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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Received June 17, 2013; accepted September 18, 2013

ABSTRACT

Angiotensin-(1–7) (Ang-(1–7)) is a major vasoactive metabolite of angiotensin I (Ang I), both being important components of the renin-angiotensin system (RAS). Ang-(1–7) acting via the Mas receptor was documented in kidneys, heart, liver, and gastrointestinal (GI)-tract. We studied the gastric protective activity of exogenous Ang-(1–7) in stress-induced ulcerogenesis (WRS) without or with A-779 (d-Ala7-ANG-(1–7) receptor antagonist), AVE 0991 (5-formyl-4-methoxy-2-phenyl-[4-[2-(ethylaminocarbonylsulfonamido)-5-isobutyl-3-thienyl]-phenyl]-methyl-imidazole) the activity COX-2 (rofecoxib), and L-NNA (5-formyl-4-methoxy-2-phenyl-[4-[2-(ethylaminocarbonylsulfonamido)-5-isobutyl-3-thienyl]-phenyl]-methyl-imidazole), the activator COX-2 inhibitors (N5-[iminonitroamino)methyl]-l-ornithine) reversed the reduction in lesion number and the rise in GBF evoked by d-Ala7-ANG-(1–7). Ang II augmented the WRS lesions, decreased GBF and increased the plasma IL-1α and TNF-α levels. Capsaicin denervation augmented the reduction of Ang-(1–7)-induced gastric lesions and increase in GBF; these effects were restored by supplementation with calcitonin gene-related peptide (CGRP). The cNOS mRNA was upregulated while iNOS, IL-1α 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 mediated by NO, endogenous prostaglandins, sensory neuropeptides, and anti-inflammatory action involving the inhibition of proinflammatory markers iNOS, IL-1α, and TNF-α.

Introduction

The renin-angiotensin system (RAS) is a critically important system involved in physiologic regulation of blood pressure and water-mineral balance (Paul et al., 2006). Its components of RAS appear to be functionally active in numerous organs including kidneys, heart, brain, reproductive organs, and skin. Angiotensin I (Ang I) and Angiotensin II (Ang II) play an important role in control of gastrointestinal (GI) functions such as the control and electrolyte homeostasis, maintenance of regional blood flow, mucosal absorption of 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 membrane NADPH oxidase in vascular smooth muscle cells (VSMCs), enhances the production of reactive oxygen species such as superoxide, nitric oxide, and peroxynitrite, and increases cell proliferation and migration (Riberi et al., 2006; Hasegawa et al., 1998; Lai et al., 1999; Matsuo et al., 2002). Chronic stimulation of AT1 may lead to cell death, fibrosis, and vascular remodeling and dysfunction (Lai et al., 1999).

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-[4-[2-(ethylicarbonil)sulfonamide]-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; iNOS, inducible nitric-oxide synthase; IL, interleukin; l-OMM, [-iminonitroamino)methyl]-l-ornithine; NOS, nitric-oxide synthase; PG, prostanoid; 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 (H$_{2}$O$_{2}$), and inactivates NO pathway (Mehta and Griendling, 2007). Ang II-activating phospholipase C (PLC) and protein kinase C (PKC) or phospholipase A$_{2}$ 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 (Bregenzo 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 AT$_{1}$ and AT$_{2}$ were detected in the human esophageal, gastric, small intestinal, and colonic mucosa (Hirasawa et al., 2002; Casselbrant et al., 2009; Hallersund et al., 2009). The antagonists of Ang II AT$_{1}$ receptors attenuated gastric injury induced by ischemia-reperfusion, cold stress, and indomethacin-induced damage in rodents due to an inhibition of sympathetic nerve activity and the attenuation of vasoconstrictor and proinflammatory activity of Ang II (Pavel et al., 2004; Morsy et al., 2009; Gemici et al., 2010; Saavedra et al., 2011).

