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

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ABSTRACT

Angiotensin-(1–7) [Ang-(1–7)] is a major vasoactive metabolite of the renin-angiotensin system (RAS). Ang-(1–7) acting via Mas receptor was documented in kidneys, heart, lungs, 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)], an antagonist of Ang-(1–7) receptor, AVE 0991 (5-formyl-4-methoxy-2-phenyl-1[4-[2-(ethylaminocarbonylsulfonamido)-5-isobutyl-3-thienyl]-phenyl]-methyl-imidazole), the antagonist of Ang-(1–7) receptor as well as the inhibition of nitric-oxide (NO) synthase, the activity COX-2 (rofecoxib), and renin-angiotensin system (RAS) apparent components of WRS ulcerogenesis. The involvement of cyclo-oxygenase (COX)-1 (indomethacin, SC-560 [5-(4-chloro-phenyl)-1-(4-methoxyphenyl)-3-trifluoromethyl-pyrazole]), the activity COX-2 (rofecoxib), and denervation with capsaicin. The mRNA expression of constitutively expressed nitric-oxide synthase (cNOS), inducible nitric-oxide synthase (iNOS), interleukin (IL)-1β, and tumor necrosis factor (TNF)-α was analyzed by reverse transcription polymerase chain reaction. The WRS lesions were dose-dependently reduced by pre-treatment with Ang-(1–7)b, which also induced an increase in gastric blood flow (GBF) and luminal content of NO. COX-1 and COX-2 inhibitors restored GBF and NO levels (N,N-diethylaminomethyl-l-ornithine) reversed the reduction in lesion number and the rise in GBF evoked by Ang-(1–7) in pretreated rats. We conclude that Ang-(1–7) protects against WRS ulcerogenesis via an increase in GBF, these effects were restored to supplementation with calcitonin gene-related peptide (CGRP). The cNOS mRNA was upregulated while iNOS, IL-1β and TNF-α mRNAs were downregulated in Ang-(1–7) pretreated rats. We conclude that Ang-(1–7), in contrast to Ang II which worsened WRS ulcerogenesis, affords potent gastroprotection against WRS ulcerogenesis via an increase in GBF, restored GBF and NO levels, induced iNOS, and inhibited proinflammatory markers iNOS, IL-1β, and TNF-α.

ABBREVIATIONS: A-779, d-Ala7-ANG-(1–7); ACE, angiotensin-converting enzyme; Ang II, angiotensin II; Ang-(1–7), angiotensin-(1–7) peptide; 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; iNOS, inducible nitric-oxide synthase; IL, interleukin; l-NNa, N5-[iminonitroaminomethyl]-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.

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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 (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 nervous system activation, homolog ACE2 or neutral endopeptidase (NEP, also known as neprilysin). 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., 2003; 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 vasodilatory, antihypertensive, cardio-protective, and anti-inflammatory effects. Ang I is quickly degraded to Ang-(1–7) by neutral endopeptidase (NEP).

Angiotensin-(1–7) [Ang-(1–7)] is known to be a biologically active peptide generated from angiotensin I through angiotensin-converting enzyme (ACE) homolog ACE2 or neutral endopeptidase (NEP), also known as nephrilysin, raising the question of Ang-(1–7) in the rat stomach, the formation of Ang-(1–7) by dysfunction of AT$_1$ receptors (Santos et al., 2003). Mas-receptor knockout mice developed hypertension due to dysfunction of AT$_1$ receptors (Santos et al., 2003; Xu et al., 2011). The involvement of endogenous Ang-(1–7) on stress ulcerogenesis is currently not well understood. The decrease in NO synthesis, and secretion of eNOS expression, suggesting a link between Ang-(1–7) and Mas receptor (Xu et al., 2008). The vasoconstrictive effect of Ang II in hypertension is limited by vasoactive Ang-(1–7) and bradykinin (Oliveira et al., 2001; Vara de et al., 2006; Sampaio et al., 2007). Ang-(1–7) exhibited endothelial protection against reflux esophagitis (Burger 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 so far.

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 (cNOS), inducible nitric-oxide synthase (iNOS), proinflammatory cytokines interleukin (IL)-1$\beta$ and tumor necrosis factor (TNF)$\alpha$, and plasma levels of these cytokines during stress ulcerogenesis.

Materials and Methods

Animals. Male Sprague–Dawley rats total 250 g in weight were used in the study. Rats were fasted for 24 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 was conducted in accordance with the statements of the Helsinki Declaration regarding handling of experimental animals.

