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 angiotensin I (Ang I), both being important components of the renin-angiotensin system (RAS). Ang-(1–7) acting via AT1 receptor was documented in kidneys, heart, brain, and gastrointestinal (GI)-tract. We studied the gastroprotective activity of exogenous Ang-(1–7) in rats exposed to water immersion and restraint stress (WRS) without or with A-779 [d-Ala7-Ang-(1–7)] (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; iNOS, inducible nitric-oxide synthase; IL, interleukin; 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.

Intragastric or subcutaneous administration of Ang-(1–7) in WRS rats significantly reduced gastric lesions and increased gastric blood flow (GBF). Ang-(1–7) pretreatment with Ang-(1–7) or capsaicin resulted in the suppression of cyclo-oxygenase (COX)-2 activity and in the inhibition of nitric-oxide synthase (iNOS), interleukin (IL)-1β, and tumor necrosis factor (TNF)α. These effects were restored by supplementation with calcitonin gene–related peptide (CGRP). The cNOS mRNA was upregulated while iNOS, IL-1β, and TNFα mRNAs were downregulated in Ang-(1–7)-pretreated rats. We conclude that Ang-(1–7), in contrast to Ang II, which worsened WRS ulcerogenesis, affords potent gastroprotection against WRS ulcerogenesis via an increase in GBF and NO production, which results in reduced gastric lesion number and the rise in GBF.

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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-(ethyleniminocarbonylsulfonamido)-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-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., 2009). 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 adrenergic 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; Santos et al., 2011).

Antagonists of AT₁ receptor candesartan and losartan prevented stress-induced gastric lesions (Konturek et al., 2003,2004; Merai et al., 2009). Angiotensin-(1–7) [Ang-(1–7)] is a downstream peptide generated from angiotensin I through ACE2, the angiotensin-converting enzyme (ACE) homolog ACE₂ or neutral endopeptidase (NEP), also known as nephrilysin. Since the discovery of Ang-(1–7) in 1976, the presence of this heptapeptide has been detected in brain, blood vessels, heart, kidney, liver, and stomach (Santos et al., 2005; Xie et al., 2011). Ang-(1–7) acting via its own G protein-coupled receptor, called Mas (Santos et al., 2001; Stegbauer et al., 2008) exhibit the vasodilatory, antihypertensive, cardioprotective, and anti-thrombotic effects. Ang I is quickly degraded to Ang-(1–7) in the rat stomach and the formation of Ang-(1–7) may even precede Ang I conversion to Ang II (Groszczynski et al., 2009). Mas receptor knock-out mice show an increased vulnerability to ischemia, decrease in NO synthesis, and accumulation of eNOS expression, suggesting a link between AT₁ and Mas receptor (Xu et al., 2008). The vasoconstrictive fraction of Ang II in hypertension is limited by vasoactive Ang-(1–7) and bradykinin (Oliveira et al., 2004; Sampaio et al., 2007). Ang-(1–7) exerted endothelial protection against reflux esophagitis (Pinheiro SV et al., 2004; Santos and Fereira, 2006) and reduced 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 were used in the study. Rats were fasted for 4 hours with free access to drinking 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 conducted according to the guidelines for the use of experimental animals.

**Stress-Induced Gastric Lesions, Chemicals, and Drug Application.** To induce gastric lesions, rats were immobilized in an individual Bolman cages and immersed in ice water (23°C) for 3.5 hours of WRS or AVE 0991 (50 g/kg i.p.), the long-lasting ACE inhibitor (Jawien et al., 2012). The antagonists of AT₁ receptor antagonistic and agonistic activities were determined in a separate group of rats (series D) treated with A-779 (5 mg/kg i.p.), the selective Ang-(1–7) receptor agonist (Bayorh et al., 1999; Santos et al., 2006) or with the combination of Ang-(1–7) and Mas receptor antagonist (Bayorh et al., 1999; Santos et al., 2006) or with the combination of i-nNOS (20 mg/kg i.p.), the selective inhibitor of NO-synthase activity, on WRS lesions and NO activity in the G group determined.

