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 renin-angiotensin system (RAS). Ang-(1–7) acting via Ang-(1–7) receptor was documented in kidneys, heart, liver, 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) Mas receptors, AVE 0991, [5-formyl-4-methoxy-2-phenyl-[4-[2-(ethylaminocarbonylsulfonamido)-5-isobutyl-3-thienyl]-phenyl]-methyl]-imidazole, the agonist of Ang-(1–7) receptors as well as the inhibition of nitric-oxide synthase, the production 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 assayed by reverse transcription polymerase chain reaction. The WRS lesions were dose-dependently reduced by Ang-(1–7) treatment, which also induced an increase in gastric blood flow (GBF) and renal content of NO. COX-1 and COX-2 inhibitors [5-imino(nitroamino)methyl-L-ornithine] 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 augmented the reduction of Ang-(1–7)-induced gastric lesions and decrease 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 neuropeptides, 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 (Saul et al., 2006). The components of RAS appear to be functionally active in numerous organs including kidneys, heart, liver, 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 malabsorption and electrolyte homeostasis, maintenance of normal GBF 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 NADPH oxidase in vascular smooth muscle cells (VSMCs), enhances the production of reactive oxygen species such as NO, and platelet aggregation.

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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-[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-OMA, L-ornithine; NO, nitric-oxide synthase; PG, prostaglandin; RAS, renin-angiotensin system, SC-560, [5-(4-chloro-phenyl)-1-(4-methoxyphenyl)-3-trifluoromethyl-pyrazole]; TNF, tumor necrosis factor; WRS, water immersion and restraint stress.
superoxide and hydrogen peroxide (H$_2$O$_2$), and inactivates NO pathway (Mehta and Griendling, 2007). Ang II-activating phospholipase C (PLC) and protein kinase C (PKC) or phospholipase A$_2$ enhanced synthesis of vasoconstrictive leukotrienes and smooth muscle cell contraction (Mehta and Griendling, 2007; Lemarie et al., 2009). Increased reactive oxygen species (ROS) and decreased blood flow play fundamental roles in the pathogenesis of GI mucosal injury (Bregenzo et al., 2003; Nakagiri et al., 2010).

Exposure to stress is commonly recognized as a risk factor of microbleeding and gastric mucosal injury. Reaction to stress is mediated via two distinct but unrelated systems: the hypothalamic-pituitary-adrenocortical (HPA) system and the sympathoadrenal system (Goldstein and McEwen, 2002; Saavedra et al., 2006). Ang II receptor subtypes AT$_1$ and AT$_2$ were detected in the human esophageal, gastric, small intestinal, and colonic mucosa (Hirasawa et al., 2002; Casselbrant et al., 2009; Hallersund et al., 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 and the attenuation of vasoconstrictor and proinflammatory activity of Ang II (Pavel et al., 2003; Mory et al., 2009; Gemici et al., 2010; Saavedra et al., 2006; Santos et al., 2011).

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

Angiotensin-(1–7) (Ang-(1–7)) has been shown to be a potent vasoactive agonist generated from angiotensin I through angiotensin-converting enzyme (ACE) homolog ACE$_2$ 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; Xu et al., 2011). Ang-(1–7) acting via its own G protein-coupled receptor, called Mas (Santos et al., 2005; Stegall et al., 2009) exhibits the vasodilatory, antihypertensive, cardioprotective, and anti-inflammatory effects, Ang I is quickly degraded by ACE in the rat stomach, leading to the formation of Ang-(1–7) in the rat stomach. The formation of Ang-(1–7) in the rat stomach is independent of the Ang II (Czarniecki et al., 2009). Mas receptor knockout mice exhibited increased NO production due to dysfunction of eNOS, suggesting a link between Ang-(1–7) and Mas receptor (Xu et al., 2008). The vasoconstrictive function of Ang II in hypertension is limited by vasoactive Ang-(1–7) and bradykinin (Oliveira et al., 2002; Sampaio et al., 2007). Ang-(1–7) exhibits ex vivo gastroprotection against reflux esophagitis (Pompili et al., 2012). Whether Ang-(1–7) protects the gastric mucosa against stress injuries due to an increase in NO and the action of prostaglandin (PG)/COX-1 and PG/COX-2 pathways and sensory nerves has not been extensively studied.

