50, and 50 mg/kg s.c. approximately 2 weeks before the experiment to induce the functional ablation of sensory nerves as described previously (Konturek et al., 2009; Kwiecien et al., 2012a). In separate subgroup of series G with capsaicin denervation, the involvement of calcitonin gene–related peptide (CGRP), the major rat neuropeptide released from sensitive afferent nerve endings in protective action of exogenously administered Ang-(1–7) against WRS lesions, was determined. In one of the subgroups of series G, the capsaicin-denervated rats received supplementation with exogenous CGRP (10 μg/kg s.c.) combined with Ang-(1–7) and 30 minutes later were exposed to onset of WRS as in other groups described above.

All tested drugs and compounds were of analytical grade and were purchased from Sigma-Aldrich Laborchemikalien (Schelldorf, Germany) except of SC-560 and rofecoxib purchased from Cayman.
Chemical (Ann Arbor, MI) and Pfizer (Illeitessen, Germany), respectively.

Measurement of GBF and Determination of Gastric Lesion Number. At the termination of 3.5 hours WRS, rats were anesthe-
tized with pentobarbital (60 mg/kg i.p.), the abdomen was opened, and GBF measured by means of H2-gas clearance technique as reported before (Brzozowski et al., 2004, 2006; Kwiecien et al., 2007). The GBF was measured in the fundic part of the gastric mucosa not involving mucosal lesions. Average values of three measurements were determined and expressed as a percentage of the value determined in intact rat stomach. Gastric lesions number was determined on photographed stomachs with computerized planimetry (Morphomat, Carl Zeiss, Berlin, Germany) (Kwiecien et al., 2012a) by a blinded investigation.

Determination of Luminal NO Content and Plasma Level of IL-1β and TNF-α. The luminal concentration of NO was quantified indirectly as nitrate (NO3−) and nitrite (NO2−) levels in the gastric contents using the nitrate/nitrite kit purchased from Cayman Chemical as detailed in our previous studies (Brzozowski et al., 2008; Pawlik et al., 2011; Kwiecien et al., 2012b).

The blood samples (~3 ml) were taken from the vena cava for the measurement of plasma proinflammatory cytokines IL-1β and TNF-α as described previously (Kwiecien et al., 2012b). In brief, the plasma TNF-α and IL-1β was determined by a solid-phase sandwich enzyme-linked immunosorbent assay (ELISA; BioSource International Inc., Camarillo, CA) according to the manufacturer’s instructions. Each sample (50 μl) was incubated with biotinylated antibodies specific to TNF-α and IL-1β, washed three times with assay buffer, and finally conjugated with streptavidin peroxidase to form a complex with a stabilized chromogen as described previously (Kwiecien et al., 2012b).

The expression mRNA of cNOS, iNOS, IL-1β, and TNF-α in the rat gastric mucosa determined by reverse transcriptase-polymerase chain reaction (RT-PCR) with specific primers. Mucosal specimens were scraped onto a glass slide and immediately snap-frozen in liquid nitrogen and stored at −80°C until analysis. Total RNA was extracted from mucosal samples by a guanidium isocyanate-phenol-chloroform method using a kit from Stratagene (La Jolla, CA) according to the manufacturer’s instructions. The total RNA concentration in each sample was determined by 1% agarose-formaldehyde gel electrophoresis and ethidium bromide staining. Aliquoted RNA samples were stored at −80°C until analysis.

Extraction of RNA. The total RNA concentration in each sample was determined by 1% agarose-formaldehyde gel electrophoresis and ethidium bromide staining. Aliquoted RNA samples were stored at −80°C until analysis.

Measurement of plasma proinflammatory cytokines IL-1β and TNF-α was determined by a solid-phase sandwich enzyme-linked immunosorbent assay (ELISA; BioSource International Inc., Camarillo, CA) according to the manufacturer’s instructions. Each sample (50 μl) was incubated with biotinylated antibodies specific to TNF-α and IL-1β, washed three times with assay buffer, and finally conjugated with streptavidin peroxidase to form a complex with a stabilized chromogen as described previously (Kwiecien et al., 2012b).