Endogenous angiotensin II (AII) or vehicle (control) was investigated in rats (F) 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; Satoh et al., 2013). 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 admin-istered 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 (Ilertissen, 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 mucusal 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 luminal content 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 sample (5 μl) was diluted with biotinylated antibodies specific for 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 before (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 A-779. Total RNA was extracted from mucosal tissues by using guanidium isocyanate phenol-chloroform method using kit from Stratagene (La Jolla, CA). RNA concentration in each sample was determined spectrophotometrically at 260/280 nm. Two micrograms of total RNA was uncoiled by heating (65°C for 5 minutes) and then reversed transcribed into complementary DNA (cDNA) in a 20-μl reaction mixture that contained 50 IU of Moloney murine leukemia virus reverse transcriptase (MMLV-RT), 0.3 μg of oligo(dT) primer, 1 μl of RNase block ribonuclease inhibitor (40 IU/μl), 2 μl of a 100 mM mixture of deoxynucleoside triphosphate (dATP), deoxythymidine triphosphate (dTTP), deoxyguanosine triphosphate (dGTP), and deoxythymidine triphosphate (dCTP), 5 μl 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 25-μl reaction volume containing 0.3 μl (2.5 IU) Taq polymerase, 1 mM (each) dNTP (Pharmacia, Munich, Germany), 1.5 mM MgCl2, 5 μl Taq polymerase reaction buffer (50 mM KCl, 10 mM Tris-HCl, pH 8.3), 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 overlaid in a DNA thermal cycler (Perkin-Elmer/Cetus, Norwalk, CT) in the area dedicated for performing PCR reaction. The nucleotide sequences of the primers for cNOS, iNOS, IL-1β, TNF-α, and β-actin are presented in Table 1 was constructed based on published cDNA for these factors. The primers were synthesized by Invitrogen/Life Technologies (Eggenstein, Germany).

Polymerease chain reaction products were detected by electrophoresis in a 1.5% agarose gel containing ethidium bromide. Location of predicted products was confirmed by using Human Ladder marker as a standard size marker. The intensity bands was quantified using densitometry (LK3 Ultrascan, PharmaNova, Upplands Väsby, Sweden) as described in detail in our previous studies (Brzozowski et al., 2008; Pawlik et al., 2011). The signals for cNOS, iNOS, IL-1β, and TNF-α were standardized against the β-actin signal for each sample, and results were expressed as fold change of cNOS, iNOS, IL-1β, and TNF-α mRNA/β-actin.

Statistical Analysis. Results of the experiment 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 in mean 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 in 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 purchase, gender, age, weight, diet, and housing conditions. There was no individual pairing of animals, the paired statistical tests were 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 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.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer Sequence</th>
<th>Annealing Temperature</th>
<th>Size of PCR Product</th>
</tr>
</thead>
<tbody>
<tr>
<td>cNOS</td>
<td>Forward: 5′- TAC GGA GCA GCA AAT CCA C-3′, Reverse: 5′- CAG GCT GCA CTC CTG TTA TC-3′</td>
<td>63.5°C</td>
<td>540 bp</td>
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<tr>
<td>IL-1β</td>
<td>Forward: 5′- GCT ACC ATT GTC TGG CCC GT-3′, Reverse: 5′- GAC CAT TGG TTC TCT CTA GGC-3′</td>
<td>62°C</td>
<td>543 bp</td>
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<tr>
<td>TNF-α</td>
<td>Forward: 5′- TAC TGA ACT TCG GGG TGA TGG TGT CTC-3′, Reverse: 5′- CAG CTC TGG CTC TGG AAG AGA ACC-3′</td>
<td>56°C</td>
<td>295 bp</td>
</tr>
<tr>
<td>β-Actin</td>
<td>Forward: 5′- TGT TAA CCA ACT GGG ACG ATA TGG-3′, Reverse: 5′- GAT CTT CAT CCT CAT GGT GCT AGG-3′</td>
<td>54°C</td>
<td>764 bp</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 TC-3′</td>
<td>60°C</td>
<td>397 bp</td>
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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 diminished WRS-induced gastric lesions, while producing a significant dose-dependent increase in GBF and luminal NO concentration (Fig. 2). The dose of Ang-(1–7) inhibiting WRS lesions by 50% (ID_{50}) 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 62% 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, 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. These changes were completely reversed by the pretreatment with A-779. It was found that intraperitoneal administration of Ang-(1–7) at the dose of 50 μg/kg i.p. combined with intraperitoneal treatment with Ang-(1–7) (Fig. 2; Table 2).