Stress-Induced Gastric Lesions, Cytokines, and Drugs

Application. To induce gastric lesions, rats were immobilized in individual Polman cages and immersed in a water bath (23°C) for 3.5 hours with the rat xyphoid level as reported by our group previously (Brzozowski et al., 2000, Konturek et al., 2001). In another series of experiments groups of rats (series G) were selected. Thirty minutes before exposure to water immersion and restraint stress (WRS), rats in series A–C received pretreatment with (a) exogenous Ang-(1–7) (6.25–50 μg/kg, i.p.), Ang II (α-1 antagonist), the long-lasting ACE inhibitor (Jawien et al., 2012). The angiotensin Mas receptor agonistic and antagonistic activities were determined in a separate group of animals (series D) treated with A-779 (5 mg/kg i.p.), the selective Ang-(1–7) receptor antagonist (Bayoh et al., 1999; Santos et al., 2003; Jawien et al., 2005) with or without the combination with Ang-(1–7). At 30 minutes after exposure to water immersion and restraint stress (WRS), the neuropeptide Ang-(1–7) receptor agonist capsaicin (Pinheiro SV et al., 2004; Santos and Fereira, 2006) was administered.

Ang-(1–7) and Ang II as a treatment with Ang-(1–7) or perindopril, with or without the combination with l-NNA (20 mg/kg i.p.), selective inhibitors of NO-synthase activity, on WRS lesions and alterations in the GBF were determined.

The involvement of endogenous PG in the gastroprotective effects of Ang-(1–7) on 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 (Brazowska 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 sensory afferent nerve endings in protective action of exogenously administered Ang-(1–7) against WRS lesions, was determined. In one of the subgroups of series G, the capsaicin-denervated rats received supplementation with exogenous CGRP (10 μg/kg s.c.) combined with Ang-(1–7) and 30 minutes later were exposed to onset of WRS as in other groups described above.

All tested drugs and compounds were of analytical grade and were purchased from Sigma-Aldrich Laborchemikalien (Schelldorf, Germany) except of SC-560 and rofecoxib purchased from Cayman.
Chemical (Ann Arbor, MI) and Pfizer (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 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., 2012b) 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 samples were incubated with biotinylated antibodies specific to rat 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) with specific primers. Mucosal specimens were scraped off using a glass slide and immediately snap-frozen in liquid nitrogen and stored at –80°C until analysis. Total RNA was extracted from mucosa samples by a guanidium isothiocyanate-phenol-chloroform method and reverse transcriptase-polymerase chain reaction (RT-PCR) was performed using standard conditions. The resultant cDNA (2 µl) was amplified in a 50-µl reaction mixture that contained 2 µl of cDNA, 1.5 mM/l MgCl₂, 5 µl of a 100 mM mixture of deoxyadenosine triphosphate (dATP), deoxythymidine triphosphate (dTTP), deoxyguanosine triphosphate (dGTP), and deoxycytidine 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 a 50-µl reaction volume containing 0.3 ml (2.5 IU) Tag polymerase, 0.5 µM (each) dNTP (Pharmacia, Germany), 1.5 mM/l MgCl₂, 5 µl Taq polymerase reaction buffer (50 mM KCl, 10 mM Tris-Cl, pH 8.3), and primers used at final concentration of 0.5 µM. The reaction mixture was overlaid with 25 µl of mineral oil to prevent evaporation. Polymerase chain reaction mixture was amplified on a DNA thermal cycler (Perkin-Elmer/Cetus, Norwalk, CT) in the area dedicated for performing PCR reaction. The identity of cDNAs used as the primers for cNOS, iNOS, IL-1β, TNF-α, and β-actin presented in Table 1 was constructed based on published cDNAs of these factors. The RNA was synthesized at Invitrogen/Life Technologies (Eggenstein, Germany).

Polymerase chain reaction products were detected by electrophoresis on a 1.5% agarose gel containing ethidium bromide. Location and predicted products was confirmed by using DNA 100 bp ladder as a standard size marker. The intensity was quantified using densitometry (LK9 Ultrascan, Pharmacia, Uppl, 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 arbitrary units (AU), and TNF-α mRNA/β-actin mRNA ratio.

**Statistical Analysis.** Results of each 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 the 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 value for lesion number. The control rats did not differ from experimental groups in terms of relevant characteristics, such as source of pigs, gender, age, weight, diet, and housing conditions.