Angiotensin-(1–7) (Ang-(1–7)) is a downstream peptide generated from angiotensin I (Ang I) by kininogenase converting enzyme (ACE) homolog A(1)C (ACE homologous neutral endopeptidase, NEP), also known as nephrilysin (Loeb, 1976). The presence of Ang-(1–7) in the rat stomach and the esophagus was previously detected by immunohistochemical staining (Olszanecki et al., 2009; Konturek et al., 2012). The involvement of endogenous Ang-(1–7) in the gastroprotective effects of exogenous Ang-(1–7) in rats exposed 30 minutes later to 3.5 hours of water immersion and restraint stress (WRS), rats in series A–C received pretreatment with either: A) exogenous Ang-(1–7) (6.25–50 mg/kg i.p.), the nonpeptide Ang-(1–7) receptor antagonist (Bayorh et al., 1999; Santos et al., 2005; Santos and Fereira, 2006) with or without the combination with Ang-(1–7) (5 mg/kg i.p.), the selective Ang-(1–7) Mas receptor antagonist (Bayorh et al., 1999; Santos et al., 2005) or with the combination with Ang-(1–7) (5 mg/kg i.p.) and Ang-(1–7) receptor agonist (Pinheiro SV et al., 2004; Santos and Fereira, 2006), respectively.

In rats, the effects of pretreatment with Ang-(1–7) or perindopril, with or without the combination with l-NNA (20 mg/kg i.p.), the selective inhibitor of NO-synthase activity, on WRS lesions and adrenals in the GI tract were determined.

The experimental design and the endogenous PG in the gastroprotective effects of Ang-(1–7) or vehicle (control) was investigated in rats treated with indomethacin (5 mg/kg i.p.), the nonselective Cox-1 and Cox-2 inhibitor, or SC-560 (5 mg/kg i.p.), the selective inhibitor of COX-1, and rofecoxib (10 mg/kg i.p.), the selective inhibitor of COX-2 activity as reported in our previous studies (Bregenzo et al., 2000, 2006; Satoh et al., 2013). In another subgroup with COX-1 and COX-2 inhibitors, rats of series F were coadministered with exogenous prostaglandin E$_{2}$ (PGE$_{2}$; 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.

Materials and Methods

Animals. Male Wistar rats (total 254) with weight averaging about 250 g were used for the 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 was conducted according to the guidelines of the Helsinki Declaration regarding animal research.
of Chemical (Ann Arbor, MI) and Pfizer (Ilertissen, Germany), respectively.

**Measurement of GFB and Determination of Gastric Lesion Number.** At the termination of 3.5 hours WRS, rats were anesthetized with pentobarbital (60 mg/kg i.p.), the abdomen was opened, and GFB measured by means of H2-gas clearance technique as reported before (Brzozowski et al., 2004, 2006; Kwiecien et al., 2007). The GFB 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 change 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 described 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. A control sample (50 μl) was incubated with biotinylated antibodies specific for TNF-α and IL-1β, washed three times with assay buffer and finally conjugated with streptavidin peroxidase complex with a stabilized chromogen as described previously (Kwiecien et al., 2012b).

The expression mRNA of cNOS, iNOS, and TNF-α in the rat gastric mucosa determined by reverse transcriptase-polymerase chain reaction (RT-PCR) was measured in each group. Mucosal specimens were scraped using a glass slide and immediately snap-frozen in liquid nitrogen and stored at −80°C until analysis. Total RNA was extracted from each sample by a guanidium isothiocyanate-phenol-chloroform method using a kit from Stratagene (La Jolla, CA) according to the manufacturer's instructions. RNA concentration in each sample was determined by 1% agarose gel electrophoresis and ethidium bromide staining. Aliquots RNA samples were used at final concentration of 0.5 μg/μl. Total RNA (5 μg) was incubated with biotinylated antibodies specific for TNF-α and IL-1β, washed three times with assay buffer and finally conjugated with streptavidin peroxidase complex with a stabilized chromogen as described previously (Kwiecien et al., 2012b).

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**Statistical Analysis.** Results of all measurements were expressed as mean ± S.E.M. and the statistical analysis was performed with two-way analysis of variance (ANOVA) test and Tukey post hoc test where appropriate. Differences between estimates of effects were considered significant at P < 0.05. All results in the treated animals were compared with the appropriate control group, which had been established for each set of experiments. Dependent variables were expressed both in percentage of control for GBF and in absolute values for lesion number. The control rats did not differ from experimental groups in terms of relevant characteristics, such as source of pair, gender, age, weight, diet, and housing conditions. There was no individual pairing of animals, the paired statistical test was not used.

**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 GFB (Fig. 1). The pretreatment with Ang II applied in a dose of 5 μg/kg failed to significantly affect the mean lesion number and GFB compared with vehicle-control.

