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 the Mas receptor was documented in kidneys, heart, lung, and a gastrointestinal (GI)-tract. We studied the gastroprotective activity of exogenous Ang-(1–7) in rats exposed to water immersion and restraint stress (WRS), a widely used model of ulcerogenesis. Ang-(1–7) stimulated gastric blood flow (GBF) and luminal content of NO. COX-1 and COX-2 inhibitors or L-NNA (Nω-nitro-L-arginine methyl ester) reversed the reduction in lesion number and the rise in GBF evoked by Ang-(1–7). Ang II augmented the WRS lesions, decreased GBF and increased the plasma IL-1β and TNF-α levels. Capsaicin denervation attenuated the reduction of Ang-(1–7)-induced gastric lesions and rise in GBF; these effects were restored by supplementation with calcitonin gene-related peptide (CGRP). The cNOS mRNA was upregulated while iNOS, IL-1β, and TNF-α mRNAs were downregulated in Ang-(1–7) pretreated rats. We conclude that Ang-(1–7), in contrast to Ang II, which worsened WRS ulcerogenesis, affords potent gastroprotection against WRS ulcerogenesis via an increase in GBF mediated by NO, endogenous prostaglandins, sensory neurotransmitters, 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 physiologic regulation of blood pressure and water and mineral balance (Paul et al., 2006). Its components of RAS appear to be functionally active in numerous organs including kidneys, heart, lung, reproductive organs, and skin. Angiotensin I (Ang I) and Angiotensin II (Ang II) play an important role in control of gastrointestinal (GI) functions such as fluid and electrolyte homeostasis, maintenance of functional arterial flow, mucosal absorption of 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 vasoconstriction, inflammation, and carcinogenesis (Fandriks 2011; Garg et al., 2012).

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ABBRVIEATIONS: 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; iNOS, inducible nitric-oxide synthase; IL, interleukin; L-NNA, N5-[iminonitroamino(methyl)]-l-ornithine; NOS, nitric-oxide synthase; PG, prostaglandin; RAS, renin-angiotensin system; SC-560, [5-(4-chloro-phenyl)-1-(4-methoxyphenyl)-3-trifluoromethyl-pyrazole]; TNF, tumor necrosis factor; WRS, water immersion and restraint stress.
superoxide and hydrogen peroxide (H₂O₂), and inactivates NO pathway (Mehta and Griendling, 2007). Ang II-activating phospholipase C (PLC) and protein kinase C (PKC) or phospholipase A₂ enhanced synthesis of vasoconstrictive leukotrienes and smooth muscle cell contraction (Mehta and Griendling, 2007; Lemarie et al., 2009). Increased reactive oxygen species (ROS) and decreased blood flow play fundamental roles in the pathogenesis of GI mucosal injury (Bregenzio et al., 2003; Nakagiri et al., 2010).

Exposure to stress is commonly recognized as a risk factor of microbleeding and gastric mucosal injury. Reaction to stress is mediated via two distinct but unrelated systems: the hypothalamic-pituitary-adrenocortical (HPA) system and the sympahtoadrenal system (Goldstein and McEwen, 2002; Saavedra et al., 2006). Ang II receptor subtypes AT₁ and AT₂ were detected in the human esophageal, gastric, small intestinal, and colonic mucosa (Hirasawa et al., 2002; Casselbrant et al., 2009; Hallersund et al., 2011). The antagonists of Ang II AT₁ receptors attenuated gastric injury induced by ischemia-reperfusion, cold stress, and indomethacin-induced damage in rodents due to an inhibition of sympathetic nerve activity and the attenuation of vasoconstrictor and proinflammatory activity of Ang II (Pavel et al., 2007; Morsy et al., 2009; Gemici et al., 2010; Saavedra et al., 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 angiotensin-converting enzyme (ACE) homolog Ang-(1–7) converting enzyme (NEP), also known as neprilysin, since the discovery of Ang-(1–7) in 1976, the presence of this hectapeptide 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., 2005; Stegbauer et al., 2011), exhibit the vasodilatory, antihypertensive, cardiotonic, anti-inflammatory, and antifibrotic effects. Ang I is quickly converted to Ang-(1–7) in the rat stomach during the formation of Ang II (Gnanasek et al., 2009). Mas receptor knockout mice show increased vascular, cardiac, and renal injury due to dysfunctional Ang II octapeptide. Ang-(1–7) loss results in decreased NO synthesis, and alterations 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., 2006; Benci et al., 2006; Sampaio et al., 2007). Ang-(1–7) exhibited a protective effect against reflux esophagitis (Pawlik 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.

