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) that acts via Mas receptor. The study aimed at investigating the prevention of stress-ulcerogenesis. The WRS lesions were dose-dependently reduced by pre-treatment with Ang-(1–7), which also increased the gastric blood flow (GBF) and renal content of NO. COX-1 and COX-2 inhibitors, Nω-nitro-L-arginine-methyl ester (l-NAME) and L-arginine (L-Arg), nitric-oxide synthase, inducible nitric-oxide synthase, and nitric-oxide synthase, the suppression of cyclo-oxygenase (COX)-1 and COX-2 (rofecoxib), and denervation with capsaicin. The mRNA expression of constitutively expressed nitric-oxide synthase (cNOS), inducible nitric-oxide synthase (iNOS), and tumor necrosis factor (TNF-α) was assessed by reverse transcription polymerase chain reaction. The WRS ulcerogenesis was sustained by NO, endogenous prostaglandins, sensory neuro-peptides, and anti-inflammatory action involving the inhibition of proinflammatory markers iNOS, IL-1β, and TNF-α.

Introduction

The renin-angiotensin system (RAS) is a classic endocrine system involved in the regulation of blood pressure and glucose, gastrointestinal motility, mucosal secretion, gastric inflammation, and carcinogenesis (Fandriks 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, and serves as a major source of reactive oxygen species.

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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; i.g., intragastric; iNOS, inducible nitric-oxide synthase; IL, interleukin; l-NAME, Nω-nitro-L-arginine(methyl)-l-ornithine; NO, 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 (H$_2$O$_2$), and inactivates NO pathway (Mehta and Griendling, 2007). Ang I-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 (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 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., 2011). 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 thalamroadrenal axis and the attenuation of vasoconstrictor and proinflammatory activity of Ang II (Pavel et al., 2003; Morsy et al., 2009; Gemici et al., 2010; Saavedra et al., 2011).

Antagonists of AT$_1$ receptor cadoxen and candoxan prevented stress-induced gastric lesions (Tejera et al., 2003,2004); Merai et al. (2009).

Angiogenin-1 (Ang-1) is a known proangiogenic factor generated from angiogenin I through the renin-converting enzyme (ACE) homolog ACE2 or neutral endopeptidase (NEP), also known as nephrinase because the discovery of Ang-1 in 1976, the presence of this heptapeptide has been detected in brain, blood vessels, heart, kidney, liver, and stomach (Santos et al., 2005; Xu et al., 2011). Ang-1 (7) acting via its own G protein-coupled receptor called Mas (Santos et al., 2003; Stegbauer et al., 2004) exhibit the vaso dilatory, antihypertensive, cardioprotective, and antifibrotic effects. Ang I is quickly converted to Ang-1 (7) in the rat stomach, as indicated by the formation of Ang-1 (7) in the stomach of Ang-1 (7) transgenic (Giannetti et al., 2009). Mas receptor knockout mice show that the Ang-1 (7) increase in NO synthesis, and the decrease of eNOS expression, suggesting a link between Ang-(1–7) and Mas receptor (Xu et al., 2008). The vasoconstrictive action of Ang II in hypertension is limited by vasoactive Ang-(1–7) and bradykinin (Oliveira et al., 2009; Incalleco et al., 2009; Sampaio et al., 2007). Ang-1 (7) exhibited endothelial protection against reflex esophagitis (Prior et al., 2012). Whether Ang-1 (7) protects the gastric mucosa against stress lesions due to an increase of NO and the activity of prostaglandin (PG)COX-1 and PG/COX-2 pathways and sensory nerves has not been extensively studied before.

We compared the effects of endogenous Ang-(1–7) and Ang II on stress-induced gastric lesions and accompanying changes in the gastric blood flow (GBF). The involvement of endogenous PG and NO as well as the activity of afferent sensory nerves in the mechanism of gastroprotection induced by Ang-(1–7) was investigated by testing the effect of exogenous Ang-(1–7) against stress ulcerogenesis in the presence of NO-synthase inhibitor L-NNA, nonselective and selective COX-1 and COX-2 inhibitors, as well as in rats with capsaicin denervation. We also assessed the effect of Ang-(1–7) on the expression of mRNA for constitutively expressed nitric-oxide synthase (eNOS), 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 rats total 25−50 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 statements of the Helsinki Declaration regarding handling and use of animals. The dissections of the stomach and intestinal segments were performed according to the guidelines set by the Institutional Animal Care and Use Committee of Jagiellonian University Medical College.

