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 MAS receptor was documented in kidneys, heart, liver, and gastrointestinal (GI)-tract. We studied the gastroprotective activity of exogenous Ang-(1–7) in restrained rats under water immersion and restraint stress (WRS) without or with AVE 0991 (5-formyl-4-methoxy-2-phenyl-1[4-[2-(ethylaminocarbonylsulfonamido)-5-isobutyl-3-thienyl]-phenyl]-methyl-imidazole), the agonist of Ang-(1–7) receptor, as well as the inhibition of nitric-oxide synthase (NO) synthase, the suppression of cyclo-oxygenase (COX)-1 and COX-2 (rofecoxib), and denervation with capsaicin. The mRNA expression of constitutively expressed nitric-oxide synthase (cNOS), inducible nitric-oxide synthase (iNOS), IL-1b and TNF-α was assessed by reverse transcription polymerase chain reaction. The WRS lesions were dose-dependently reduced by pretreatment with Ang-(1–7), which also caused an increase in gastric blood flow (GBF) and muscle content of NO. COX-1 and COX-2 inhibitors (Nω-nitro-L-arginine-methyl ester)-l-omithine 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 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 ulcers, affords potent gastroprotection against WRS ulcerogenesis via an increase in GBF mediated by NO, endogenous prostaglandins, sensory neuroprotection 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-mineral balance (Paul 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 motility and electrolyte homeostasis, maintenance of normal blood 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...
superoxide and hydrogen peroxide (H_2O_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 (Bregonzio 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 neuronal control and attenuation of vasoconstriction and proinflammatory activity of Ang II (Pavel et al., 2009; Mory et al., 2009; Gemici et al., 2010; Saavedra et al., 2006; Simões et al., 2011).

Antagonists of AT_1 receptor candesartan and losartan prevented stress-induced gastric lesions (Bregonzio 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 AT_2 receptor 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, kidneys, liver, and stomach (Santos et al., 2002; Stegbauer et al., 2011). Ang-(1–7) acting via its own G protein-coupled receptor, called Mas (Santos et al., 2002; Stegbauer et al., 2011), exhibit the vasodilatory, antihypertensive, cardioprotective, and antimicrobial effects, and I is quickly degraded by ACE in the rat stomach in the formation of Ang-1–7–precedes even AT_1 receptors in the formation of Ang II (Czarniecki et al., 2009). Mas-related knockout mice exhibited a depletion due to dysfunction of endothelial nitric oxide synthase, 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; Czarniecki et al., 2006; Sampaio et al., 2007). Ang-(1–7) exhibited endopeptidase protection against reflex esophagitis (Parrish et al., 2012). Whether Ang-(1–7) protects the gastric mucosa against stress lesions due to an increase of NO and the action of prostaglandin (PG)/COX-1 and PG/COX-2 pathways and sensory nerves has not been extensively studied before.

We compared the exogenous 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 aferrent 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)-1β and tumor necrosis factor (TNF)-α, and plasma levels of these cytokines during stress ulcerogenesis.

Materials and Methods

Animals. Male Sprague rats total 25 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 with the statements of the Helsinki Declaration regarding handling experimental animals.

Stress-Induced Gastric Lesions, Chemicals, and Drug Application. To induce gastric lesions, rats were immobilized in individual Polman cages and immersed in ice water (23°C) for 3.5 hours. The rats were separated into groups previously described (Brzozowski et al., 2000, Konturek et al., 2001). Three major subgroups of rats (ann G) were selected. Thirty minutes before exposure to water immersion and restraint stress (WRS), rats in series A–C received pretreatment with either: A) exogenous Ang-(1–7) (6.25–50 μg/kg i.g.); B) exogenous AT_1 receptor agonist (Pinheiro SV et al., 2004; Santos and Fereira, 2006), 30 minutes of WRS or AVE 0991 (50 μg/kg i.p.), the nonpeptide ACE inhibitor captopril (5 mg/kg i.p.); C) perindopril (8 mg/kg i.p.), the nonpeptide ACE inhibitor. (Jawien et al., 2012). The angiotensin 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 AT_1 receptor antagonist (Bayorh et al., 1999; Santos et al., 2002, 2005) or with the combination with Ang-(1–7) for 30 minutes later to 3.5 hours of WRS, the group E AVE 0991 (50 μg/kg i.p.), the nonpeptide Ang-(1–7) receptor antagonist, Pinheiro SV et al, 2004; Santos and Fereira, 2006), respectively.

In series G, the effects of pretreatment with Ang-(1–7) or perindopril (10) or with the combination with i-NNA (20 mg/kg i.p.), perindopril (5 mg/kg i.p.), and competitive inhibitor of NO-synthase activity, on WRS lesions and afferent nerves in the GBF were determined.