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**TABLE 1**
The annealing temperature, nucleotide sequence primers, and size of products used for RT-PCR determination

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer Sequence</th>
<th>Annealing Temperature</th>
<th>Size of PCR Product</th>
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<tr>
<td>c-NOS</td>
<td>Forward: 5′- TAC GGA GCA GCA AAT CCA C-3′, Reverse: 5′- CAG GCT GCA GTC CTT TGA TC-3′</td>
<td>63.5</td>
<td>540</td>
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<tr>
<td>IL1-β</td>
<td>Forward: 5′- GCT ACC TAT GTC TTT CCC GT-3′, Reverse: 5′- GAC CAT TGC TGT TTC CTA GG-3′</td>
<td>62</td>
<td>543</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Forward: 5′- TAC TGA ACT TCG GGG TGA TGT GTC C-3′, Reverse: 5′- CAG CCT TGC TTT AAG AGA ACC-3′</td>
<td>56</td>
<td>295</td>
</tr>
<tr>
<td>β-Actin</td>
<td>Forward: 5′- TGT TAA CCA ACT GGG ACG ATA TGG-3′, Reverse: 5′- GAT CTT GAT CTT CAT GGT GCT AGG-3′</td>
<td>54</td>
<td>764</td>
</tr>
<tr>
<td>iNOS</td>
<td>Forward: 5′- CCA CAA TAG TAC AAT ACT AC-3′, Reverse: 5′- ACG AGG TGT TCA GCG TGC TCG-3′</td>
<td>60</td>
<td>397</td>
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**Results**
Mean Lesion Number and GBF in Rats Pretreated with Ang II or Ang-(1-7). Exposure of vehicle-pretreated control rats to 3.5 hours of WRS caused gastric mucosal lesions (hemorrhagic erosions) accompanied by a significant fall in GBF (Fig. 1). The pretreatment with Ang II applied in a dose of 5 μg/kg failed to significantly affect the mean lesion number and GBF compared with vehicle-control.
The administration of Ang II in higher doses ranging from 6.25 to 40 μg/kg dose-dependently increased the mean lesion number and produced a significant dose-dependent decrease in GBF (Fig. 1). The pretreatment with Ang-(1–7) administered i.p. in graded doses ranging from 6.25 to 50 μg/kg, dose-dependently diminished WRS-induced gastric lesions, while producing a significant and dose-dependent increase in GBF and luminal NO content (Fig. 2). The dose of Ang-(1–7) inhibiting WRS lesions by 50% (ID₅₀) was 27 μg/kg. Since the dose of 50 μg/kg afforded the maximal protective response (P < 0.05), this dose of Ang-(1–7) was used in all our determinations. The absolute values for GBF expressed in ml/min per 100 g are presented in Table 2. Exposure to WRS in rats pretreated with vehicle-control significantly decreased the GBF by 22% compared with the values in the intact gastric mucosa. This decrease in GBF under WRS conditions was significantly worsened by the pretreatment with Ang II. In contrast, pretreatment with Ang-(1–7) resulted in a significant increase in the GBF (P < 0.05) compared with the vehicle-pretreated group. The Ang-(1–7)-induced protection of the peptic lesions was accompanied by a rise in the GBF and luminal NO content observed at the 50 μg/kg dose of this peptide. These changes were completely reversed by the pretreatment with A-779 (50 μg/kg i.p.) combined with intraperitoneal treatment with Ang-(1–7) (50 μg/kg) (Fig. 2; Table 2).

Effect of AVE 0991, the Agonist of Ang-(1–7) Mas Receptor, on WRS-Induced Gastric Lesions and Alterations in the GBF. As shown in Fig. 3, the pretreatment with AVE 0991 (50 μg/kg i.p.) significantly reduced the mean lesion number (P < 0.05) and caused a significant increase in the GBF (P < 0.05) compared with the respective values in vehicle-pretreated rats. The decrease in lesion number and an increase in the GBF induced by AVE 0991 were completely abolished in rats treated with the combination of A-779 and AVE 0991 (Fig. 3).