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 ulcogeneration 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 Wistar rats, total 254 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 was conducted in accordance with the statements of the Helsinki Declaration regarding handling of experimental animals.

Assessing Gastric Lesions, Chemicals, and Drugs

Ang-(1–7) Mas receptor agonistic and antagonistic 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) Mas receptor antagonist (Bayohr et al., 1999; Santos et al., 2006; Pinheiro SV et al., 2004) with or without the combination with Ang-(1–7) Mas receptor agonist (Bayohr et al., 2007) or control group (series D). After the induction of stress ulcers, rats were killed, gastric sections were prepared, and lesions were analyzed.

In the study, the effects of pretreatment with Ang-(1–7) or perindopril, with or without the combination with l-NNA (20 mg/kg i.p.), and superoxide-dismutase (SOD) on the protective impact of NO-synthase activity, on WRS lesions and NO pathway in the stomach were determined.

The involvement of endogenous PG in the gastroprotective effects of Ang-(1–7) (vehicle) or vehicle (control) was investigated in rats treated with capsaicin (total 254 mg/kg i.p.). Male Wistar rats about 250 g were used in this study. Rats were fasted for 24 hours with free access to water before the 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.

Pretreatment with capsaicin (10 mg/kg i.p.) 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.

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 GFB and Determination of Gastric Lesion Number. At the termination of 3.5 hours WRS, rats were anesthetized with pentobarbital (60 mg/kg i.p.), the abdomen was opened, and GFB measured by means of H2-gas clearance technique as reported before (Brzozowski et al., 2004, 2006; Kwiecien et al., 2007). The GFB was measured in the fundic part of the gastric mucosa not involving mucosal lesions. Average values of three measurements were determined and expressed as a percentage of change of the value determined in intact rat stomach. Gastric lesions number was determined on photographed stomachs with computerized planimetry (Morphonat, 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 assay kit purchased from Cayman Chemical as described in our previous study (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 for rat TNF-α and IL-1β, washed three times with assay buffer, and finally conjugated with streptavidin peroxidase to form an enzyme complex with a stabilized chromogen 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) with specific primers. Mucosal specimens were scraped using a glass slide and immediately snap-frozen in liquid nitrogen and stored at –80°C until analysis. Total RNA was extracted from mucosal samples by a guanidium isothiocyanate (phenol:chloroform:isoamyl alcohol) method using a kit from Stratagene (La Jolla, CA) according to the manufacturer’s instructions. One microgram of RNA was covalently bound to an oligo(dT) primer and then uncoiled by heating (65°C for 5 minutes) and then reversed transcribed into complementary DNA (cDNA) in a 50-μl reaction mixture that contained 50 IU of Moloney murine leukemia virus reverse transcriptase (MMLV-RT), 0.3 mg of oligo(dT) primer, 1 ml of RNase block ribonuclease inhibitor (40 IU/μl), 2 ml of a 100 mM mixture of deoxyadenosine triphosphate (dATP), deoxythymidine triphosphate (dTTP), deoxyguanosine triphosphate (dGTP), deoxycytidine triphosphate (dTCTP), 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 used in a 25-μl 2 × Taq polymerase reaction volume containing 0.3 ml (2.5 IU) Taq polymerase, 1 ml of each dNTP (Pharmacia, Germany), 1.5 mM MgCl2, 5 ml of Taq polymerase chain 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 and then exposed to 35 cycles of polymerase chain reaction with a profile of 1 min at 95°C, 1 min at 56°C, and 1 min at 72°C, followed by a 10 min extension at 72°C (Elmer/Cetus, Norwalk, CT). The area dedicated for performing PCR reaction. The densities of the bands were normalized against the β-actin mRNA on agarose gels, and results were expressed as cNOS, iNOS, IL-1β, TNF-α mRNA/β-actin mRNA ratio following densitometry (LKB Ultrascan, Pharmacia, Uppsala, Sweden) as described in detail in our previous studies (Brzozowski et al., 2008; Konturek et al., 2009). The signals for cNOS, iNOS, IL-1β, and TNF-α were standardized against the β-actin loading control for each sample, and results were expressed as cNOS, iNOS, IL-1β, and TNF-α mRNA/β-actin mRNA ratio.