### Table 1

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer Sequence</th>
<th>Annealing Temperature °C</th>
<th>Size of PCR Product bp</th>
</tr>
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<tbody>
<tr>
<td>c-NOS</td>
<td>Forward: 5'- TAC GGA GCA GCA AAT CCA C-3', Reverse: 5'- CAG COT GCA GTG CT TGA TC-3'</td>
<td>63.5</td>
<td>540</td>
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<tr>
<td>IL-1β</td>
<td>Forward: 5'- GCT ACC TAT GTG TTT CCC GT-3', Reverse: 5'- GAC CAT TGC TGT TTC CTA GG-3'</td>
<td>62</td>
<td>543</td>
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<tr>
<td>TNF-α</td>
<td>Forward: 5'- TAC TGA ACT TCC GGG TGA TTG TGC C-3', Reverse: 5'- CAG CCT TGG CCC TTT AAG AGG ACC-3'</td>
<td>56</td>
<td>295</td>
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<tr>
<td>β-Actin</td>
<td>Forward: 5'- TTG TAA CCA ACT GGG ACG ATA TGG-3', Reverse: 5'- GAT CTT CAT GTT GCT GTT 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 TGG TCA GCG TGC TC-3'</td>
<td>60</td>
<td>397</td>
</tr>
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</table>
The administration of Ang II in higher doses ranging from 6.25 to 40 μg/kg dose-dependently increased the 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 reduced WRS-induced gastric lesions, while producing a significant and a 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 (P < 0.05) compared with the values in the intact gastric mucosa. This fall in GBF under WRS conditions was significantly worsened by the pretreatment with Ang II. In contrast, the pretreatment with Ang-(1–7) resulted in a significant increase in the GBF (P < 0.05) compared with the pretreatment with vehicle. The Ang-(1–7)-induced protection was accompanied by the rise in the GBF and luminal NO content observed at the 50 μg/kg dose of this peptide, as completely evoked by the pretreatment with A-779 (50 μg/kg i.p.) combined with intraperitoneal AVE 0991 with Ang-(1–7) (Fig. 2; Table 2).

**Effect of Ang-(1–7) on Induction of Gastric Damage and Alterations in 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 produced a significant decrease 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 evoked by the combination of A-779 and AVE 0991 (P < 0.05).

**Effect of Suppression of NO-Synthase on Ang-(1–7)- and Perindopril-Induced Gastroprotection 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. 3. The pretreatment with perindopril (5 mg/kg i.p.) also significantly decreased the number of WRS-induced gastric lesions (P < 0.05) and significantly increased 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 Ang-(1–7) or perindopril evoked by Ang-(1–7) or perindopril (Fig. 4).

**Effect of COX-1/PG and COX-2/PG Suppression on Ang-(1–7)-Induced Gastroprotection against WRS-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 lesion number compared to vehicle-control and Ang-(1–7) alone (P < 0.05).

**Table 2**

Effect of pretreatment with vehicle (Veh), Ang II (40 μg/kg i.p.), and Ang-(1–7) (50 μg/kg i.p.) with or without combination with A-779 (50 μg/kg i.p.) on changes in GBF expressed in absolute values (ml/min per 100 g) in gastric mucosa of rats exposed to WRS

<table>
<thead>
<tr>
<th>Type of Test</th>
<th>GBF</th>
<th>ml/min per 100 g</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intact</td>
<td>46</td>
<td>± 2.8</td>
</tr>
<tr>
<td>Veh + WRS</td>
<td>27</td>
<td>± 2.2</td>
</tr>
<tr>
<td>Ang II + WRS</td>
<td>21</td>
<td>± 1.6**</td>
</tr>
<tr>
<td>Ang-(1–7) + WRS</td>
<td>35</td>
<td>± 2.7**</td>
</tr>
<tr>
<td>A-779 + Ang-(1–7) + WRS</td>
<td>26</td>
<td>± 2.2*</td>
</tr>
</tbody>
</table>

Results are mean ± S.E.M. from seven animals per each experimental group. Asterisk and cross indicate a significant change (P < 0.05) below or above values obtained in rats pretreated with Ang II and Ang-(1–7). Cross indicates a significant change (P < 0.05) compared with the value in Ang-(1–7) alone.
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 seven animals per each experimental group. The values in AVE 0991 group were compared with vehicle-controls and with those in AVE 0991 treated group with concomitant treatment with COX-1 and COX-2 inhibitors. Cross indicates a significant change (P < 0.05) compared with respective values in vehicle-control group. Results are mean ± S.E.M. from seven rats per each experimental group. The effect of Ang-(1–7) lesion number and produced a significant fall in GBF compared with vehicle-controls and with those in AVE 0991 group. Cross indicates a significant change (P < 0.05) compared with respective values in vehicle-control group.