We compared the 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)-1ß and tumor necrosis factor (TNF)-a, 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 the study. Rats were fasted for 24 hours with free access to water before exposure to WRS. The study was approved by the Institutional Animal Care and Use Committee of Jagiellonian University Medical College in Cracow and was conducted in accordance with the statements of the Helsinki Declaration regarding handling of experimental animals.

**Assessing Stress-Induced Gastric Lesions, Chemicals, and Drugs.** To induce gastric lesions, rats were immobilized in individual Bolman cages and immersed in the cold water (23°C) for 3.5 hours or until the rat xyphoid level as reported by our group previously (Brzozowski et al., 2000, 2006; Satoh et al., 2013). In one group, major sensory nerves (atlanto-G) were cut. 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.), B) Ang II (5 mg/kg i.p.), or C) perindopril (5 mg/kg i.p.), the nonpeptide Ang-(1–7) Mas receptor antagonist (Bayorh et al., 1999; Santos et al., 2010) with or without the combination with Ang-(1–7) in rats exposed 30 minutes later to 3.5 hours of WRS. In the group A, the nonpeptide Ang-(1–7) receptor agonist (Pinheiro SV et al., 2004; Santos and Fereira, 2006), respectively.

**In vitro.** The effects of cotreatment with Ang-(1–7) or perindopril, with or without the combination with L-NNA (20 mg/kg i.p.), the competitive inhibitor of NO-synthase activity, on WRS lesions and alterations in the GBF were determined.

**In vivo.** The exogenous PG in the gastroprotective effects of Ang-(1–7) or vehicle (control) was investigated in rats treated with indomethacin (5 mg/kg i.p.), the selective 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 E$_2$ (PGE$_2$, 5 μg/kg i.g.) in the presence of Ang-(1–7).

In series F, 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 sensory nerves in the mechanism of gastroprotection induced by 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 He2-gas clearance technique as reported before (Brzozowski et al., 2004, 2006; Kwiecien et al., 2007). The GBF was measured in the fundic part of the gastric mucosa not involving mucosal lesions. Average values of three measurements were determined and expressed as a percentage of the value determined in intact rat stomach. Gastric lesions number was determined on photographed stomachs with computerized planimetry (Morphomat, Carl Zeiss, Berlin, Germany) (Kwiecien et al., 2012a) by a blinded investigation.

**Determination of Luminal NO Content and Plasma Level of IL-1β and TNF-α.** The luminal concentration of NO was quantified indirectly as nitrate (NO3–) and nitrite (NO2–) levels in the gastric luminal fluid. Aliquots of gastric luminal fluid were treated with a stabilized chromogen as described before (Kwiecien et al., 2008; Pawlik et al., 2011; Kwiecien et al., 2012b). In brief, the plasma and TFN-α and IL-1β was determined by a solid-phase sandwich enzyme-linked immunosorbent assay (ELISA; BioSource International Inc., Camarillo, CA) according to the manufacturer’s instructions. Each sample (5 μl) was incubated with biotinylated antibodies specific for TFN-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 above (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. The stomachs were removed from rats exposed to WRS without or with the pretreatment with Ang-(1-7) alone or combined with A-779. RT was done as described previously (Kwiecien et al., 2012b). In brief, the plasma and TFN-α and IL-1β was determined by a solid-phase sandwich enzyme-linked immunosorbent assay (ELISA; BioSource International Inc., Camarillo, CA) according to the manufacturer’s instructions. Each sample (5 μl) was incubated with biotinylated antibodies specific for TFN-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 above (Kwiecien et al., 2012b).

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**Statistical Analysis.** All 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 between estimates of effects were considered significant at P < 0.05. 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 experiment rats in terms of relevant characteristics, such as source of the rat, gender, age, weight, diet, and housing conditions. No animals were used in the study. The paired statistical test was not used in this case.