The involvement of endogenous PG in the gastroprotective effects of Ang-(1–7) or vehicle (control) was investigated in rats (F) treated with indomethacin (5 mg/kg i.p.), the nonselective COX-1 and COX-2 inhibitor, or SC-560 (5 mg/kg i.p.), the selective inhibitor of COX-1, and rofecoxib (10 mg/kg i.p.), the selective inhibitor of COX-2 activity as reported in our previous studies (Brzozowski et al., 2000, 2006; Satoh et al., 2013). In another subgroup with COX-1 and COX-2 inhibitors, rats of series F were coadministered with exogenous prostaglandin E_2 (PGE_2; 5 μg/kg i.g.) in the presence of Ang-(1–7).

In series G, the effect of blockade of sensory nerves induced by large dose of capsaicin (total 125 mg/kg s.c.) on the protective and hyperemic activity of Ang-(1–7) was examined. Capsaicin was injected for 3 consecutive days at a respective dose of 25, 50, and 50 mg/kg s.c. approximately 2 weeks before the experiment to induce the functional ablation of sensory nerves as described previously (Konturek et al., 2009; Kwiecien et al., 2012a). In separate subgroup of series G with capsaicin denervation, the involvement of calcitonin gene–related peptide (CGRP), the major rat neuropeptide released from sensitive afferent nerve endings in protective action of exogenously administered Ang-(1–7) against WRS lesions, was determined. In one of the subgroups of series G, the capsaicin-denervated rats received supplementation with exogenous CGRP (10 μg/kg s.c.) combined with Ang-(1–7) and 30 minutes later were exposed to onset of WRS as in other groups described above.

All tested drugs and compounds were of analytical grade and were purchased from Sigma-Aldrich Laborchemikalien (Schelldorf, Germany) except of SC-560 and rofecoxib purchased from Cayman.
Chemical (Ann Arbor, MI) and Pfizer (Ille<ref>lcalcogeneous, 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 was measured by means of $H_2$-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 change of the value determined in intact rat stomach. Gastric lesions number was determined on photographed stomachs with computerized planimetry (Morphomat, Carl Zeiss, Berlin, Germany) (Kwiecien et al., 2012a) by a blinded investigation.

**Determination of Luminal NO Content and Plasma Level of IL-1β and TNF-α.** The luminal concentration of NO was quantified indirectly as nitrate (NO$_3$) and nitrite (NO$_2$) levels in the gastric contents using the nitrate/nitrite kit purchased from Cayman Chemical as described in detail in our previous studies (Brzozowski et al., 2008; Pawlik et al., 2011; Kwiecien et al., 2012b).

The blood samples (~3 ml) were taken from the vena cava for the measurement of plasma proinflammatory cytokines IL-1β and TNF-α as described previously (Kwiecien et al., 2012b). In brief, the plasma TNF-α and IL-1β was determined by a solid-phase sandwich enzyme-linked immunosorbent assay (ELISA; BioSource International Inc., Camarillo, CA) according to the manufacturer’s instructions. Plasma sample (50 μl) was 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 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. The stomachs were removed from rats exposed to WRS without the pretreatment with Ang-(1-7) alone or combined with Ang II. The determination mRNA expression of cNOS, iNOS, IL-1β, and TNF-α 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 each sample by a guanidium isothiocyanatephenol-chloroform method using a kit from Stratagene (La Jolla, CA). The RNA concentration in each sample was determined using 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 mRNA were standardized against the β-actin signal for each sample, and results were expressed as cNOS/a-β, iNOS/a-β, and TNF-α mRNA/β-actin values.