**Effect of Ang-(1–7) Mas Receptor, on WRS-Induced Gastric Damage and Alterations in the GBF.** As shown in Fig. 2, the pretreatment with AVE 0991 (50 μg/kg i.p.) significantly reduced the mean lesion number (P < 0.05) and produced a significant increase in the GBF (P < 0.05) compared with the respective values in vehicle-control pretreated rats. This 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 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 effect being similar to the respective values presented in Fig. 2. The pretreatment with perindopril (5 mg/kg i.p.) also significantly reduced 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.) resulted in a significant reduction in lesion number and caused a significant decrease 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 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 lesions (2.2* vs. 0.8**, 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.

**TABLE 2**

<table>
<thead>
<tr>
<th>Type of Test</th>
<th>GBF (ml/min per 100 g)</th>
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<tbody>
<tr>
<td><strong>Intact</strong></td>
<td>46 ± 2.8</td>
</tr>
<tr>
<td>Veh + WRS</td>
<td>27 ± 2.9**</td>
</tr>
<tr>
<td>Ang II + WRS</td>
<td>21 ± 1.6**</td>
</tr>
<tr>
<td>Ang-(1–7) + WRS</td>
<td>35 ± 2.7**</td>
</tr>
<tr>
<td>A-779 + Ang-(1–7) + WRS</td>
<td>26 ± 2.2**</td>
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**Fig. 1.** Mean lesion number and the changes in the GBF in rats pretreated intraperitoneally with vehicle (saline; Veh) or Ang II (Ang II) administered intraperitoneally in graded doses ranging from 5 to 40 μg/kg. Results are mean ± S.E.M. from seven animals per each experimental group. The Ang II-pretreated groups were compared with vehicle-pretreated intraperitoneally with vehicle (saline; Veh) or angiotensin II (A-779) at 50 μg/kg without the combination with A-779.

**Fig. 2.** Mean lesion number and the changes in the GBF in rats pretreated intraperitoneally with vehicle (saline; Veh) or Ang II (Ang-(1–7)) in graded doses ranging from 6.25 to 50 μg/kg or in those with administration of A-779 (50 μg/kg i.p.), the Mas receptor antagonist, combined with Ang-(1–7). Results are mean ± S.E.M. from eight animals per each experimental group. The groups receiving Ang-(1–7) in graded doses were compared with vehicle-pretreated values for lesion number, GBF, and NO content. The Ang-(1–7) group at the dose of 50 μg/kg, which afforded the maximal protection, was compared with that treated with the combination of Ang-(1–7) at 50 μg/kg with A-779, as indicated under Materials and Methods. Asterisk indicates a significant change (P < 0.05) compared with the respective values of lesion number, GBF, and luminal NO in vehicle-controls. Cross indicates a significant change (P < 0.05) compared to the values obtained in Ang-(1–7) administered intraperitoneally at the dose of 50 μg/kg without the combination with A-779.

**Fig. 3.** Mean lesion number and the changes in the GBF in rats pretreated intraperitoneally with vehicle (saline; Veh) or Ang II (Ang-(1–7)) in graded doses ranging from 6.25 to 50 μg/kg or in those with administration of A-779 (50 μg/kg i.p.), the Mas receptor antagonist, combined with Ang-(1–7). Results are mean ± S.E.M. from seven animals per each experimental group. The Ang II-pretreated groups were compared with vehicle-pretreated intraperitoneally with vehicle (saline; Veh) or angiotensin II (A-779) at 50 μg/kg without the combination with A-779.
gastric lesions accompanied by a significant rise in the GBF as presented in Fig. 2. The pretreatment with COX-1 and COX-2 inhibitors alone significantly increased the lesion number and produced a significant rise 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 (10 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 additional 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 recorded in Ang-(1–7)-treated animals without concomitant treatment with COX inhibitors. Double asterisks 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) but without combination with PGE2.

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 recorded in Ang-(1–7)-treated animals without concomitant treatment with COX inhibitors. Double asterisks 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) but without 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-1α and TNF-α were negligible in intact rats not exposed to WRS. In contrast, the plasma TNF-α and
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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 a total dose of 125 mg/kg s.c. for 3 consecutive days at a respective dose 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. Asterisk and cross indicate a significant change (P<0.05) compared to the respective values in Ang-(1–7)–pretreated rats.