Effect of Suppression of NO-Synthase on Ang-(1–7)- and Perindopril-Induced Gastric Protection and Alterations in GBF in Rats Exposed to WRS. Figure 4 shows that pretreatment with Ang-(1–7) (50 μg/kg i.p.) significantly reduced the WRS-induced gastric lesions and increased GBF, with the effects similar to the respective values presented in Fig. 2. The pretreatment with perindopril (5 mg/kg i.p.) also significantly increased the number of WRS-induced gastric lesions (P < 0.05) but did not significantly increase GBF compared to vehicle-control. Administration of L-NNA (20 mg/kg i.p.), which itself failed to significantly affect the lesion number and GBF, compared to vehicle-treated control, reversed the protective action in lesion number and the rise in GBF evoked by Ang-(1–7) or perindopril (Fig. 4).

Effect of COX-1/PG and COX-2/PG Suppression on Ang-(1–7)-Induced Gastric Damage and Alteration in GBF. As shown in Fig. 5, the pretreatment with Ang-(1–7) (50 μg/kg i.p.) caused a similar decrease in the mean number of WRS-induced lesions compared with those pretreated with Ang-(1–7) alone.

### Table 2

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<th>Type of Test</th>
<th>GBF (ml/min per 100 g)</th>
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<tr>
<td>Intact</td>
<td>46 ± 2.8</td>
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<tr>
<td>Veh + WRS</td>
<td>27 ± 2.2</td>
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<tr>
<td>Ang II + WRS</td>
<td>21 ± 1.6**</td>
</tr>
<tr>
<td>Ang-(1–7) + WRS</td>
<td>35 ± 2.7**</td>
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<tr>
<td>A-779 + Ang-(1–7) + WRS</td>
<td>26 ± 2.2**</td>
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The addition of PGE2 (5 mg/kg i.p.), rofecoxib (10 mg/kg i.g.), and SC-560 (5 mg/kg i.g.) significantly attenuated by pretreatment with indomethacin (INDO) and exposed to WRS. Results are mean ± S.E.M from seven animals per each experimental group. The values in AVE 0991 group were compared with vehicle-controls and with those in AVE 0991 given in combination with A-779. Asterisk indicates a significant change (P < 0.05) compared with the respective values in vehicle-control group. Cross indicates a significant change (P < 0.05) compared to values obtained in AVE 0991-treated rats without concomitant treatment with A-779 treatment.

As shown in Fig. 6, the pretreatment with Ang-(1–7) (50 µg/kg i.p.) caused a similar decrease in the number of WRS-induced gastric lesions accompanied by a significant rise in the GBF as presented in Figs. 4 and 5. The exogenous administration of CGRP (10 µg/kg s.c.) in rats with intact sensory nerves resulted in a significant decrease of WRS-induced gastric damage (P < 0.05) and significant increase in the GBF (P < 0.05) compared with respective values achieved with Ang-(1–7) (Fig. 6). The capsaicin denervation tended to increase the mean lesion number and to decrease GBF compared to rats with intact sensory nerves. The reduction in lesion number and an increase in the GBF caused by Ang-(1–7) in rats with intact sensory innervation were almost completely lost in those with capsaicin denervation. The concurrent administration of CGRP combined with Ang-(1–7) significantly reduced the mean lesion number (P < 0.05) and significantly increased GBF in capsaicin-denervated rats (P < 0.05); however, these values were still significantly different from those attained with Ang-(1–7) in rats with intact sensory nerves (Fig. 6).

**Effect of Pretreatment with Ang-(1–7) or Ang II on Plasma Levels of Proinflammatory Cytokines IL-1β and TNF-α in Rats Exposed to WRS.** As shown in Fig. 7, the plasma levels of IL-1β and TNF-α were negligible in intact rats not exposed to WRS. In contrast, the plasma TNF-α and