There was not a random 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>c-NOS</td>
<td>Forward: 5'- TAC GGA GCA GCA AAT CCA C-3', Reverse: 5'- CAG GCT GCA GTC CTT TGA TC-3'</td>
<td>63.5</td>
<td>540</td>
</tr>
<tr>
<td>IL-1β</td>
<td>Forward: 5'- GCC ACC ACC GCT TGG CTC TT-3', Reverse: 5'- GAC CAT TCC TCT TGC CTA GG-3'</td>
<td>62</td>
<td>543</td>
</tr>
<tr>
<td>α-NOS</td>
<td>Forward: 5'- TAC TGA ACT TCG GGG TGA TGG GTC C-3', Reverse: 5'- CAG CCT TGG CTC TGG AAG AGA ACC-3'</td>
<td>56</td>
<td>295</td>
</tr>
<tr>
<td>β-Actin</td>
<td>Forward: 5'- TTAG TAA CCA ACT GGG ACG ATA TG-3', Reverse: 5'- GAT TCT GAT CCT CAT GGT GCT AGG-3'</td>
<td>54</td>
<td>764</td>
</tr>
<tr>
<td>iNOS</td>
<td>Forward: 5'- CCA CAA TAG TAC AAT ACT AC-3', Reverse: 5'- AGG TGG TCA GCG TGC AC-3'</td>
<td>60</td>
<td>397</td>
</tr>
</tbody>
</table>
The administration of Ang II in higher doses ranging from 62.5 to 400 μg/kg dose-dependently increased the lesion number and produced a significantly dose-dependent decrease in GBF (Fig. 1). The pretreatment with Ang-(1-7) administered i.p. in graded doses ranging from 5 to 40 μg/kg resulted in a significant and a dose-dependent decrease in GBF and luminal NO concentration (Fig. 2). The dose of Ang-(1-7) inhibiting WRS lesions by 50% (ID50) was 720 Magierowski et al.

**Effect of pretreatment with vehicle (Veh), ANG II (40 μg/kg i.p.), and Ang-(1-7) (50 μg/kg i.p.) or in those with 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.) 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-pretreated rats. The decrease in lesion number and an increase in the GBF induced by AVE 0991 were completely abolished in rats treated with the combination of A-779 and AVE 0991 (P < 0.05).

**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 (P < 0.05) compared with the respective values in intact gastric mucosa. 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).

**Materials and Methods**

Asterisk indicates a significant change (P < 0.05) below or above values obtained in rats pretreated with Ang II and Ang-(1-7).

**TABLE 2**

<table>
<thead>
<tr>
<th>Type of Test</th>
<th>GBF (ml/min per 100 g)</th>
</tr>
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<tbody>
<tr>
<td>Intact</td>
<td>46 ± 2.8</td>
</tr>
<tr>
<td>Veh + WRS</td>
<td>27 ± 2.2*</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>
</tr>
</tbody>
</table>

**Fig. 2.** Mean lesion number and the changes in the GBF in rats pretreated intraperitoneally with vehicle (saline; Veh) or AngII or Ang-(1-7) (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 animals as indicated under **Materials and Methods**. Asterisk indicates a significant change (P < 0.05) compared with the respective values in Veh-controls.

**Fig. 3.** Mean lesion number and the changes in the GBF in rats pretreated intraperitoneally with vehicle (saline; Veh) or Ang-(1-7) (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 animals as indicated under **Materials and Methods**. Asterisk indicates a significant change (P < 0.05) compared with the respective values in Veh-controls.

**Fig. 4.** Mean lesion number and the changes in the GBF in rats pretreated intraperitoneally with vehicle (saline; Veh) or Ang-(1-7) (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 animals as indicated under **Materials and Methods**. Asterisk indicates a significant change (P < 0.05) compared with the respective values in Veh-controls.

**Fig. 5.** Mean lesion number and the changes in the GBF in rats pretreated intraperitoneally with vehicle (saline; Veh) or Ang-(1-7) (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 animals as indicated under **Materials and Methods**. Asterisk indicates a significant change (P < 0.05) compared with the respective values in Veh-controls.
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 mean lesion number and produced a significant rise in the GBF compared with vehicle-treated animals exposed to WRS (data not shown). The reduction of lesion number by Ang-(1–7) (50 μg/kg i.p.) was significantly attenuated by pretreatment with indomethacin (INDO), SC-560 (SC), and rofecoxib (ROFE) with and without the supplementation with prostaglandin E2 (PGE2). Results are mean ± S.E.M from seven animals per each experimental group. The values in AVE 0991-treated group were compared with vehicle-controls and with those in AVE 0991 group given in combination with A-779. Asterisk indicates a significant change (P < 0.05) compared with the respective values in vehicle-control group. Cross indicates a significant change (P < 0.05) compared to values obtained in AVE 0991-treated rats without concomitant treatment with A-779 treatment.