Statistical Analysis. All results of our 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 between estimates of effects were taken as significant at P < 0.05. All results in the treated animals were compared with the appropriate control group, which had been established for each set of experiments. Dependent variables were expressed both in percentage of control for GBF and in absolute values for lesion number. The control rats did not differ from experimental rats in terms of relevant characteristics, such as source of pigs, gender, age, weight, diet, and housing conditions. Since there was no individual pairing of animals, the paired statistical test was not used. Results

Mean Lesion Number and GFB in Rats Pretreated with Ang II or Ang-(1–7). Exposure of vehicle-pretreated control rats to 3.5 hours of WRS caused gastric mucosal lesions (hemorrhagic erosions) accompanied by a significant fall in GFB (Fig. 1). The pretreatment with Ang II applied in a dose of 5 μg/kg failed to significantly affect the mean lesion number and GFB compared with vehicle-control.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer Sequence</th>
<th>Annealing Temperature (°C)</th>
<th>Size of PCR Product (bp)</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 ATT GTC TGG CCC GTG-3', Reverse: 5'- GAC CAT TGC TGG TTC CTA GG-3'</td>
<td>62</td>
<td>543</td>
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<tr>
<td>TNF-α</td>
<td>Forward: 5'- TAC TGA ACT TGG GCG TTA TGG TGC C-3', Reverse: 5'- CAG CCT TGG CTC TTA AAG AAG GAC ACC-3'</td>
<td>56</td>
<td>295</td>
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<tr>
<td>β-Actin</td>
<td>Forward: 5'- TGG TAA CCA ACT GGG AGC ATA TGG-3', Reverse: 5'- GAT CTT GAT CTT CAT GCT AGG ACT-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'- AGC AGG TGT TCA GCG TGC-3'</td>
<td>60</td>
<td>397</td>
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</table>
Fig. 1. Mean lesion number and the changes in the GBF in rats pretreated intraperitoneally with vehicle (saline; Veh) or angiotensin II (Ang II) administered intraperitoneally in graded doses ranging from 6.25 to 50 μg/kg. Results are mean ± S.E.M. from seven animals per each experimental group. The Ang II-pretreated groups were compared with those of 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. The administration of Ang II in higher doses ranging from 6.25 to 40 μg/kg dose-dependently increased the mean lesion number and produced a significant dose-dependent decrease in GBF (Fig. 1). The pretreatment with Ang-(1–7) (50 μg/kg, dose-dependently increased the lesions in intact gastric mucosa, 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% (ID₅₀) was 27 μg/kg. Since the dose of 50 μg/kg afforded the maximal protective response (P < 0.05), this dose of Ang-(1–7) was used in all our determinations. The absolute values for GBF expressed in ml/min per 100 g are presented in Table 2. Exposure to WRS in rats pretreated with vehicle-control significantly decreased the GBF of 60% compared with the values in the intact gastric mucosa. This decrease in GBF under WRS conditions was significantly worsened by the pretreatment with Ang II. In contrast, pretreatment with Ang-(1–7) resulted in a significant increase in the GBF (P < 0.05) compared with that pretreated with vehicle. The Ang-(1–7)-induced protection was accompanied by the accompanying rise in the GBF and luminal NO content observed at the 50 μg/kg dose of this peptide, as completely evoked by the pretreatment with A-779 (50 μg/kg i.p.) combined with intraperitoneal AVE 0991 with Ang-(1–7) (Fig. 2; Table 2).