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<th>Fig. 3. Mean lesion number and the alterations in GBF in gastric mucosa pretreated with vehicle (Veh; control) or AVE 0991 (50 µg/kg i.p.), the Ang-(1–7) receptor agonist, without or with A-779 (the antagonist of Mas receptors; 50 µg/kg i.p.) and exposed to WRS. Results are mean ± S.E.M from six animals per each experimental group. The values in AVE 0991 group were compared with vehicle-controls and with those in AVE 0991 given in combination with A-779. Asterisk indicates a significant change (P &lt; 0.05) compared with the respective values in vehicle-control group. Cross indicates a significant change (P &lt; 0.05) compared to values obtained in AVE 0991-treated rats without concomitant treatment with A-779 treatment.</th>
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<td>Fig. 4. Mean lesion number and the alterations in GBF in gastric mucosa pretreated with vehicle, Ang-(1–7), or perindopril with or without NOSynthase inhibitor (L-NNA, 20 mg/kg i.p.) and exposed to WRS. Results are mean ± S.E.M from six animals per each experimental group. The values in Ang-(1–7) or perindopril groups were compared with vehicle (Veh)-controls and with those administered with Ang-(1–7) or perindopril with concurrent treatment with L-NNA. Asterisk indicates a significant change (P &lt; 0.05) compared with the respective values in vehicle-control group. Cross indicates a significant change (P &lt; 0.05) compared to values obtained in rats without L-NNA treatment.</td>
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<td>Fig. 5. Mean lesion number and the alterations in GBF in gastric mucosa pretreated with Ang-(1–7) without or with the combination of COX-1 and COX-2 inhibitors alone significantly increased the mean lesion number and produced a significant increase in GBF compared with vehicle-treated animals exposed to WRS (data not shown). The reduction of lesion number by Ang-(1–7) (50 µg/kg i.p.) was significantly attenuated by pretreatment with indomethacin (INDO; SC-560 (SC), and rofecoxib (ROFE) without or with the supplementation with prostaglandin E2 (PGE2). Results are mean ± S.E.M from seven animals per each experimental group. The values in Ang-(1–7)-treated animals without concomitant treatment with COX inhibitors were significantly different from those attained with Ang-(1–7) treatment with COX inhibitors. Double crosses indicate a significant change (P &lt; 0.05) compared to the values obtained in AVE 0991-treated rats without concomitant treatment with PGE2. Ang-(1–7) restored the gastroprotective effect of this peptide in the presence of COX-1 and COX-2 inhibitors (P &lt; 0.05), and these effects were accompanied by an increase in GBF similar to that recorded in Ang-(1–7)-treated animals without concomitant treatment with COX inhibitors. Double crosses indicate a significant change (P &lt; 0.05) compared to the values obtained in groups pretreated with INDO, SC, and ROFE in the presence of Ang-(1–7) but without combination with PGE2. <strong>Effect of Pretreatment with Ang-(1–7) or Ang II on Plasma Levels of Proinflammatory Cytokines IL-1β and TNF-α in Rats Exposed to WRS.</strong> As shown in Fig. 7, the plasma levels of IL-1β and TNF-α were negligible in intact rats not exposed to WRS. In contrast, the plasma TNF-α and</td>
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cNOS mRNA was significantly decreased (Fig. 8, right panel). A weak signal of cNOS mRNA was observed in the vehicle-control gastric mucosa exposed to WRS (Fig. 8, right panel). Ratio of cNOS mRNA over β-actin confirmed that mRNA for cNOS was significantly in- decreased in Ang-(1–7)-pretreated animals (P < 0.05) compared to the values obtained in animals treated with Ang-(1–7) alone. Ratio of cNOS mRNA over β-actin confirmed that mRNA for cNOS was significantly decreased (P < 0.05) in rats treated with Ang-(1–7) alone. Ratio of cNOS mRNA over β-actin confirmed that mRNA for cNOS was significantly in- decreased in Ang-(1–7)-pretreated animals (P < 0.05) compared to the values obtained in animals treated with Ang-(1–7) alone. mRNA for cNOS and β-actin were detected as strong signals in gastric mucosa and gastric mucosa. These effects were significantly attenuated with cotreatment with exogenous CGRP and with or without capsaicin denervation as indicated under Materials and Methods.}