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 asterisk indicates 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.

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 group given in combination with A-779. Asterisk indicates a significant change (P < 0.05) compared with the respective values in vehicle-control group. Cross indicates a significant change (P < 0.05) compared to values obtained in AVE 0991-treated rats without concomitant treatment with A-779 treatment.

Fig. 4. Mean lesion number and the alterations in GBF in gastric mucosa pretreated with vehicle, Ang-(1–7), or perindopril with or without NOSynthase inhibitor (L-NNA, 20 mg/kg i.p.) and exposed to WRS. Results are mean ± S.E.M from six animals per each experimental group. The values in Ang-(1–7) or perindopril groups were compared with vehicle (Veh)-controls and with those administered with Ang-(1–7) or perindopril with concurrent treatment with L-NNA. Asterisk indicates a significant change (P < 0.05) compared with the respective values in vehicle-control group. Cross indicates a significant change (P < 0.05) compared to values obtained in rats without L-NNA treatment.

Fig. 5. Mean lesion number and the alterations in GBF in gastric mucosa pretreated with Ang-(1–7) without or with combination with indomethacin (INDO), SC-560 (SC), and rofecoxib (ROFE) with and without the supplementation with prostaglandin E2 (PGE2). Results are mean ± S.E.M from seven rats per each experimental group. The effect of Ang-(1–7) on lesion number and GBF was compared with that achieved with COX-1 and COX-2 inhibitors in the absence or the presence of PGE2. Asterisk indicates a significant change (P < 0.05) compared with the respective values in vehicle-control group. Cross indicates a significant change (P < 0.05) compared to values obtained in Ang-(1–7)-treated rats without concomitant treatment with COX inhibitors. Double asterisk indicates 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.

Fig. 6. Effect of capsaicin Denervation with or without Exogenous CGRP on Ang-(1–7)-Afforded Gastroprotection and Hyperemia against WRS-Induced Gastric Damage. As shown in Fig. 6, the pretreatment with Ang-(1–7) (50 μg/kg s.c.) caused a similar decrease in the number of WRS-induced gastric lesions accompanied by a significant rise in the GBF as presented in Figs. 4 and 5. The exogenous administration of CGRP (10 μg/kg s.c.) in rats with intact sensory nerves resulted in a significant decrease of WRS-induced gastric damage (P < 0.05) and significant increase in the GBF (P < 0.05) compared with respective values achieved with Ang-(1–7) (Fig. 6). The capsaicin denervation tended to increase the mean lesion number and to decrease GBF compared to rats with intact sensory nerves. The reduction in lesion number and an increase in the GBF caused by Ang-(1–7) in rats with intact sensory innervation were almost completely lost in those with capsaicin denervation. The concurrent administration of CGRP combined with Ang-(1–7) significantly reduced the mean lesion number (P < 0.05) and significantly increased GBF in capsaicin-denervated rats (P < 0.05); however, these values were still significantly different from those attained with Ang-(1–7) in rats with intact sensory nerves (Fig. 6).

Effect of Pretreatment with Ang-(1–7) or Ang II on Plasma Levels of Proinflammatory Cytokines IL-1β and TNF-α in Rats Exposed to WRS. As shown in Fig. 7, the plasma levels of IL-1β and TNF-α were negligible in intact rats not exposed to WRS. In contrast, the plasma TNF-α and
cNOS mRNA was significantly decreased (P < 0.05) 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 experiments. Results are mean ± S.E.M from six rats per each experimental group. Statistical comparisons were made between Ang-(1–7) group with or without cotreatment with exogenous CGRP and with or without capsaicin denervation as indicated under Materials and Methods. Asterisk indicates a significant change (P < 0.05) compared with respective values in vehicle-control group. Asterisk and cross 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 with the values in Ang-(1–7)-pretreated rats. Double crosses indicate a significant change (P < 0.05) compared with CGRP-treated group with capsaicin denervation.