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. of rats not exposed to WRS. In contrast, the plasma TNF-α and interleukin-1β (IL-1β) levels were significantly reduced in the Ang-(1–7) group compared to the values obtained in group treated with INDO, SC, and ROFE in the presence of Ang-(1–7) but without combination with PGE2.

Fig. 5. Mean lesion number and the alterations in GBF in gastric mucosa pretreated with vehicle (Veh; control) or Ang-(1–7) without or with concomitant treatment with COX-1 and COX-2 inhibitors alone significantly increased the mean lesion number and produced a significant fall in GBF compared with vehicle-treated animals exposed to WRS (data not shown). The reduction in lesion number by Ang-(1–7) (100 μg/kg i.p.) pretreated with vehicle was significantly attenuated by pretreatment with indoethacin (INDO) or SC-560 (SC), and rofecoxib (ROFE) without or with the combination of Ang-(1–7) (50 μg/kg i.p.). Results are mean ± S.E.M. from seven rats per each experimental group. The effect of Ang-(1–7) on lesion number and with COX-1 and COX-2 inhibitors in the presence or the absence of Ang-(1–7) restored the gastroprotective effect of this peptide in the presence of COX-1 and COX-2 inhibitors (P < 0.05), and these effects were accompanied by an increase in GBF similar to that observed in Ang-(1–7)-treated animals without concomitant treatment with COX-inhibitors. Double crosses indicate a significant change (P < 0.05) compared to the values obtained in group treated with INDO, SC, and ROFE in the presence of Ang-(1–7) without or with the combination with PGE2.

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 interleukin-1β (IL-1β) levels were significantly reduced in the Ang-(1–7) group compared to the values obtained in group treated with INDO, SC, and ROFE in the presence of Ang-(1–7) but without combination with PGE2.

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IL-1β levels were significantly increased in vehicle-pretreated rats exposed to WRS (P < 0.02). The further significant rise in plasma levels of IL-1β and TNF-α was observed in the group administered with Ang II (50 mg/kg i.p.) compared with those pretreated with vehicle and exposed to WRS (Fig. 8). In contrast, Ang-(1–7) (50 mg/kg i.p.) significantly decreased (P < 0.05) compared with the respective values in Ang-(1–7)–pretreated group (Fig. 8, left panel). The values in Ang-(1–7)– and Ang II–pretreated rats were compared with those in intact or vehicle-pretreated and exposed to WRS. Asterisk indicates a significant change (P < 0.05) compared to the values obtained in animals treated with Ang-(1–7)–pretreated group with capsaicin denervation without cotreatment with exogenous CGRP and with or without capsaicin denervation as indicated under Materials and Methods. Cross indicates a significant change (P < 0.05) compared with the respective values in Ang-(1–7)–pretreated group. The values in Ang-(1–7)– and Ang II–pretreated groups with or without cotreatment with exogenous CGRP and with or without capsaicin denervation were significantly attenuated in Ang-(1–7)–pretreated rats compared with those administered with Ang-(1–7) alone (Fig. 8, right panel).

In contrast, strong signals for IL-1β and TNF-α mRNAs were observed when rats received the combination of A-779 and Ang-(1–7) compared with those treated with Ang-(1–7) alone (Fig. 7, left panel). The ratio of IL-1β and TNF-α over β-actin confirmed that Ang-(1–7) significantly decreased expression of mRNAs for IL-1β and TNF-α and this effect was reversed in animals administered with the combination of A-779 and Ang-(1–7) (Fig. 8, right panel).

Figure 9 (upper panel) demonstrates that the signal for iNOS mRNA was negligible in the intact gastric mucosa, but mRNA for iNOS was detected as strong signal in gastric mucosa exposed to WRS, and this effect was significantly decreased in those pretreated with Ang-(1–7). The ratio of iNOS mRNA over β-actin confirmed that mRNA for iNOS was significantly increased in rats exposed to WRS when compared with that in the intact gastric mucosa and this effect was significantly attenuated in those pretreated with Ang-(1–7) (Fig. 9, lower panel). The decrease in iNOS mRNA expression observed in Ang-(1–7)-pretreated animals was reversed in those concomitantly treated with A-779. The ratio of iNOS mRNA over β-actin confirmed that mRNA for iNOS was significantly increased when A-779 was combined with Ang-(1–7) (Fig. 9, lower panel).