**Figure 8.** Mean lesion number and the changes in GBF in rats pretreated with vehicle, Ang-(1–7) (50 μg/kg i.p.), or CGRP (10 μg/kg s.c.) in rats with or without denervation. GBF was measured before and 30 minutes after the exposure to 3.5 hours of WRS. Results are mean ± S.E.M from 10 rats per each experimental group. The asterisk indicates a significant change (P < 0.05) compared with the respective values in Ang-(1–7)-pretreated rats. The cross indicates a significant change (P < 0.02) compared with those in intact or vehicle-pretreated and exposed to WRS. Asterisk and cross indicate a significant change (P < 0.05) compared with the respective values in Ang-(1–7)–pretreated rats. The double cross indicates a significant change (P < 0.05) compared with the respective values in Ang-(1–7)–pretreated rats treated with the combination of A-779 and Ang-(1–7). The values in Ang-(1–7)–pretreated animals administered with the combination of A-779 and Ang-(1–7) are mean ± S.E.M from 10 rats per each experimental group. The asterisk indicates a significant change (P < 0.05) compared with the respective values in Ang-(1–7)–pretreated rats. The cross indicates a significant change (P < 0.02) compared with those in intact or vehicle-pretreated and exposed to WRS.
Fig. 9. Determination of cNOS mRNA, IL-1β, and TNF-α expression by RT-PCR (left panel) and the ratio of cNOS, IL-1β, and TNF-α mRNAs over β-actin mRNA (right panel) in the vehicle (Veh)-control gastric mucosa (lane 1) and in those pretreated with Ang-(1–7) (50 μg/kg i.p.) (lane 2), and A-779 (50 μg/kg i.p.) combined with Ang-(1–7) (50 μg/kg i.p.) (lane 3) and exposed to WRS for 3.5 hours; M, DNA size marker. Mean ± S.E.M. of four determinations in four rats per group. Analysis of the values of the ratio of cNOS, IL-1β, and TNF-α mRNAs over β-actin mRNA expression in gastric mucosa was performed between values in Ang-(1–7)-pretreated and in those treated with combination of A-779 and Ang-(1–7) versus Ang-(1–7) alone. Asterisk indicates a significant change (P < 0.05) compared with vehicle-control gastric mucosa. Cross indicates a significant change (P < 0.05) compared with Ang-(1–7) alone.

Discussion

Our study indicates for the first time that Ang-(1–7), one of the major metabolites of Ang II, contributes to the mechanism of gastroprotection against gastric lesions induced by stress, which is one of the important risk factors for peptic ulcer, hemorrhagic erosions, and microbleedings in animals and humans (Pavel et al., 2015; Konturek et al., 2011). We have shown that parenteral administration of Ang-(1–7) ameliorated in a dose-dependent manner the severity of WRS-induced gastric lesions and this effect was accompanied by the increase in GBF and rise in luminal NO content. Blockade of Mas receptor by A-779 inhibited the Ang-(1–7)-induced protection of superpermeability, while AVE 0991, the agonist of Ang-(1–7) receptors, mimicked the gastroprotective and hyperemic actions of Ang-(1–7). Our results provide the evidence that NO-NOS system and PG-COX pathways could be involved in the protective and hyperemic activities of this Ang I metabolite because this protection and an increase in GBF were reversed by the NOS activity inhibitor L-NNA, and by either nonselective or selective COX-1 and COX-2 inhibitors. We have demonstrated that these protective and hyperemic effects of Ang-(1–7), which disappeared in COX-1- and COX-2-treated animals, have been restored by PGE2 coadministered with this peptide in the presence of COX-1 and COX-2 inhibitors. The involvement of NO in gastroprotection and the hyperemic actions of Ang-(1–7) is further supported by the fact that expression of cNOS was upregulated while expression of iNOS, considered as proinflammatory marker, was downregulated in the gastric mucosa of Ang-(1–7)-pretreated and vehicle-pretreated rats. This gastroprotective and hyperemic effect of Ang-(1–7) was similar to those exhibited by perindopril, a long lasting ACE inhibitor. The protective and hyperemic effects of Ang-(1–7) were lost in rats with capsaicin denervation consistent with the notion that this peptide may trigger the sensory afferent endings to release vasodilatory and protective CGRP. Indeed, the pretreatment with CGRP coadministered with Ang-(1–7) enhanced the protective activity of this Ang I metabolite, resulting in gastric hyperemia but also counteracted the capsaicin-induced gastric impairment and the accompanying fall in the gastric GBF observed in...
Ang-(1–7)-treated rats with deactivated sensory nerves. These findings indicate that sensory neuropeptide CGRP can cooperate with PG and NO in the mediation of Ang-(1–7)-induced gastroprotection and gastric hyperemia against WRS-induced gastric lesions (Fig. 10).

