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

Marcin Magierowski, Katarzyna Jasnos, Michal Pawlik, Grzegorz Krzysiek-Maczka, Agata Ptak-Belowska, Rafał Olszanecki, Sławomir Kwicien, Ryszard Korbut, and Tomasz Brzozowski

Department of Physiology (M.M., K.J., M.P., G.K.-M., A.P.-B., S.K., T.B.) and Department of Pharmacology (R.O., R.K.), Jagiellonian University Medical College, Cracow, Poland

Received June 17, 2013; accepted September 18, 2013

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) acts via the Mas receptor was documented in kidneys, heart, liver, and a gastrointestinal (GI) tract. We studied the gastroprotective activity of exogenous Ang-(1–7) in the WRS-induced gastric lesions evoked by 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-1-[4-[2-(ethylaminocarbonylsulfonamido)-5-isobutyl-3-thienyl]-phenyl]-methyl]-imidazole, the activity COX-2 (rofecoxib), and NOS, nitric-oxide synthase (cNOS), inducible nitric-oxide synthase (iNOS), interleukin (IL)-1β, and tumor necrosis factor (TNF-α) was assessed by reverse transcription polymerase chain reaction (RT-PCR) in kidneys, heart, liver, 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 glucose, gastrointestinal motility, mucosal secretion, gastric inflammation, and carcinogenesis (Pandriks 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 glucose, gastrointestinal motility, mucosal secretion, gastric inflammation, and carcinogenesis (Pandriks 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 glucose, gastrointestinal motility, mucosal secretion, gastric inflammation, and carcinogenesis (Pandriks 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 glucose, gastrointestinal motility, mucosal secretion, gastric inflammation, and carcinogenesis (Pandriks 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 glucose, gastrointestinal motility, mucosal secretion, gastric inflammation, and carcinogenesis (Pandriks 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).
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 (Bregnonio et al., 2003; Nakagiri et al., 2010).

Exposure to stress is commonly recognized as a risk factor of microbleeding and gastric mucosal injury. Reaction to stress is mediated via two distinct but unrelated systems: the hypothalamic-pituitary-adrenocortical (HPA) system and the sympathoadrenal system (Goldstein and McEwen, 2002; Saavedra et al., 2006). Ang II receptor subtypes AT₁ and AT₂ were detected in the human esophageal, gastric, small intestinal, and colonic mucosa (Hirasawa et al., 2002; Casselbrannt 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 nervous axis and the attenuation of vasoconstrictor and proinflammatory activity of Ang II (Pavel et al., 2003; Morsy et al., 2009; Gemici et al., 2010; Saavedra et al., 2011).

Antagonists of AT₁ receptor candesartan and losartan prevented stress-induced gastric lesions (Bregnonio et al., 2003,2004; Merai et al., 2009). Angiotensin-(1–7) [Ang–(1–7)] is a downstream peptide generated from angiotensin I by kininogen converting enzyme (ACE) homologs angiotensin-converting enzyme (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., 2003; Stegbauer et al., 2004) exhibit the vaso dilatory, anti hypertensive, cardio protective, antiinfective effect, and Ang I is quickly degraded in the plasma. Ang–(1–7) in the rat stomach, the formation of Ang–(1–7) in the stomach, Ang–(1–7) may even precede Ang I conversion to Ang II (Castro et al., 2005). Mas receptor knock out mice and Ang–(1–7) administration due to dysfunction of endothelial NO synthase expression, suggesting a link between Ang–(1–7) and Mas receptor (Xu et al., 2008). The vasoconstrictive effects of Ang II in hypertension is limited by vasoactive Ang–(1–7) and bradykinin (Oliveira et al., 2005; Sampaio et al., 2007). Ang–(1–7) exhibited antiapoptotic protection against reflux esophagitis (Pallarés 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 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 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)-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 performed with the stated approval of the Helsinki Declaration regarding the handling and use of experimental animals.