Effect of AVE 0991, the Agonist of Ang-(1–7) Mas Receptor, on WRS-Induced Gastric Protection and Alterations in the GBF. As shown in Fig. 3, pretreatment with AVE 0991 (50 μg/kg i.p.) significantly reduced the mean lesion number (P < 0.05) and caused a significant decrease 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 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 Suppression of NO-Synthase on Ang-(1–7)- and Perindopril-Induced Gastric Protection and Alterations in GBF in Rats Exposed to WRS. Figure 4 shows that pretreatment with Ang-(1–7) (50 μg/kg i.p.) significantly reduced the WRS-induced gastric lesions and increased GBF, with the extent similar to the respective values presented in Fig. 3. The pretreatment with perindopril (5 mg/kg i.p.) also significantly increased the number of WRS-induced gastric lesions (P < 0.05) and significantly increased GBF compared with 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 decrease 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 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). 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 of 60% compared with the values in the intact gastric mucosa. This decrease in GBF under WRS conditions was significantly worsened by the pretreatment with Ang II. In contrast, pretreatment with Ang-(1–7) resulted in a significant increase in GBF (P < 0.05) compared with that pretreated with vehicle. The Ang-(1–7)-induced protection was accompanied by an accompanying rise in GBF and luminal NO content observed at the 50 μg/kg dose of this peptide, as completely evoked by the pretreatment with A-779 (50 μg/kg i.p.) combined with intraperitoneal AVE 0991 with Ang-(1–7) (Fig. 2; Table 2).

**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>

Asterisk indicates a significant change (P < 0.05) below or above values obtained in rats pretreated with Ang II and Ang-(1–7). Cross indicates a significant change (P < 0.05) compared with the value in Ang-(1–7) alone.
Fig. 3. Mean lesion number and the alterations in GBF in gastric mucosa pretreated with vehicle (Veh; control) or AVE 0991 (50 μg/kg i.p.), the Ang-(1–7) receptor agonist, without or with A-779 (the antagonist of Mas receptors; 50 μg/kg i.p.) and exposed to WRS. Results are mean ± S.E.M from seven animals per each experimental group. The values in AVE 0991 group were compared with vehicle-controls and with those in AVE 0991 + A-779 group. 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 with the respective values in vehicle-high-control group. Group designated with 1.50 mg/kg i.p.) and exposed to WRS. Results are mean ± S.E.M from six animals per each experimental group. The values in AVE 0991-treated rats without concomitant treatment with A-779 restoration of lesion number by Ang-(1–7) (50 μg/kg i.p.) and exposed to WRS (data not shown). The results of these experiments are shown in Fig. 5. The pretreatment with COX-1 and COX-2 inhibitors 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 (10 mg/kg i.g.), and SC-560 (5 mg/kg i.g.) (P<0.05), and these effects were accompanied by 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 observed in Ang-(1–7)-treated animals without concomitant treatment with COX inhibitors (Fig. 5). Cross 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.

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.) 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 (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 neurons, 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 hours before the experiment. 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. Cross indicates a significant change (P < 0.05) compared to the values obtained in animals with intact sensory nerves treated with Ang-(1–7). Double crosses indicate a significant change (P < 0.05) compared with CGRP-treated group 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 II (50 μg/kg i.p.) compared with those pretreated with vehicle and exposed to WRS (Fig. 7). In contrast, the plasma levels of IL-1β and TNF-α in Ang-(1–7)-pretreated vehicle-control group and Ang II-pretreated group (50 μg/kg i.p.) significantly decreased (P < 0.05) compared with those in intact rats. 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 < 0.05) compared with the respective values in intact rats. Asterisk and cross indicates a significant change (P < 0.05) compared with respective values in Ang-(1–7)-pretreated rats. Double cross indicates a significant change (P < 0.05) compared with the values in vehicle-control and Ang II groups.