Since stress causes gastric damage of poorly recognized mechanism and etiology, and RAS has been implicated in the pathogenesis of gastric mucosal integrity (Brzozowski et al., 2012) and stress ulcerogenesis (Ender et al., 1993; Kwiecien et al., 2007; Konturek et al., 2011), we determined the effect of vasoactive Ang-(1–7) against stress-induced gastric lesions and compared it with that of Ang II. In clear contrast to Ang-(1–7), the pretreatment with Ang II failed to exert gastroprotection and exacerbated the WRS-induced gastric lesions accompanied by the fall in the GBF. Moreover, Ang-(1–7)—markedly decreased the expression and release of proinflammatory cytokines IL-1β and TNF-α (Szalachci et al., 2013)—suggesting that the anti-inflammatory properties of Ang-(1–7) contribute to protective activity of this Ang I metabolite in the rat stomach (see Fig. 10).

Previous studies documented that AT1-receptor antagonists help to maintain the proper gastric blood perfusion via the reduction of sympathetic neural activity and modulation of inflammatory mediators (Ender et al., 1993; Chung et al., 2010; Garg et al., 2012). Bregonzio et al. (2004) observed that AT1-receptor antagonists help to maintain the proper gastric blood perfusion via the reduction in mucosal neutrophil infiltration and expression of gastric intercellular adhesion molecule 1 and TNF-α (Saavedra et al., 2005, 2006). It is not excluded that the beneficial effect of AT1-receptor antagonists could depend on enhancement of the concentration of angiotensin metabolites Ang-(1–7) and Ang-(9–17) (Neves et al., 2000; Olzanecki et al., 2009), but this hypothesis requires further studies.

Our results show that WRS—increased the expression and plasma levels of TNF-α and IL-1β—and that the plasma level of these proinflammatory cytokines was significantly decreased by Ang II, suggesting that Ang-(1–7) (Ang II, known as a potent vasoconstrictor, exacerbated WRS-induced gastric damage due to its proinflammatory action. This is corroborative with the observations that high levels of circulating Ang-(1–7) ameliorated the metabolic stress induced by high-fat diet via decreased in the proinflammatory profile (adipose tissue cytokines) (Santos et al., 2012). Ang-(1–7) decreases body weight, increased HDL cholesterol, and decreased the expression of COX-2 and IL-1β in abdominal fat of overweight rats (Santos et al., 2012). Moreover, Clarke et al. (2001) reported the direct binding of Ang-(1–7) to the AT1 receptor 1L, resulting in down-regulation of these receptors. In keeping with these findings, we observed decreased expression and plasma levels of IL-1β and TNF-α in rats pretreated with Ang-(1–7), indicating that the decreased difference between Ang-(1–7) and Ang II with respect to proinflammatory cytokines. Moreover, the endogenous Ang II could contribute to pathogenesis of cold-restraint stress ulcer in obstructive jaundice rats (Mou et al., 1998). Enalapril, an inhibitor of ACE, reduced both the gastric and mucosal Ang II level, decreased gastric blood flow, and increased the extent of mucosal damage (Mou et al., 1998). Furthermore, Ang-(1–7) activated an endogenous inhibitor of ACE, enhanced the vasodilatation dose of bradykinin (Tom et al., 2003). In our study, peritoneal significantly decreased WRS-induced gastric lesions and increased GBF with an extent similar to that of Ang-(1–7). L-NNA reduced the gastroprotective and hyperemic activity of perindopril, suggesting that this extension and rise in the GBF caused by ACE inhibitor might be dose mediated by NO. Finally, the luminal content of NO and gastric mucosal expression of mRNA for cNOS were both increased by Ang-(1–7), suggesting that NO derived from cNOS pathway contributes to the beneficial effect of Ang-(1–7) against stress ulcerogenesis. In contrast, the mRNA expression of iNOS was downregulated in these rats, which is consistent with the notion that Ang-(1–7) inhibits WRS lesions due to its potent anti-inflammatory activity. We clearly demonstrated that Ang-(1–7) significantly and dose-dependently attenuated WRS-induced gastric damage while increasing GBF, and these effects were abolished by D-Ala7-Ang-(1–7) (A-779), the selective antagonist of AT1 receptors. Interestingly, the antagonist A-779 has been shown to inhibit most of the physiologic effects of Ang-(1–7) (Santos et al., 2003). Liao et al. (2011) revealed that cardioprotective effect of Ang-(1–7) against ischemia-reperfusion damage is mediated by COX2/PG system responsible for the attenuation of malondialdehyde content and rise in superoxide dismutase activity. The intestinal mucosal COX-2 expression is regulated by both AT1 and AT2 receptors (Tani et al., 2008). Ang-(1–7) stimulated PGE2 release from spontaneously hypertensive rat vascular smooth muscle cells (Jaiswal et al., 1993). In our study, the gastroprotection and increase of