**Application.** To induce gastric lesions, rats were immobilized in an individual Polman cages and immersed in an ice-water (23°C) for 3.5 hours. The mean xyphoid level was reported as in our previous studies (Brzozowski et al., 2000, Konturek et al., 2001). In separate major experiments, rats were divided into three groups. For instance, in G1–5 were selected. Thirty minutes before exposure to water immersion and restraint stress (WRS), rats in series A–C received pretreatment (with one of: A) exogenous Ang-(1–7) (6.25–50 μg/kg i.p.), B) Ang-(1–7) Mas receptor antagonist (Bayorh et al., 1999; Santos et al., 2005), or C) perindopril (5 mg/kg i.p.), the Mas receptor antagonist (Bayorh et al., 2012). The angiotensin Mas receptor agonistic and agonistic activities were determined in a separate group of rats (series D) treated with A–779 (5 mg/kg i.p.), the selective AT$_2$ receptor antagonist (Bayorh et al., 1999; Santos et al., 2005). With or without the combination with Ang-(1–7), rats were exposed 30 minutes later to 3.5 hours of WRS. In another series of rats, AVE 0991 (2 μg/kg i.p.), the nonpeptide Ang-(1–7) receptor agonist (Pinheiro SV et al., 2004; Santos and Fereira, 2006), respectively.

In order to assess the effects of cotreatment with Ang-(1–7) or perindopril, with or without the combination with l-NNA (20 mg/kg i.p.), a competitive inhibitor of NO-synthase activity, on WRS lesions and acute stress-provoked changes in COX-2, were determined.

The involvement of endogenous PG in the gastroprotective effects of Ang-(1–7) vehicle (control) was investigated in rats (series E) 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 (Brzozowski et al., 2000, 2006; Sato 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 Labormchemikalien (Schelldorf, Germany) except of SC-560 and rofecoxib purchased from Cayman.
Chemical (Ann Arbor, MI) and Pfizer (Illertissen, Germany), respectively.

**Measurement of GBF 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 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 described in detail 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. The 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 conjugate and streptavidin with a stabilized chromagen as described elsewhere (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).** The stomachs were removed from rats exposed to WRS without or with the pretreatment with Ang-(1–7) alone or combined with Ang II. Total RNA was extracted from the tissue samples by a guanidium isothiocyanate/phenol chloroform method using a kit from Stratagene (Heidelberg, Germany). RNA was treated with a mixture of deoxyadenosine triphosphate (dATP), deoxyribothymidine triphosphate (dTTP), deoxyguanosine triphosphate (dGTP), and deoxycytidine triphosphate (dCTP), 5 ml of 10 × RT buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl, 5 mM MgCl₂). The resultant cDNA (2 μl) was amplified in a 20 μl reaction volume containing 0.3 ml (2.5 IU) Taq polymerase, 0.1 mM (each) dNTP (Pharmacia, Germany), 1.5 mM μl MgCl₂, 5 ml Taq polymerase chain reaction buffer (50 mM KCl, 10 mM Tris-HCl, pH 7.5), and primers used at final concentration of 0.5 μM. The mixture was overlaid with 25 μl of mineral oil to prevent evaporation. The polymerase chain reaction mixture was amplified in a DNA thermal cycler (Perkin-Elmer/Cetus, Norwalk, Conn) in the area dedicated for performing PCR reaction. The quality of DNA as the source for primers for cNOS, IL-1β, TNF-α, and β-actin as presented in Table 1 were constructed based on published cDNA for these factors. The primers were synthesized by Life/ThermoFisher Scientific Technologies (Eggenstein, Germany) with specific primers for cNOS, iNOS, IL-1β, and TNF-α mRNA.