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The alterations in plasma IL-1β and TNF-α levels in intact sensory nerves were significantly upregulated in WRS-induced gastric mucosa. These effects were significantly attenuated when rats received 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 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 gastroprotective effect of Ang-(1–7) pretreatment was accompanied by the increase in gastric blood flow and rise in luminal NO content. Blockade of Mas receptor by A-779 inhibited the Ang-(1–7)-induced gastroprotective effect of this peptide, while AVE 0991, the agonist of AT1 receptor, 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 GSBF 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 (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 attenuation of inflammatory mediators (Ender et al., 1993; Chung et al., 2003). Liao et al. (2011) revealed that cardioprotective effect of Ang-(1–7) against ischemia-reperfusion damage is mediated by the cNOS pathway, suggesting that the beneficial effect of Ang-(1–7) is dependent on endothelial NO synthase activity. 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 inflammatory infiltration and expression of gastric intercellular adhesion molecule 1 and TNF-α (Saavedra et al., 2005, 2006). It is not excluded that the beneficial effect of AT1-receptor antagonists could depend on enhancement of the concentration of angiotensin metabolites Ang-(1–7) and Ang-(1–9) (Neves et al., 2000; Olszanecki et al., 2009), but this hypothesis requires further studies.

Our results show that WRS increased the expression and plasma levels of TNF-α and IL-1β, and that plasma level of these proinflammatory cytokines was reduced by Ang II, suggesting that Ang-(1–7), known as a potent vasoconstrictor, aggravated WRS-induced gastric damage due to its proinflammatory action. This is corroborative with our observations that high levels of circulating Ang-(1–7) contributed to inflammatory profile in adipose tissue (Santos et al., 2012). Ang-(1–7) decreased body weight, increased HDL cholesterol and decreased expression of COX-2 and IL-1β in abdominal fat of overweight rats (Santos et al., 2012). Moreover, Clark et al. (2001) reported the direct binding of Ang-(1–7) to the AT1-receptor, 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 WRS-induced gastric damage due to its potent anti-inflammatory action. This is in line with the notion that 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) acts as a potent endogenous inhibitor of ACE, enhanced the vasodilator effects of bradykinin (Tom et al., 2003). In our study, pretreatment significantly decreased WRS-induced gastric damage and increased GBF with an extent similar to that observed with Ang-(1–7). L-NNA reduced the gastroprotective and hemorheic activity of perindopril, suggesting that this attenuation and rise in the GBF caused by ACE inhibitor might be at least 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.

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 mucoprotective mechanisms due to an activation of NONOS and COX/PG systems, sensory neuropeptides such as CGRP released from sensory nerves, and the potent inhibition of proinflammatory cytokines and gastric mucosal inflammation.
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 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 of the 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) (Bregonzio 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, exogenous 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 GBF was significantly less 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 protective factor in the stomach, can cooperate with Ang-(1–7) in this protection.

In summary, Ang II and Ang-(1–7) showed an opposite action against stress ulcers. As Ang-(1–7) enhanced stress ulcerogenesis but attenuated stress protection against stress lesions. The mechanism of Ang-(1–7)-induced protection against stress may involve activation of NO/cNOS and PG, COX system and mediators and gastroprotective sensory neuropeptides, though CGRP as GRP may be a candidate for the anti-inflammatory properties of Ang-(1–7) exhibit only in the rat. Further studies in experimental animals are warranted to further elucidate the potential efficacy of Ang-(1–7) in various pathological and clinical disorders.

Authorship Contributions

As part of the research conducted by Brzozowski, Kwiecien, and Brzozowski. Conducted experiments: Magierowski, Pawlik, Kwiecien. Performed data analysis: Kryszkie-Matzka, Olzanecki, Korbus. Wrote or contributed to the writing of the manuscript: Magierowski, Kwiecien, Brzozowski.

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Bregonzio C, Armando I, Ando H, Jezova M, Biaiardi G, and Saavedra JM (2004) Angiotensin-1-7 gastrin releasing protein released from rat sensory nerve enteral, also mediates Ang-(1–7)-induced protection and hyperemia. Exogenous 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 GBF was significantly less 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 protective factor in the stomach, can cooperate with Ang-(1–7) in this protection.

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