IL-1β levels were significantly increased in vehicle-pretreated rats exposed to WRS (P < 0.02). Thereafter significant rise in plasma levels of 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 (P < 0.05). In contrast, plasma levels of IL-1β and TNF-α observed in Ang-(1–7)-pretreated group and Ang II-pretreated group were significantly increased compared with vehicle-control group and Ang II-pretreated group alone. The alterations in plasma IL-1β and TNF-α were significantly upregulated in WRS-induced gastric mucosa. These effects were significantly attenuated when rats received the combination of Ang-(1–7) and Ang-(1–7) alone (Fig. 8, right panel). In contrast, strong signals for IL-1β and TNF-α mRNAs were observed in intact animals administered with Ang-(1–7) and Ang-(1–7) alone (Fig. 7, left panel). The ratio of IL-1β and TNF-α over β-actin confirmed that this effect was significantly decreased in intact gastric mucosa and this effect was significantly attenuated in those pretreated with Ang-(1–7) alone (Fig. 7, right panel). 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) alone (Fig. 7, right 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).
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 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 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 (Szalachiec 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 attenuation of inflammatory mediators (Ender et al., 1993; Chung et al., 2004; Bregonzio et al., 2004) 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).

Fig. 10. Proposed mechanisms and factors that may contribute to the gastroprotective action of vasoactive angiotensin metabolite, Ang-(1–7). RAS metabolite Ang-(1–7) acts via specific Mas receptor and stimulates mucoepithelial properties via activation of NO/NOS and COX/PG systems, sensory neuropeptides such as CGRP released from sensory nerves, and the potent inhibition of proinflammatory cytokines and gastric mucosal inflammation.

Our results show that WRS increased the expression and plasma levels of TNF-α and IL-1β, and that a plasma level of these proinflammatory cytokines was increased by Ang II, suggesting that Ang-(1–7) and Ang II, known as a potent vasoconstrictor, aggravated WRS-induced gastric damage due to its proinflammatory action. This is corroborative with our observations: high levels of circulating Ang-(1–7) of untreated the gastric stress induced in rats via deactivate in the proinflammatory profile adipose tissue cytokine (Szlachcic et al., 2013). Ang-(1–7) decreases visceral fat, increased HDL cholesterol, and decreased expression of COX-2 and IL-1β in abdominal fat of overweight rats (Sanchez et al., 2012). Moreover, Chandrashekar et al. (2001) reported a direct binding of Ang-(1–7) to the Ang 1 receptor in vitro, resulting in downregulation of these receptors. In keeping with these findings, we observed decreased expression of iNOS and increased plasma levels of IL-1β and TNF-α in rats pretreated with Ang-(1–7), indicating that 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-restraint-stress ulcer in obstructive jaundice rats (Mou et al., 1998). Enalapril, an inhibitor of ACE, reduced both the plasma 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) activity was not endogenous inhibitor of ACE, enhanced the vasodilatory effects of bradykinin (Tom et al., 2003). In our study, perindopril significantly decreased WRS-induced gastric damage and raised GBF with an extent similar to that of Ang-(1–7). L-NNA reduced the gastroprotective and hyperemic activity of perindopril, suggesting that this protection and rise in the GBF caused by ACE inhibitor might be cause 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
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 COX/PG 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 allfferent sensory fibers and the major sensory neuropeptide CGRP released from rat sensory nerves endogenously might 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 with capsaicin denervation; however, this increase in GFB was significantly more pronounced in capsaicin-denervated rats compared with those with intact sensory nerves. Thus, it is reasonable to consider CGRP, which is a potent vasodilator and a pro-inflammatory mediator in the stomach, can cooperate with Ang-(1–7) in this protection.

In summary, Ang II and Ang-(1–7) are opposite action against stress ulcerogenesis because Ang-(1–7) enhanced stress ulcerogenesis but Ang-(1–7) alleviated protection against stress lesions. The mechanism of Ang-(1–7)-induced protection against stress may involve activation of NO/cGMP/PKG and COX systems and mediators and gastroprotective sensory neuropeptides. This study established a pan-anti-inflammatory property of Ang-(1–7) exhibited by multiple sensory afferent nerves via an anti-inflammatory property that is not expression of the proinflammatory mediator TNF-α and NF-κB. Further studies in experimental settings are warranted to further understand the peripheral efficacy of Ang-(1–7) in the central regulation of stress.

Authorship Contributions

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Conducted experiments: Magierowski, Kwiecien, Pawlik, Kwiecien. Contributed data or analytic tools: Kryszek–Maczka, Olszanecki, Korbut.
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