Stress-Induced Gastric Lesions, Histological, and Biochemical Application. To induce gastric lesions, rats were immobilized in individual Polman cages and immersed in water at 36°C for 3.5 hours or to the rat xyphoid level as reported by our group previously (Brzozewski et al., 2000, Konturek et al., 2001). The rats in series A, B, C, D, E, F, G were selected. Thirty minutes before exposure to water immersion and restraint stress (WRS), rats in series A–C received pretreatment with one of the following: A) exogenous Ang–(1–7) (6.25–50 μg/kg s.c.), B) Ang II (1 μg/kg s.c.), and C) perindopril (8 mg/kg i.p.), the subcutaneous ACE inhibitor (Santos et al., 2012). The Angiotensin Mas receptor agonistic and antagonist activities were determined in a separate group of rats (series D) treated with A–779 (5 mg/kg i.p.), the selective AT₁ receptor antagonist (Bayohr et al., 1999; Santos et al., 2003; Calixto et al., 2005) with or without the combination of Ang–(1–7) rats exposed 30 minutes later to 3.5 hours of WRS with or without AVE 0991 (5 μg/kg i.p.), the nonpeptide Ang–(1–7) receptor antagonist (Pinheiro SV et al., 2004; Santos and Ferreira, 2006), respectively.

In series A, the effects of cotreatment with Ang–(1–7) or perindopril, with or without combination with 1-NNA (20 mg/kg i.p.), the selective inhibitor of NO-synthase activity, on WRS lesions and the activities in the other groups were determined.

The involvement of endogenous PG in the gastroprotective effects of exogenous Ang–(1–7) or vehicle (control) was investigated in rats 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 (Brzozewski et al., 2000, 2006; Satoh et al., 2013). In another subgroup with COX-1 and COX-2 inhibitors, rats of series F were coadministered with exogenous prostaglandin E₂ (PGE₂; 5 μg/kg i.g.) in the presence of Ang–(1–7).

In series G, the effect of blockade of sensory nerves induced by large dose of capsaicin (total 125 mg/kg s.c.) on the protective and hyperemic activity of Ang–(1–7) was examined. Capsaicin was injected for 3 consecutive days at a respective dose of 25, 50, and 50 mg/kg s.c. approximately 2 weeks before the experiment to induce the functional ablation of sensory nerves as described previously (Konturek et al., 2009; Kwiecien et al., 2012a). In separate subgroup of series G with capsaicin denervation, the involvement of calcitonin gene–related peptide (CGRP), the major rat neuropeptide released from sensitive afferent nerve endings in protective action of exogenously 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 (Ilternissen, Germany), respectively.

Measurement of GBF and Determination of Gastric Lesion Number. At the termination of 3.5 hours WRS, rats were anesthetized with pentobarbital (60 mg/kg i.p.), the abdomen was opened, and GBF measured by means of H₂-gas clearance technique as reported before (Brzozowski et al., 2004, 2006; Kwiecien et al., 2007). The GBF was measured in the fundic part of the gastric mucosa not involving mucosal lesions. Average values of three measurements were determined and expressed as a percentage of the value determined in intact rat stomach. Gastric lesions number was determined on photographed stomachs with computerized planimetry (Morphomat, Carl Zeiss, Berlin, Germany) (Kwiecien et al., 2012a) by a blinded investigation.

Determination of Luminal NO Content and Plasma Level of IL-1ß and TNF-α. The luminal concentration of NO was quantified indirectly as nitrate (NO₃⁻) and nitrite (NO₂⁻) levels in the gastric luminal 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-1ß and TNF-α as described previously (Kwiecien et al., 2012b). In brief, the plasma sample (50 μl) was incubated with biotinylated antibodies specific for TNF-α and IL-1ß, washed three times with assay buffer, and finally conjugated with streptavidin peroxidase to form a complex with a stabilized chromogen as described previously (Kwiecien et al., 2012b).