**Statistical Analysis.** Results of the experiment were expressed as mean ± S.E.M. and the statistical analysis was performed with two-way analysis (variance ANOVA) test and Tukey post hoc test where appropriate. Differences between estimates of effects were considered significant at $P<0.05$. All results in the treated animals were compared with the appropriate control group, which had been established 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 purchase, gender, age, weight, diet, and housing conditions. There was no individual pairing of animals, the paired statistical test therefore 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>Forward Primer Sequence</th>
<th>Reverse Primer Sequence</th>
<th>Annealing Temperature °C</th>
<th>Size of PCR Product bp</th>
</tr>
</thead>
<tbody>
<tr>
<td>c-NOS</td>
<td>5'- TAC GGA GCA GCA AAT CCA C-3', Reverse: 5'- CAG GCT GCA GTC CIT TGA TC-3'</td>
<td>63.5</td>
<td>540</td>
<td></td>
</tr>
<tr>
<td>IL-1β</td>
<td>5'- GCT ACC TAT GTC TGG CCC GT-3', Reverse: 5'- GAC CAT TGC TGT TTC CTA GG-3'</td>
<td>62</td>
<td>543</td>
<td></td>
</tr>
<tr>
<td>TNF-α</td>
<td>5'- TAC TGA ACT TCG GGG TGA TTG GTC C-3', Reverse: 5'- CAG CCT TGG CCT TGG AAG AGA ACC-3'</td>
<td>56</td>
<td>295</td>
<td></td>
</tr>
<tr>
<td>β-Actin</td>
<td>5'- TGG TAA CCA ACT GGG ACG ATA TGG-3', Reverse: 5'- GAT CTT GAT CTT CAT GGT GCT AGG-3'</td>
<td>54</td>
<td>764</td>
<td></td>
</tr>
<tr>
<td>iNOS</td>
<td>5'- CCA CAA TAG TAC AAT ACT AC-3', Reverse: 5'- ACG AGG TGT TCA GCG TGC TC-3'</td>
<td>60</td>
<td>397</td>
<td></td>
</tr>
</tbody>
</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 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. 2. Mean lesion number and the changes in the GBF in rats pretreated intraperitoneally with vehicle (saline; Veh) or Ang-(1–7) in graded doses ranging from 6.25 to 50 μg/kg, dose-dependently attenuated WRS-induced gastric lesions, while producing a significant and a 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 compared with the values in the intact gastric mucosa. This fall in GBF under WRS conditions was significantly reversed 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.) compared with intraperitoneal treatment with Ang-(1–7) (Fig. 2; Table 2).

Effect of A-779, 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 AVE 0991 and A-779 (P < 0.05).

Effect of Suppression of NO-Synthase on Ang-(1–7)- and Perindopril-Induced Gastroprotection and Alterations in GBF. As shown in Fig. 4, the pretreatment with AVE 0991 (50 μg/kg i.p.) significantly reduced the WRS-induced gastric lesions and increased GBF, with the effects similar to the respective values presented in Fig. 2. The pretreatment with perindopril (5 mg/kg i.p.) also significantly increased the number of WRS-induced gastric lesions by 20% (P > 0.05) and significantly increased GBF compared to vehicle-control. Administration of L-NNA (20 mg/kg i.p.), which itself failed to significantly affect the lesion number and GBF, compared to vehicle-treated control, reversed the effect of AVE 0991 on lesion number and the rise in GBF evoked by Ang-(1–7) or perindopril (Fig. 4).

Table 2

<table>
<thead>
<tr>
<th>Type of Test</th>
<th>GBF (ml/min per 100 g)</th>
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<tbody>
<tr>
<td>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>

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 gastric lesions compared to vehicle-control and reversed the decrease in GBF and luminal NO content observed at the 50 μg/kg dose of Ang-(1–7) alone.

Materials and Methods

Animals. Male Sprague-Dawley rats weighing 250–300 g were used. The animals were housed in a climate-controlled environment (23 ± 2°C and 50 ± 10% humidity) on a 12-h light-dark cycle. The rats were provided with water and rat chow ad libitum.

Induction of WRS. WRS was used to induce gastric lesions as described elsewhere (7). Briefly, 0.5 ml of 10% trinitrobenzene sulfonic acid (98%) in 95% ethanol was administered by a single intragastric injection into fasted rats (15 min after food deprivation). The control animals received an equivalent volume of saline.

Effect of Pretreatment with Ang-(1–7) and Perindopril on WRS-Induced Gastric Lesions and Alterations in GBF.

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) administered i.p. in graded doses ranging from 6.25 to 50 μg/kg, dose-dependently reduced WRS-induced gastric lesions, while producing a significant and a 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.
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 in Ang-(1–7)-treated animals without concomitant treatment with COX inhibitors. Double asterisks indicate a significant change (P < 0.05) compared to the values obtained in Ang-(1–7)–treated animals without concomitant treatment with COX inhibitors. Asterisk indicates a significant change (P < 0.05) compared with respective values achieved with Ang-(1–7) treatment. Cross indicates a significant change (P < 0.05) compared with the respective values in vehicle (Veh)-control group.

