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 Krzysztof-Maczka, Agata Ptak-Belowska, Rafal Olszanecki, Sławomir Kwiecień, Ryszard Korbut, and Tomasz Brzozowski

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

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ABSTRACT

Angiotensin-(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 gastric protective activity of exogenous Ang-(1–7) in water-immersed rats with water restriction. The lesions were dose-dependently reduced by pretreatment with Ang-(1–7) which also caused an increase in gastric blood flow (GBF) and plasma content of NO. COX-1 and COX-2 inhibitors (N enterosubstituted L-ornithine) and l-ornithine reversed the reduction in lesion number and the increase in GBF evoked by Ang-(1–7). Ang II augmented the WRS lesions, decreased GBF and increased the plasma IL-1α and TNF-α levels. Capsaicin denervation augmented the reduction of Ang-(1–7)-induced gastric lesions and increase in GBF; these effects were restored by supplementing with calcitonin gene-related peptide (CGRP). The cNOS mRNA was upregulated while iNOS, IL-1α and TNF-α mRNAs were downregulated in Ang-(1–7)-pretreated rats. We conclude that Ang-(1–7), in contrast to Ang II, which worsens WRS ulcerogenesis, affords potent gastroprotection against WRS ulcergenesis 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

Renin-angiotensin system (RAS) is a critical homeostatic system involved in physiologic regulation of blood pressure and water balance (Paul et al., 2006). Its 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 the control of fluid and electrolyte homeostasis, maintenance of normal GBF (muscular type), mucosal absorption of 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 potenti 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 (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 (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 AT₁ and AT₂ were detected in the human esophageal, gastric, small intestinal, and colonic mucosa (Hirasawa et al., 2002; Casselbrant et al., 2009; Hallersund et al., 2011). The antagonists of Ang II AT₁ receptors attenuated gastric injury induced by ischemia-reperfusion, cold stress, and indomethacin-induced damage in rodents due to an inhibition of sympathetic outflow to the stomach and the attenuation of vasococontractor activity of Ang II (Pavel et al., 2007; Morsy et al., 2009; Gemici et al., 2010; Saavedra et al., 2010; Santos et al., 2011).

Antagonists of AT₁ receptor candesartan cilexetil and irbesartan prevented stress-induced gastric lesions (Magierowski et al., 2003, 2004; Merai et al., 2009).

Angiotensin-(1-7) [Ang-(1-7)] is a downstream peptide generated from angiotensin I by a converting enzyme (ACE) homolog ACE2 or neutral endopeptidase (NEP) and isomerized from angiotensin-I through angiotensin-converting enzyme (ACE) homolog ACE2 or neutral endopeptidase (NEP) (2003, 2004; Merai et al., 2009). Ang-(1-7) may even precede Ang I conversion to Ang II (Czarniecki et al., 2009). Mas-receptor knockout mouse exhibited viscidulatory, antihypertensive, cardioprotective, and antifibrotic effects (Ang I is quickly degraded in the rat stomach; the formation of Ang-(1-7) in the rat stomach is not possible) (Boyarshir et al., 1999; Santos et al., 2003; Castro et al., 2005) with or without the Ang-(1-7) Mas receptor antagonist (Bayorshir et al., 1999; Santos et al., 2003; Castro et al., 2005) with or without the combination with Ang-(1-7) Mas agonist AVE 0991 (50 mg/kg s.c.), the nonpeptide Ang-(1-7) receptor agonist perindopril (5 mg/kg i.p.), or Ang-(1-7) Mas receptor antagonist (Bayorshir et al., 1999; Santos et al., 2003; Castro et al., 2005) with or without the combination with Ang-(1-7) Mas agonist AVE 0991 (50 mg/kg s.c.), the nonpeptide Ang-(1-7) receptor agonist perindopril (5 mg/kg i.p.), or Ang-(1-7) Mas receptor antagonist (Bayorshir et al., 1999; Santos et al., 2003; Castro et al., 2005) with or without the combination with Ang-(1-7) Mas agonist.
Chemical (Ann Arbor, MI) and Pfizer (Ilертissen, 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 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 (NO₃⁻) and nitrite (NO₂⁻) levels in the gastric contents using the nitrate/nitrite kit purchased from Cayman Chemical as described in detail in our previous studies (Brzozowski et al., 2008; Pawlik et al., 2011; Kwiecien et al., 2012b). The blood samples (~3 ml) were taken from the vena cava for the measurement of plasma proinflammatory cytokines IL-1β and TNF-α as described previously (Kwiecien et al., 2012b). In brief, the plasma 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. Each sample (50 μl) was incubated with biotinylated antibodies specific to 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 previously (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 to determine 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 washing a glass slide and immediately snap-frozen in liquid nitrogen and stored at −80°C until analysis. Total RNA was extracted from the tissue samples by a guanidinium isocyanate-phenol-chloroform method using a kit from Stratagene (La Jolla, CA). RNA was quantified using a spectrophotometer. The RNA purity and integrity were assessed by 1% agarose-formaldehyde gel electrophoresis and ethidium bromide staining. Aliquots of total RNA (1 μg) were used for cDNA synthesis using MMLV-RT, 0.3 mg of oligo(dT) primer, 1 ml of RNase block ribonuclease inhibitor (40 IU/ml), 2 ml of a 100 mM mixture of deoxyadenosine triphosphate (dATP), deoxythymidine triphosphate (dTTP), deoxyguanosine triphosphate (dTTP), and deoxyctydine triphosphate (dCTP), 5 ml of 10× RT buffer (10 mM Tris-HCl, pH 8.3, 5 ml KCl, 5 mM MgCl₂). The resultant cDNA (2 μl) was amplified in a 5-μl reaction volume containing 0.3 ml (2.5 IU) Taq polymerase, 2.5 ml of each (dNTP (Pharmacia, Germany), 1.5 mM MgCl₂, 5 ml Taq polymerase chain reaction buffer (50 mM KOH, 10 ml 1× Tris-HCl, pH 8.3), and primers used at final concentration of 0.5 μM. The mixture was overlaid with 25 μl of mineral oil to prevent evaporation. The polymerase chain reaction mixture was amplified with a DyeDeoxy thermal cycler (Perkin-Elmer/Cetus, Norwalk, CT). The area dedicated for performing PCR reaction. The electrophoresis was used for primers for cNOS, iNOS, IL-1β, TNF-α and β-actin, these are Table 1 was constructed based on published cDNA for these factors. The primers synthesized by Invitrogen/Life Technologies (Eggcnstein, CA, USA).