Fig. 10. Proposed mechanisms and factors that may contribute to the gastroprotective action of vasoactive angiotensin metabolite, Ang-(1–7). RAS metabolite Ang-(1–7) acts via specific Mas receptor and stimulates mucosoprotective mechanisms due to an activation of NONOS and COX/PG systems, sensory neuropeptides such as CGRP released from sensory nerves, and the potent inhibition of proinflammatory cytokines and gastric mucosal inflammation.

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GBF evoked by Ang-(1-7) were counteracted by pretreatment with COX-1 and COX-2 inhibitors. For many years, PGs have been considered major cytoprotective mediators that play an important role in various aspects of gastroduodenal protection and ulcer healing (Robert, 1979; Tarnawski et al., 1988; Brzozowski et al., 2006; Takeuchi, 2010). Youssif et al. (2012) revealed that PGs are important intermediaries of the beneficial effects of Ang-(1-7) in cardiac recovery and vascular reactivity in diabetes. Herein, exogenous PGE2 added to Ang-(1-7) in the presence of COX-1 and COX-2 inhibitors restored the gastroprotective and hyperemic activities of this metabolite. Thus, the mechanism through which the Ang-(1-7)/Mas receptor axis induced gastroprotection depends on the activation of COXPG system and endogenous PG.

Sensory nerves were implicated in the mechanism of gastroprotection against various gastric damaging factors, including stress and Helicobacter pylori lipopolysaccharide (LPS) (Brzozowski et al., 2004; Kwiecien et al., 2007). The gastroprotective and hyperemic activities of Ang-(1-7) were markedly impaired in rats with capsaicin-induced functional denervation of sensory nerves. This indicates that besides NO and PG affenter sensory fibers and the major sensory neuropeptide CGRP released from rat sensory nerve endings might markedly impaired in rats with capsaicin-induced functional denervation of sensory nerves. This indicates that besides NO and PG affenter sensory fibers and the major sensory neuropeptide CGRP released from rat sensory nerve endings might markedly impaired in rats with capsaicin-denervated rats compared with those with intact sensory nerves. Thus, it is reasonable to consider that CGRP, which is a potent vasodilator and a neurotransmitter in the stomach, can cooperate with Ang-(1-7) in this protection.

In summary, Ang II and Ang-(1-7) show opposite action against stress ulcerogenesis; because Ang II enhanced stress ulcerogenesis but Ang-(1-7) afforded protection against stress lesions. The mechanism of Ang-(1-7)-induced protection against stress may involve activation of NO/cGMP and PGs system and anti-inflammatory and anti-inflammatory neuropeptides such as CGRP. In contrast to Ang II, Ang-(1-7) exhibits an anti-inflammatory profile that is not expressed in protection of proinflammatory TGF-β1 and NF-κ. Further studies in experimental settings are warranted to further evaluate the potential efficacy of Ang-(1-7) in various experimental models of stress-induced gastric lesions.

Authorship Contributions

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