As shown in Fig. 6, the 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
Fig. 6. Mean lesion number and the changes in GBF in rats pretreated with vehicle, Ang-(1–7) (50 μg/kg i.p.), or CGRP (10 μg/kg s.c.), in rats with intact sensory nerves and in those with functional ablation of sensory nerves by capsaicin (capsaicin denervation) and exposed to 3.5 hours of WRS. To induce the functional ablation of sensory nerves, 25 rats were injected with capsaicin in 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 exposure experiments. Results are mean ± S.E.M from six rats per each experimental group. The asterisk indicates a significant change (P < 0.05) compared with those in intact rats. Asterisk and cross indicates a significant change (P < 0.05) compared to the values obtained in animals with intact sensory nerves and 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). The further 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 (P < 0.05) (Fig. 7). In contrast, the plasma levels of IL-1β and TNF-α of intact vehicle-control group and Ang II-pretreated group were significantly lower than those observed in Ang-(1–7)-pretreated group (P < 0.05) (Fig. 7). The alterations in plasma IL-1β and TNF-α levels in intact sensory nerves and those pretreated with vehicle (Veh; control), angiotensin II (ANG II, 40 μg/kg i.p.) for Ang-(1–7) (50 μg/kg i.p.) before the exposure to 3.5 hours of WRS. Results are mean ± S.E.M from 10 rats per each experimental group. The values in Ang-(1–7)-pretreated rats were compared 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 the respective values in Ang-(1–7)-pretreated rats. Cross indicates a significant change (P < 0.05) compared with the values in vehicle-control and Ang II groups.

The ratio of IL-1β mRNA over β-actin confirmed that IL-1β mRNA was significantly increased when A-779 was combined with Ang-(1–7) alone (Fig. 8, right panel).

A-779 and TNF-α mRNAs were significantly downregulated 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 in those 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) protective effects. This indicates 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 and by either nonselective or selective 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 counteracted the capsaicin-induced gastric impairment and the accompanying fall in the gastric GBF observed in a retraction notice.
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 (Szalachci et al., 2015) 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; Konturek et al., 2011; Garg et al., 2012). Bregenzo et al. (2001) observed that AT1 blockade led to increase in adrenal cortical tone, reduction in TNF-α and interleukin-1β (IL-1β) expression, and neutrophil infiltration in stressed animals. However, the blockade of AT1R does not influence gastroprotective action of corticosterone released during stress (Filaretova et al., 1998; Level et al., 2008). Similarly, AT1-receptor antagonists dose-dependently attenuated gastric ulcers induced by rottent (Merai et al., 2009; Morsy et al., 2009) and counteracted the effects of ischemia and inflammation on the reductions of mucosal neutrophil 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) (Neves et al., 2000; Olszanecki et al., 2009), but this hypothesis requires further studies.

Our results show that WRS decreased the expression and plasma levels of TNF-α and IL-1β, and that the plasma level of these proinflammatory cytokines was increased by Ang II, suggesting that the absence of Ang-(1–7) and Ang II, known as a potent vasoactive, aggravated WRS-induced gastric damage due to its proinflammation reaction. This is corroborative with our observations that high levels of circulating Ang-(1–7) significantly reduced the gastric stress-induced cytokine profile, proadipogenic cytokine (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, Clarin et al. (2001) reported a direct binding of Ang-(1–7) to the Ang II receptor and, thus, resulting in downregulation of these receptors. In keeping with these findings, we observed decreased expression and plasma levels of IL-1β and TNF-α in rats pretreated with Ang-(1–7), indicating that the interplay difference between Ang-(1–7) and Ang II with respect to proinflammatory cytokines. Moreover, the endogenous Ang II could contribute to pathogenesis of cold-straint stress ulcer in obstructive jaundice rats (Mou et al., 1998). Enalapril, an inhibitor of ACE, reduced both the stress 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 vasodilator effects of bradykinin (Tom et al., 2003). In our study, peritoneally administrated Ang-(1–7) significantly decreased WRS-induced gastric lesions and increased GBF with an extent similar to that of rats co-treated with 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) 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

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**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 mucosoprotective mechanisms due to an 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.
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 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 denervation of sensory fibers. This indicates that besides NO and PG affenter sensory fibers and the major sensory neuropeptide CGRP released from rat sensory nerve endings might mediate Ang-(1–7)-induced protection and hyperemia. Endogenous CGRP in the presence of Ang-(1–7) restored these protective effects 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 conclude that CGRP, which is a potent vasodilator and a growth factor in the stomach, can cooperate with Ang-(1–7) in this protection.

In summary, Ang II and Ang-(1–7) are opposite active against stress ulcerogenesis because Ang II enhances stress ulcerogenesis but Ang-(1–7) afforded protection against stress lesions. The mechanism of Ang-(1–7)-induced protection against stress may involve activation of NO/c-NOS and PG COX system and mediolateral and gastroprotective sensory neuropeptides such as CGRP. In contrast to Ang II, Ang-(1–7) exhibited strong anti-inflammatory properties that involve expression suppression of proinflammatory cytokines such as TNF-α. Further studies in experimental rats are warranted to further establish the efficacy of Ang-(1–7) in various other experimental animal models.

Authorship Contributions

Participated in research design: Ptak-Belowska, Kwiecien, Brzozowski.

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