Fig. 7. Mean lesion number and the changes in GBF in rats pretreated with vehicle, Ang-(1–7) (50 μg/kg i.p.), or Ang II (40 μg/kg i.p.) for 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)–pretreated rats were compared to 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 or vehicle-pretreated 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–treated groups.

Fig. 8. The alteration of plasma IL-1β and TNF-α levels in intact and those pretreated with vehicle (Veh; control), Ang-(1–7) [Ang-(1–7)], or Ang II [Ang II] administered with 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)–pretreated rats were compared to those in intact or vehicle-pretreated rats exposed to WRS. Asterisk indicates a significant change (P<0.02) compared with the respective values in intact or vehicle-pretreated 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–treated groups.

IL-1β levels were significantly increased in vehicle-pretreated rats exposed to WRS (P<0.02). The further significant rise in plasma level of IL-1β and TNF-α was observed in the group administered with Ang II (50 μg/kg i.p.) compared with those pretreated with vehicle and exposed to WRS (P<0.05) (Fig. 7). In contrast, Ang-(1–7) (50 μg/kg i.p.) significantly decreased expression of IL-1β and TNF-α mRNAs observed in vehicle-control group and Ang II-pretreated group (P<0.05) (Fig. 9).

Expression of cNOS mRNA and TNF-α mRNAs in Rats Treated with Ang-(1–7) without or with the Combination with Ang II Figure 8 (left panel) shows the expression of cNOS mRNA in gastric mucosa of vehicle-pretreated rats and those administered Ang-(1–7) with or without the combined administration of Ang-779 and then exposed to WRS. Vehicle-pretreated rats exposed to WRS, the signal for cNOS mRNA was weak compared with that observed in intact gastric mucosa. In contrast, the strong signal for cNOS mRNA was observed in rats pretreated with Ang-(1–7) (50 μg/kg i.p.) and exposed 30 minutes later to WRS compared with those pretreated with vehicle. Ratio of cNOS mRNA over β-actin confirmed that cNOS mRNA was significantly increased in Ang-(1–7)-pretreated gastric mucosa over that observed in the vehicle-control gastric mucosa exposed to WRS (Fig. 8, right panel). A weak signal of cNOS mRNA was recorded in rats with combined administration of A-779 and Ang-(1–7) compared with that in animals treated 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).

A strong signal 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 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).
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., 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 GBF and rise in luminal NO content. Blockade of Mas receptor by A-779 inhibited the Ang-(1–7)-induced protection of 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 NOS-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 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 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 mechanisms 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) 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 by attenuating the reduction of gastric blood flow (Filaretova et al., 1998; Pavel et al., 2008). Similarly, AT1-receptor antagonists dose-dependently attenuated gastric lesions due to its anti-inflammatory action. This is corroborative with the observations that high levels of circulating Ang-(1–7) and Ang-(1–7) ameliorated the metabolic stress induced by a high-fat diet via decrease in the proinflammatory profile adipose tissue cytokines (Santos et al., 2012). Ang-(1–7) decreased body weight, increased HDL cholesterol levels, and decreased expression of COX-2 and IL-1β in abdominal fat of overweight rats (Santos et al., 2012). Moreover, Clarin et al. (2001) reported a direct binding of Ang-(1–7) to the Ang II receptor subtype 1, resulting in 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), which further ties the 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) actions as an endogenous inhibitor of ACE, enhanced the vasodilator effects of bradykinin (Tom et al., 2003). In our study, perindopril significantly decreased WRS-induced gastric lesions and raised GFB with an extent similar to that observed with Ang-(1–7). L-NNA reduced the gastroprotective and hemorheic activity of perindopril, suggesting that this protection and rise in the GFB caused by ACE inhibitor might be 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 GFB, 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
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). 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; 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 REGULATORY ROLE OF CAPSAICIN IN THE GASTROINTESTINAL TRACT.

GBF evoked by Ang-(1–7)–induced protection and hyperemia can be abolished by capsaicin in the presence of Ang-(1–7) restored this protection in part, and gastric hyperemia in rats with sensory denervation; however, this increase in GBF was significantly less pronounced after the pretreatment with 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). 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.

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