The expression mRNA of cNOS, iNOS, IL-1ß, and TNF-α in the rat gastric mucosa determined by reverse transcription-polymerase chain reaction (RT-PCR) of the stomachs were removed from rats exposed to WRS without or with the pretreatment with Ang-(1-7) alone or combined with A-779. Total RNA was extracted from mucosal samples by a guanidium isothiocyanate-phenol-chloroform method using a kit from Stratagene (La Jolla, CA). Total RNA concentration in each sample was determined by means of 1% agarose gel electrophoresis and ethidium bromide staining. Aliquots of RNA samples were reverse transcribed into cDNA (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), and deoxycytidine triphosphate (dCTP), 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 amplification in a 25-μl reaction volume containing 0.3 ml (2.5 IU) Taq polymerase, 200 mM (each) dNTP (Pharmacia, Germany), 1.5 mM MgCl₂, 5 ml Taq polymerase chain reaction buffer (50 mM KCl, 10 mM Tris-HCl, pH 8.3), and primers used at final concentrations 25 ng/μl of total RNA mixture, were overlaid with 25 μl of mineral oil to prevent evaporation. Polymerase chain reaction mixture was amplified in a DNA thermal cycler (Perkin-Elmer/Cetus, Norwalk, CT) in the area dedicated for performing PCR reaction. The electrophoretical analysis of primers for cNOS, iNOS, IL-1ß, TNF-α, and β-actin presented in Table 1 was constructed based on published characteristics. The primers were synthesized by Bioneer/Life Technologies (Egggenstein, Germany).

Polymerase chain reaction products were detected by electrophoresis on a 1.5% agarose gel containing ethidium bromide. Location of predicted products was confirmed by using DNA 100-bp ladder (Gibco/Life Technologies, Eggenstein, Germany) as a standard size marker. The intensity bands were quantified using densitometry (LBK Ultrascan, Pharmacia, Upplands V, Sweden) as described in detail in our previous studies (Brzozowski et al., 2008; Brzozowski et al., 2009). The signals for cNOS, iNOS, IL-1ß, and TNF-α were standardized against the β-actin signal for each sample, and results were expressed as OD of cNOS, iNOS, IL-1ß, and TNF-α mRNA/β-actin mRNA.

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:
The annealing temperature, nucleotide sequence primers, and size of products used for RT-PCR determination

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer Sequence</th>
<th>Annealing Temperature</th>
<th>Size of PCR Product</th>
</tr>
</thead>
<tbody>
<tr>
<td>c-NOS</td>
<td>Forward: 5'- TAC GGA GCA GCA AAT CCA C-3', Reverse: 5'- CAG CTT GCA GTC CTT TGA TC-3'</td>
<td>63.5, 640</td>
<td></td>
</tr>
<tr>
<td>IL-1ß</td>
<td>Forward: 5'- GCT ACC ATT GTC TGG CTC GT-3', Reverse: 5'- GAC CAT TGC TGT TCT CTA GG-3'</td>
<td>62, 543</td>
<td></td>
</tr>
<tr>
<td>TNF-α</td>
<td>Forward: 5'- TAC TGA ACT TCG GGG TGA TGG TGC C-3', Reverse: 5'- CAG CCT TGC TGG TCT AAG AGA ACC-3'</td>
<td>56, 295</td>
<td></td>
</tr>
<tr>
<td>β-Actin</td>
<td>Forward: 5'- TGT TAA CCA ACT GGG ACG ATA TGG-3', Reverse: 5'- GAT CTT GAT CTT CAT GGT GCT AGG-3'</td>
<td>54, 764</td>
<td></td>
</tr>
<tr>
<td>iNOS</td>
<td>Forward: 5'- CCA CAA TAG TAC AAT ACT AC-3', Reverse: 5'- ACG AGG TGT TCA GCG TGC TC-3'</td>
<td>60, 397</td>
<td></td>
</tr>
</tbody>
</table>
The administration of Ang II in higher doses ranging from 10 to 40 μg/kg dose-dependently increased the lesion number and produced a significant dose-dependent decrease in GBF (Fig. 1). The pretreatment with Ang-(1–7) administered i.p. in graded doses ranging from 6.25 to 50 μg/kg, dose-dependently 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. 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 drop 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 a rise in the GBF and luminal NO content observed at the 50 μg/kg dose of this peptide, as completely evinced by the pretreatment with AVE 0991 (50 μg/kg i.p.) compared with intraperitoneal saline or with Ang-(1–7) (Fig. 2; Table 2).