**Statistical Analysis.** Results of the experiment were expressed as mean ± S.E.M. and 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 by 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 rats in terms of relevant characteristics, such as source of purchase, gender, age, weight, diet, and housing conditions. There was no individual pairing of animals, the paired statistical analysis 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 GBF 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>
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<tr>
<td>c-NOS</td>
<td>Forward: 5'-TAC GGA GCA GCA AAT CCA C-3', Revers: 5'-CAG GCT GCA GTC CTT TGA TC-3'</td>
<td>63.5</td>
<td>540</td>
</tr>
<tr>
<td>IL-1β</td>
<td>Forward: 5'-GCT ACC TAT GTC TTT CCC GT-3', Revers: 5'-GAC CAT TGC TGT TTT 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 TCG GGG TGA TTC TGT C-3', Revers: 5'-CAG CCT TGG CTT AAG AGA ACC-3'</td>
<td>56</td>
<td>295</td>
</tr>
<tr>
<td>β-Actin</td>
<td>Forward: 5'-TTG TAA CCA ACT GGG ACG ATA TGG-3', Revers: 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'-CAA CAA TAG TAC AAT ACT AC-3', Revers: 5'-ACG AGG TGT TCA GCG TGC TC-3'</td>
<td>60</td>
<td>397</td>
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The administration of Ang II in higher doses ranging from 10 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 dose-dependent increase in GBF and luminal NO content (Fig. 2). The dose of Ang-(1–7) inducing a 50% reduction in lesion number (ID50) was 50 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 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, 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 GBF and luminal NO content observed at the 50 μg/kg dose of this peptide, as completely reversed by the pretreatment with A-779 (50 μg/kg i.p.) combined with intraperitoneal treatment with Ang-(1–7) (Fig. 2; Table 2).

**Effect of AVE 0991, the Agonist of Ang-(1–7) Mas Receptor, on WSS-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-control pretreated rats. The decrease in lesion number and an increase in GBF induced by AVE 0991 were completely reversed 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 Gastric Lesions 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 reduction 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 Gastroprotection Against WSS-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 to vehicle-control pretreated rats. Administration of A-779 (50 μg/kg i.p.) combined with the respective values in intact gastric mucosa. The combination of Ang-(1–7) and A-779, as indicated under **Materials and Methods.** Asterisk indicates a significant change (P < 0.05) below or above values obtained in rats treated with Ang II and Ang-(1–7).

### Table 2

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

*P < 0.05, **P < 0.005 for comparison with control groups.
gastric lesions accompanied by a significant fall in GBF as presented in Fig. 2. The pretreatment 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 of lesion number by Ang-(1–7) (50 μg/kg i.p.) was significantly attenuated by pretreatment with indomethacin (5 mg/kg i.p.), rofecoxib (5 mg/kg i.g.), and SC-560 (5 mg/kg i.g.) (P < 0.05), and these effects were accompanied by a significant fall in GBF (Fig. 5). The addition of PGE2 (5 μg/kg i.g.) to 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 control was carried out with a significant fall in GBF (Fig. 5). 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...
IL-1β levels were significantly increased in vehicle-pretreated rats exposed to WRS (P < 0.02). The further significant rise in plasma IL-1β and TNF-α was observed in the group administered with Ang-(1–7) (50 μg/kg i.p.) compared with those pretreated with vehicle and exposed to WRS (Fig. 7). In contrast, Ang-(1–7) (50 μg/kg i.p.) significantly decreased (P < 0.05) the plasma levels of IL-1β and TNF-α compared to 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 with intact sensory nerves treated with Ang-(1–7). Cross indicates a significant change (P < 0.05) compared to the values obtained in animals with intact sensory nerves treated with Ang-(1–7) and those pretreated with Ang-(1–7) alone. Double crosses indicate a significant change (P < 0.05) compared with the values in vehicle-control and ANG II groups.