**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 1**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer Sequence</th>
<th>Annealing Temperature (°C)</th>
<th>Size of PCR Product (bp)</th>
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<tr>
<td>c-NOS</td>
<td>Fw: 5′-TAC GGA GCA GCA AAT CCA C-3′, Rev: 5′-CAG CTT GCA GTC CTG TTA TC-3′</td>
<td>63.5</td>
<td>540</td>
</tr>
<tr>
<td>IL-1β</td>
<td>Fw: 5′-GCC ACC CAT TGT TCG GGC ACT-3′, Rev: 5′-GAG CAT TGC TGT TCG CTA GG-3′</td>
<td>62</td>
<td>543</td>
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<tr>
<td>TNF-α</td>
<td>Fw: 5′-TAC TGA ACT TCG GGG TGA TCG GTG C-3′, Rev: 5′-CAG CCT TGT CCG TCG AAG AGA ACC-3′</td>
<td>56</td>
<td>295</td>
</tr>
<tr>
<td>β-Actin</td>
<td>Fw: 5′-TTG TAA CCA ACT GGG ACG ATA TG-3′, Rev: 5′-GAT CTT GAT CTT CAT GGT GCT AGG-3′</td>
<td>54</td>
<td>764</td>
</tr>
<tr>
<td>iNOS</td>
<td>Fw: 5′-CCA CAA TAG TAC AAT ACT ACC-3′, Rev: 5′-ACG AGG TGT TCA GCG TGC T-3′</td>
<td>60</td>
<td>397</td>
</tr>
</tbody>
</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) (50 μg/kg, dose-dependently increased GBF of Ang II-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% (ID50) was 27 μg/kg. Since the dose of 50 μg/kg afforded the maximal protective response (P < 0.05), this dose of Ang-(1–7) was used in all our determinations. The absolute values for GBF expressed in ml/min per 100 g are presented in Table 2. Exposure to WRS in rats pretreated with vehicle-control significantly decreased the GBF (P < 0.05) compared with the values in the intact gastric mucosa. This 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, as completely reversed by the pretreatment with A-779 (50 μg/kg, i.p.) combined with intraperitoneal administration with Ang-(1–7) (Fig. 2; Table 2).

Effect of AVE 0991, the Agonist of Ang-(1–7) Mas Receptor, on WRS-Induced Gastric Lesions and Alterations in the GBF. As shown in Fig. 3, the pretreatment with AVE 0991 (50 μg/kg i.p.) significantly reduced the mean lesion number (P < 0.05) and caused a significant increase in the GBF (P < 0.05) compared with the respective values in vehicle-control pretreated rats. The decrease in lesion number and an increase in the 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 Gastric Protection and Alterations in the GBF. Exposure to WRS in rats pretreated with Ang-(1–7) or perindopril (Fig. 4). Figure 4 shows that pretreatment with AVE 0991 (50 μg/kg i.p.) significantly reduced the WRS-induced gastric lesions and increased GBF, with the effect similar to the respective values presented in Fig. 1. 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 to vehicle-control. Administration of l-NNa (20 mg/kg i.p.), which by itself failed to significantly affect the lesion number and GBF, compared to vehicle-treated control, reversed the suppression 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 Gastric Protection 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) 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>
</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 ± 2.7*</td>
</tr>
</tbody>
</table>

TABLE 2

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

Results are mean ± S.E.M. from seven animals per each experimental group. 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.
The addition of PGE2 (5 mg/kg i.p.), rofecoxib (10 mg/kg i.g.), and SC-560 (5 mg/kg i.g.) significantly attenuated by pretreatment with indomethacin (INDO), SC-560 (SC), and rofecoxib (ROFE) presented in Fig. 2. The pretreatment with COX-1 and COX-2 inhibitors alone significantly increased the mean lesion number and produced a significant fall in GBF compared with vehicle-treated animals (Fig. 5). The reduction of lesion number by Ang-(1–7)–pretreated with vehicle (Veh; control) or AVE 0991 (50 μg/kg i.p.) and exposed to WRS. Results are mean ± S.E.M from seven rats per each experimental group. The values in AVE 0991 group were compared with vehicle-controls and with those in AVE 0991–pretreated with vehicle, Ang-(1–7) (50 μg/kg s.c.), and SC-560 (5 mg/kg i.g.) given in combination with A-779. Asterisk 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)–pretreated 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 the 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)–pretreated with vehicle, Ang-(1–7) without or with the combination of indomethacin (INDO), SC-560 (SC), and rofecoxib (ROFE) with or 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 produced a significant reduction in the presence or the absence of PGE2. Asterisk indicates a significant change (P < 0.05) compared with the respective values in vehicle−control group. Cross indicates significant a 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 protective effect of this peptide in the present 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) 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
**Gastric Mucosal Expression of cNOS, iNOS, IL-1β, and TNF-α mRNAs in Rats Treated with Ang-(1-7) alone or with the Combination with A-779**

**Materials and Methods**

Angiotensin II (ANG II, 40 μg/kg i.p.) or Ang-(1-7) (50 μg/kg i.p.) before the exposure to 3.5 hours of WRS. Results are mean ± S.E.M from six 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 < 0.05) compared with the respective values in intact rats. Ang-(1-7)-pretreated rats. Double crosses indicate a significant change (P < 0.05) compared with the values in any-control group with or without denervation.