Effect of AVE 0991, the Agonist of Ang-(1–7) Mas Receptor, on WRS-Induced Gastric Damage and Alterations in the Gastrovascular Blood Flow. 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 observed 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 Gastroprotection and Alterations in GBF in Rats Exposed to WRS. Figure 4 shows that pretreatment with Ang-(1–7) (50 μg/kg i.p.) significantly reduced the WRS-induced gastric lesions and increased GBF, with the effects similar to the respective values presented in Fig. 2. The pretreatment with perindopril (5 mg/kg i.p.) also significantly decreased 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 itself failed to significantly affect the lesion number and GBF compared to vehicle-treated control, reversed the increase 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 Gastrovascular Blood Flow. 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 in rats pretreated with vehicle controls, Ang II, Ang-(1–7), or perindopril. A significant increase in GBF was observed only in control rats. The combination of Ang-(1–7) and AVE 0991 significantly increased GBF compared to control, Ang-(1–7), or AVE 0991 alone.

### Table 2

<table>
<thead>
<tr>
<th>Type of Test</th>
<th>GBF</th>
</tr>
</thead>
<tbody>
<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>AVE 0991 + Ang-(1–7) + WRS</td>
<td>26 ± 2.2*</td>
</tr>
</tbody>
</table>
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–pretreated animals without concomitant treatment with A-779. Asterisk indicates a significant change (P < 0.05) compared to the respective values in vehicle-control group. Cross indicates a significant change (P < 0.05) compared with the respective values in vehicle-vehicle control group. Double crosses indicate a significant change (P < 0.05) compared to values obtained in AVE 0991-treated rats without concomitant treatment with A-779 treatment.

gastric lesions accompanied by a significant decrease in GBF as presented in Fig. 2. The pretreatment with COX-1 and COX-2 inhibitors alone significantly increased the mean lesion number and produced a significant reduction 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 s.c.), and SC-560 (5 mg/kg i.g.) (P < 0.05), and these effects were accompanied by a significant fall in GBF (Fig. 5). The addition of PGE2 (5 μg/kg i.g.) to Ang-(1–7) restored the gastroprotective effect of this peptide in the presence of COX-1 and COX-2 inhibitors (P < 0.05), and these effects were accompanied by an increase in GBF similar to that recorded in Ang-(1–7)-treated animals without concomitant treatment with COX inhibitors. Double crosses indicate a significant change (P < 0.05) compared to the values obtained in group pretreated with COX inhibitors. Asterisk indicates a significant change (P < 0.05) compared with the respective values in vehicle-vehicle control group. Cross indicates a significant change (P < 0.05) compared with the respective values in vehicle-vehicle control group.

Effect of Pretreatment with Ang-(1–7) or Ang II on Plasma Levels of Proinflammatory Cytokines IL-1β and TNF-α in Rats Exposed to WRS. As shown in Fig. 7, the plasma levels of IL-1β and TNF-α were negligible in intact rats not exposed to WRS. In contrast, the plasma TNF-α and
cNOS mRNA was significantly decreased (P < 0.05) in rats treated 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 WRS. To induce the functional ablation of sensory nerves, 25 rats were injected with capsaicin in total dose of 125 mg/kg s.c. for 3 consecutive days at a respective doses of 25 mg/kg s.c. (day 1), 50 mg/kg s.c. (day 2), and 50 mg/kg s.c. (day 3) approximately 2 weeks before the exposure to WRS. Results are mean ± S.E.M from six rats per each experimental group. The statistical comparisons were made between Ang-(1-7) groups 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 the respective values in intact gastric mucosa, and this effect was significantly reversed in those concomitantly treated with A-779. The ratio of cNOS mRNA over -actin confirmed that mRNA for iNOS was negligible in the intact gastric mucosa, but decreased in those pretreated with Ang-(1-7) (Fig. 8, right panel). A weak signal of cNOS mRNA was recorded in rats with combined administration of A-779 and Ang-(1-7) alone. Ratio of cNOS mRNA over -actin confirmed that cNOS mRNA was significantly decreased (P < 0.05) in rats treated with the combination of A-779 and Ang-(1-7) compared with those administered with Ang-(1-7) alone (Fig. 8, right panel).