Angiotensin & Plasma Cytokine Levels in Stress Ulcerogenesis

The alterations 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.) for Ang-(1–7) (50 μg/kg i.p.) before exposure to 3.5 hours of WRS. Results are mean ± S.E.M from 10 rats per experimental group. The values in Ang-(1–7)-pretreated rats were compared with those in intact or vehicle-pretreated and exposed to WRS. Asterisk indicates a significant change (P < 0.02) compared with the respective values in intact rats. Asterisk and cross indicate a significant change (P < 0.05) compared with the respective values in Ang-(1–7)-pretreated rats. Cross indicates a significant change (P < 0.05) compared with the values in vehicle-control and ANG II groups.

Figure 8

The alteration of plasma IL-1β and TNF-α levels in intact and those pretreated with vehicle (Veh; control), Ang-(1–7) (50 μg/kg i.p.), or Ang-(1–7) alone. Ratio of iNOS mRNA over β-actin confirmed that mRNA for iNOS was significantly increased in rats exposed to WRS. The decrease in iNOS mRNA was observed when rats received the combination of A-779 and Ang-(1–7) alone and those administered with Ang-(1–7) alone (Fig. 8, right panel).

Ang-(1–7) and those pretreated with Ang-(1–7) alone (Fig. 8, 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). 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). The ratio of iNOS mRNA over β-actin confirmed that mRNA for iNOS was significantly increased in rats exposed to WRS when compared with Ang-(1–7) alone. Ratio of cNOS mRNA over β-actin confirmed that cNOS mRNA was significantly decreased (P < 0.05) in rats treated with the combination of A-779 and Ang-(1–7) compared with those administered with Ang-(1–7) alone (Fig. 8, right panel).
**Fig. 9.** Determination of iNOS 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), A-779 (50 μg/kg i.p.) (lane 3), and A-779 (50 μg/kg i.p.) combined with Ang-(1–7) (50 μg/kg i.p.) (lane 4) 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 mRNA expression in gastric mucosa was performed between values in Ang-(1–7)-pretreated and in those 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., 2011; 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 protective action in rat 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, cNOS 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 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 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 (Szlauchcic 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 activation of inflammatory mediators (Ender et al., 1993; Konturek et al., 2011). Breganzio et al. (2009) observed that AT1 blockade led to increased adrenal corticosterone, the reduction of sympathetic neural activity and by attenuants help to maintain the proper gastric blood perfusion via during stress (Filaretova et al., 1998; Pavel et al., 2008).

Influence gastroprotective action of glucocorticoids released in animals. However, the blockade of AT1 receptors does not reduce inflammation and plasma levels of IL-1α and TNF-α in rats pretreated with Ang-(1–7), in contrast, the more difference between Ang-(1–7) and Ang II with respect to proinflammatory cytokines. Moreover, the endogenous Ang II could contribute to pathogenesis of cold-trauma stress ulcer in obstructive jaundice rats (Mou et al., 1998). Enalapril, an inhibitor of ACE, reduced both the hypergastrinemia and gastric mucosal Ang II level, decreased gastric blood flow, and increased the extent of mucosal damage (Mou et al., 1998). Furthermore, Ang-(1–7) attenuates the endogenous inhibitor of ACE, enhanced the vasodilator effects of bradykinin (Tom et al., 2003). In our study, perindopril significantly decreased WRS-induced gastropathies and increased GBF with an extent similar to that observed with Ang-(1–7). L-NNA reduced the gastroprotective and hypotensive activity of perindopril, suggesting that this beneficial action 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 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., 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
Gastroprotection by Angiotensin-(1–7) Against Stress Damage

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 gastrodudodenal protection and ulcer healing (Robert, 1979; Tarnawski et al., 1988; Brzozowski et al., 2006; Takeuchi, 2010). Yousif 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 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; Kwiecień et al., 2007). The gastroprotective and hyperemic activities of Ang-(1–7) were markedly impaired in rats with capsaiacin-induced functional ablation of sensory fibers. This indicates that besides NO and PG afferent sensory fibers and the major sensory neuropeptides CGRP released from rat sensory nerve endings may mediate Ang-(1–7)-induced protection and hyperemia. Endogenous CGRP in the presence of Ang-(1–7) restored this protection in part, and gastric hyperemia in rats in capsaicin denervation; however, this increase in GBF was significantly less pronounced which is a potent vasodilator and protective factor in the stomach, however, this increase in GBF was significantly less pronounced

References