**Figure 8 (upper panel)** demonstrates that the signal for IL-1β and TNF-α mRNAs were strongly detected in vehicle-treated gastric mucosa, and the ratio of IL-1β or TNF-α mRNA over β-actin mRNA (Fig. 8, right panel) confirmed that IL-1β and TNF-α mRNAs were significantly upregulated in WRS-induced gastric mucosa. These effects were significantly attenuated when rats were pretreated with 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) (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 II 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 and vehicle-pretreated and in those treated with combination of A-779 and Ang-(1–7) versus Ang-(1–7) alone. Asterisk indicates a significant change (P < 0.05) compared with vehicle-control gastric mucosa. Cross indicates a significant change (P < 0.05) compared with Ang-(1–7) alone.

**Fig. 8.** Determination of cNOS mRNA, IL-1β, and TNF-α expressions by RT-PCR (left panel) and the ratio of cNOS, IL-1β, and TNF-α mRNAs over β-actin mRNA (right panel) in the vehicle (Veh)-control gastric mucosa (lane 1) and in those pretreated with Ang-(1–7) (50 μg/kg i.p.) (lane 2), and A-779 (50 μg/kg i.p.) combined with Ang-(1–7) (50 μg/kg i.p.) (lane 3) and exposed to WRS for 3.5 hours; M, DNA size marker. Mean ± S.E.M. of four determinations in four rats per group. Analysis of the values of the ratio of cNOS, IL-1β, and TNF-α mRNA expressions in gastric mucosa was performed between values in Ang-(1–7)-pretreated and in those treated with combination of A-779 and Ang-(1–7) versus Ang-(1–7) alone. Asterisk indicates a significant change (P < 0.05) compared with vehicle-control gastric mucosa. Cross indicates a significant change (P < 0.05) compared with Ang-(1–7) alone.
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., 2004). Bregonzio et al. (2004) observed that AT1-receptor antagonists help to maintain the proper gastric blood perfusion via the reduction of sympathetic neural activity and by attenuating gastric ulcers in rodents (Merai et al., 2009; Morsy et al., 2007; Konturek et al., 2011), we determined the effect of AT1-receptor antagonists on the WRS-induced gastric lesions. However, the blockade of AT1-R in WRS-induced gastric lesions accompanied by the fall in the GBF. Moreover, Ang-(1–7) significantly and dose-dependently attenuated gastric ulcer formation (Merai et al., 2009; Morsy et al., 2009) and counteracted the effects of ischemia and inflammationmediated by COX/PG system responsible for the attenuation of malondialdehyde content and rise in superoxide dismutase activity. The intestinal mucosal COX-2 expression is regulated by both AT1 and AT2 receptors (Tani et al., 2003). 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 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-(7–9) (Neves et al., 2000; Olszanecki et al., 2009), but the 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 is augmented by Ang II, suggesting that, in contrast to Ang-(1–7), known as a potent vasoactive (Fig. 10), WRS-induced gastric damage due to its proinflammatory reaction. This corroborative with the observation that high levels of circulating Ang-(1–7) ameliorated the metabolic stress-induced gastric damage via decrease in the proinflammatory profile adipose tissue 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) activates an endogenous inhibitor of ACE, enhanced the vasoactive effects of bradykinin (Tom et al., 2003). In our study, perindopril significantly decreased WRS-induced gastric lesions and raised GBF with an extent similar to that observed with Ang-(1–7). L-NNA reduced the gastroprotective and hyperemic activity of perindopril, suggesting that this action and rise in the GBF caused by ACE inhibitor might be at least partially 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 Ang receptors. Interestingly, the antagonist A-779 has been shown to inhibit most of the physiologic effects of Ang-(1–7) against stress ulcerogenesis. In our study, the gastroprotection and increase of
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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 sensory neuropeptides such as CGRP. In contrast to Ang II, Ang-(1–7) show opposite action against stress ulcerogenesis, because Ang II enhanced stress lesions. The mechanism of Ang-(1–7) in various gastrointestinal disorders.

Authorship Contributions

Performed experiments: Magierowski, Pawlik, Kwiecien, Brzozowski.

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References


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