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 (Fig. 7). In contrast, Ang-(1-7) (50 μg/kg s.c.) significantly decreased (P < 0.05) the plasma levels of IL-1β and TNF-α compared with vehicle-control group and Ang II-pretreated group (Fig. 8). Figure 8 (upper panel) demonstrates that the signal for cNOS mRNA in gastric mucosa of vehicle-pretreated rats and those administered Ang-(1-7) with or without the combined administration of A-779 and then exposed to WRS, and vehicle-pretreated rats exposed to WRS. The signal for cNOS mRNA was weak compared with that observed in intact gastric mucosa. In contrast, the strong signal for cNOS mRNA was observed in rats pretreated with Ang-(1-7) (50 μg/kg i.p.) and exposed 30 minutes later to 3.5 hours of WRS and compared with those pretreated with vehicle i.p. Ratio of cNOS mRNA over -actin confirmed that cNOS mRNA was significantly increased in Ang-(1-7)-pretreated gastric mucosa over that observed in the vehicle-control gastric mucosa exposed to WRS (Fig. 8, right panel). A weak signal of cNOS mRNA was recorded in rats with combined administration of A-779 and Ang-(1-7) compared with that in animals treated with Ang-(1-7) alone. Ratio of cNOS mRNA over -actin confirmed that cNOS mRNA was significantly decreased (P < 0.05) in rats treated with the combination of A-779 and Ang-(1-7) compared with those administered with Ang-(1-7) alone (Fig. 8, right panel).

As shown in Fig. 8 (left panel) both IL-1β and TNF-α mRNAs were strongly detected in vehicle-pretreated gastric mucosa, and the ratio of IL-1β or TNF-α mRNA over -actin in intact rats (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 -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 -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 -actin confirmed that mRNA for iNOS was significantly increased when A-779 was combined with Ang-(1-7) (Fig. 9, lower panel).
Fig. 9. Determination of iNOS mRNA expression by RT-PCR (left panel) and the ratio of cNOS, IL-1β, and TNF-α mRNAs over β-actin mRNA (right panel) in the intact gastric mucosa (lane 1), vehicle (Veh)-control gastric mucosa exposed to WRS (lane 2), and in those pretreated intraperitoneally with Ang-(1–7) (50 μg/kg i.p.) (lane 3), and A-779 (50 μg/kg i.p.) combined with Ang-(1–7) (50 μg/kg i.p.) (lane 4) 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 mRNAs expression 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.