**Fig. 6.** 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 intact sensory nerves and in those with functional ablation of sensory nerves by capsaicin (capsaicin denervation) and exposed to 3.5 hours of WRS. To induce the functional ablation of sensory nerves, 25 rats were injected with capsaicin in total dose of 125 mg/kg s.c. for 3 consecutive days at a respective doses of 25 mg/kg s.c. (day 1), 50 mg/kg s.c. (day 2), and 50 mg/kg s.c. (day 3) approximately 2 weeks before the experiment. Results are mean ± S.E.M from six rats per each experimental group. Statistical comparisons were made between Ang-(1–7)–pretreated gastric mucosa and the vehicle-control group. Asterisk indicates a significant change (P < 0.05) compared with the respective values in Ang-(1–7)–pretreated group. The values in Ang-(1–7)– and Ang II–pretreated rats were compared with those in intact or vehicle-pretreated and exposed to WRS. Asterisk and cross indicate a significant change (P < 0.05) compared to the values obtained in animals with intact sensory nerves treated with Ang-(1–7). Cross indicates a significant change (P < 0.05) compared with the values obtained in animals with intact sensory nerves treated with Ang-(1–7). Double crosses indicate a significant change (P < 0.05) compared with CGRP-treated group without capsaicin denervation.

**Fig. 7.** The alteration of plasma IL-1β and TNF-α levels in intact and those pretreated with vehicle (Veh; control), angiotensin II (ANG II, 40 µg/kg i.p.) or Ang-(1–7) (50 µg/kg i.p.) before the exposure to 3.5 hours of WRS. Results are mean ± S.E.M from 10 rats per each experimental group. The values in Ang-(1–7)– and Ang II–pretreated rats were compared with those in intact or vehicle-pretreated and exposed to WRS. Asterisk indicates a significant change (P < 0.05) compared with the respective values in intact rats. Cross indicates a significant change (P < 0.05) compared with the respective values in Ang-(1–7)-pretreated rats. Double crosses indicate a significant change (P < 0.05) compared with the values in vehicle-control and ANG II groups.
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., 2008; 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 GFB and rise in luminal NO content. Blockade of Mas receptor by A-779 inhibited the Ang-(1–7)-induced protection and hyperemia, 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 GFB 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 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 GFB observed in WRS-exposed rats (Fig. 8).
Ang-(1–7)-treated rats with deactivated sensory nerves. These findings indicate that sensory neuropeptide CGRP can cooperate with PG and NO in the mechanism 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 (Szlachcic et al., 2013) suggesting that the anti-inflammatory properties of Ang-(1–7) could 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 attenuation of inflammatory mediators (Ender et al., 1993; Chung et al., 2006). Similarly, AT1-receptor antagonists dose-dependently attenuated gastric ulcer formation in rodents (Merai et al., 2009; Morsy et al., 2009) and counteracted the effects of ischemia and inflammation on the reduction of 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; Olszanecki 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 indicating that a plasma level of these proinflammatory cytokines was not attenuated by Ang II, suggesting that the blockade of AT1 receptors, Ang II, known as a potent vasoconstrictor, aggravated WRS-induced gastric damage due to its proinflammatory action. This is corroborative with the observations that high levels of circulating Ang-(1–7) protected the gastric stress induced by the high-fat diet via decrease in the proinflammatory profile (adipose tissue cytokine, Santos et al., 2012). Ang-(1–7) decreased body weight, increased HDL cholesterol, and decreased expression of COX-2 and IL-1β in abdominal fat of overweight rats (Saavedra et al., 2012). Moreover, Clarke et al. (2001) reported the direct binding of Ang-(1–7) to the Ang I receptor, leading to downregulation 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), further highlighting the apparent 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) activity as an endogenous inhibitor of ACE, enhanced the vasodilator effects of bradykinin (Tom et al., 2003). In our study, perindopril, a significantly decreased WRS-induced gastritis and increased GBF with an extent similar to that observed with Ang-(1–7), L-NNA reduced the gastroprotective and hyperemic activity of perindopril, suggesting that this action and rise in the GBF caused by ACE inhibitor might be at least in part 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 Mas 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 COX/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., 2003). 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
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). Yusif 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 ablation of sensory fibers. This indicates that besides NO and PG-afferent sensory fibers and the major sensory neupeptide CGRP released from rat sensory nerve endings might cooperate with Ang-(1–7) in the gastroprotection and adaptation of gastric mucosa to Helicobacter pylori-lipopolysaccharide challenge. 

References

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