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 from hyperemia, while AVE 0991, the agonist of Ang-(1–7) receptors, mimicked the gastroprotective and hyperemic actions of Ang-(1–7). Our results provide the evidence that NO-NOS system and PG-COX pathways could be involved in the protective and hyperemic activities of this Ang 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 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; Konturek et al., 2011). Bregenzi et al. (2006) observed that AT1 blockade led to increase in adrenal cortical size, reduction in TNF-α and interleukin-2, chemokine monocyte 1 (ICAM-1) expression, and neutrophil infiltration in stressed animals. However, the blockade of AT1R does not influence gastroprotection and neuropeptide release during stress (Filaretova et al., 1997; Level et al., 2008). Similarly, AT1-receptor antagonists dose-dependently attenuated gastric ulcers and ulcers (Merai et al., 2009; Morsy et al., 2009) and counteracted the effects of ischemia and inflammation in vivo (Tom et al., 2003). Liao et al. (2011) revealed that cardioprotective effect of Ang-(1–7) against ischemia-reperfusion 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 duodenal and gastric mucosal Ang II level, decreased gastric blood flow, and increased the extent of mucosal damage (Mou et al., 1998). Furthermore, Ang-(1–7) activity as an endogenous inhibitor of ACE, enhanced the vasodilator effects of bradykinin (Tom et al., 2003). In our study, perindopril significantly decreased WRS-induced gastric lesions and increased GBF with an extent similar to that of Ang-(1–7) and Ang II, and this beneficial difference between Ang-(1–7) and Ang II might be mediated by NO. Finally, the luminal content of NO and gastric mucosal expression of mRNA for cNOS were both increased by Ang-(1–7), suggesting that both NO derived from cNOS pathway contributes to the beneficial effect of Ang-(1–7) against stress ulcerogenesis. In contrast, the mRNA expression of iNOS was downregulated in these rats, which is consistent with the notion that Ang-(1–7) inhibits WRS lesions due to its potent anti-inflammatory activity.

We clearly demonstrated that Ang-(1–7) significantly and dose-dependently attenuated WRS-induced gastric damage while increasing GBF, and these effects were abolished by d-Ala7-Ang-(1–7) (A-779), the selective antagonist of Mas receptors. Interestingly, the antagonist A-779 has been shown to inhibit most of the physiologic effects of Ang-(1–7) (Santos et al., 2003). Liao et al. (2011) revealed that cardioprotective effect of Ang-(1–7) against ischemia-reperfusion damage is mediated by COX/PG system responsible for the attenuation of malondialdehyde content and rise in superoxide dismutase activity. The intestinal mucosal COX-2 expression is regulated by both AT1 and AT2 receptors (Tani et al., 2008). Ang-(1–7) stimulated PGE2 release from spontaneously hypertensive rat vascular smooth muscle cells (Jaiswal et al., 1993). In our study, the gastroprotection and increase of

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 mucusoprotective mechanisms due to an activation of NOS/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 of 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 capsaiacin-induced functional ablation of sensory fibers. This indicates that besides NO and PG afferent sensory fibers and the major sensory neuropeptide CGRP released from rat sensory nerve endings contribute to immediate Ang-(1–7)-induced protection and hyperemia. Endogenous CGRP in the presence of Ang-(1–7) restored these protective, in part, and gastric hyperemia in rats with capsaiacin denervation; however, this increase in GBF was significantly less pronounced in capsaiacin-denervated rats compared with those with intact sensory nerves. Thus, it is reasonable to consider that CGRP, which is a potent vasodilator and protectors factor in the stomach, can cooperate with Ang-(1–7) in this protection.

In summary, Ang II and Ang-(1–7) show opposite action against stress ulcerogenesis because Ang II enhances stress ulcerogenesis but Ang-(1–7) affords protection against stress lesions. The mechanism of Ang-(1–7)-induced protection against stress may involve activation of NO/cGMP and PG, COX system and modulatory and gastroprotective sensory neuropeptides such as CGRP. In contrast to Ang II, Ang-(1–7) exhibits antinociceptive and anti-inflammatory properties that are not evident in the case of proinflammatory mediators such as NF-κα. Further studies in experimental models are warranted to further understand the therapeutic efficacy of Ang-(1–7) in the stress-induced gastric disorders.

Authorship Contributions

Participated in research design: Magierowski, Kwiecien, Brzozowski.
Conducted experiments: Magierowski, Pawlik, Kwiecien.
Contributed new reagents or analytic tools: Kryszk-Maeczka, Olzanski, Korbut.
Performed data analysis: Magierowski, Jasnos, Brzozowski.
Wrote or contributed to the writing of the manuscript: Magierowski, Kwiecien, Brzozowski.

References


Oliveira MA, Fortes ZB, Santos RA, Kosla MC, and De Carvalho MH (1999) Syner-


Sampaio SV, Simões e Silva AC, Sampaio WO, de Paula RD, Mendes EP, Bontempo