Polymerase chain reaction products were detected by ethidium bromide staining in a 1.5% agarose gel containing ethidium bromide. Location and size of predicted products were confirmed by using 1 kb ladder as a standard size marker. The intensity bands were quantified using densitometry (LKB Ultrascan, Pharmaica, Uppland, Sweden) as described in detail in our previous studies (Brzozowski et al., 2008; Pawlik et al., 2011). The signals for cNOS, iNOS, IL-1β, and TNF-α were standardized against the β-actin signal for each sample, and results were expressed as cNOS/β-actin, iNOS/β-actin, and TNF-α mRNA/β-actin.

**Statistical Analysis.** 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 in clinical estimates of effects were considered significant at P<0.05. All results in the treated animals were compared with the appropriate control group, which had been established in each set of experiments. Dependent variables were expressed as a percentage of control for GBF and in absolute values for lesion number. The control rats did not differ from experimental groups 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 testing was 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 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'</td>
<td>63.5</td>
<td>540</td>
</tr>
<tr>
<td></td>
<td>Reverse: 5'-CAG GCT GCA GTC CTT TGA TC-3'</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-1β</td>
<td>Forward: 5'-GCT ACC TAT GTG TGG CCC GT-3'</td>
<td>62</td>
<td>543</td>
</tr>
<tr>
<td></td>
<td>Reverse: 5'-GAC CAT TGC TGT TGC CTA GG-3'</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TNF-α</td>
<td>Forward: 5'-TAC TGA ACT TGC GGG TGA TTG TGC C-3'</td>
<td>56</td>
<td>295</td>
</tr>
<tr>
<td></td>
<td>Reverse: 5'-CAG CCT TGG CTT AAG AGA ACC-3'</td>
<td></td>
<td></td>
</tr>
<tr>
<td>β-Actin</td>
<td>Forward: 5'-TTG TAA CCA ACT GGG ACG ATA TGG-3'</td>
<td>54</td>
<td>764</td>
</tr>
<tr>
<td></td>
<td>Reverse: 5'-GAT CTT CAT CCT TAG CCT AGT ACG-3'</td>
<td></td>
<td></td>
</tr>
<tr>
<td>iNOS</td>
<td>Forward: 5'-CCA CAA TAG TAC AAT ACT AC-3'</td>
<td>60</td>
<td>397</td>
</tr>
<tr>
<td></td>
<td>Reverse: 5'-ACG AGG TGT TCA GCG TGC TC-3'</td>
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</table>
The administration of Ang II in higher doses ranging from 6.25 to 40 \( \mu g/kg \) dose-dependently increased the mean lesion number and produced a significant dose-dependent decrease in GBF (Fig. 1). The pretreatment with Ang-(1–7) (50 \( \mu g/kg \)) dose-dependently decreased WRS-induced gastric lesions, while producing a significant and a dose-dependent increase in GBF and luminal NO content (Fig. 2). The dose of Ang-(1–7) inhibiting WRS lesions by 50% (ID\(_{50}\)) was 27 \( \mu g/kg \). Since the dose of 50 \( \mu g/kg \) 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 decrease in GBF under WRS conditions was significantly worsened by the pretreatment with Ang II. In contrast, pretreatment with Ang-(1–7) resulted in a significant increase in the GBF compared with the pretreatment with vehicle. The Ang-(1–7)-induced protection was accompanied by a rise in the GBF in the Ang-(1–7) group at the dose of 50 \( \mu g/kg \) i.p. (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 \( \mu g/kg \) i.p.) significantly reduced the mean lesion number (\( P < 0.05 \)) and caused a significant increase in the GBF (\( P < 0.05 \)) compared with those pretreated with vehicle. The Ang-(1–7)–induced protection and the accompanying rise in the GBF and luminal NO content observed at the 50 \( \mu g/kg \) dose of this peptide were completely reversed by the pretreatment with AVE 0991 (50 \( \mu g/kg \) i.p.) combined with intraperitoneal injection with Ang-(1–7) (Fig. 2; Table 2).

Effect of Suppression of NO-Synthase on Ang-(1–7)- and Perindopril-Induced Gastroprotection and Alterations in GBF in Rats Predisposed to WRS. Figure 4 shows that pretreatment with Ang-(1–7) (50 \( \mu g/kg \) i.p.) significantly reduced the WRS-induced gastric lesions and increased GBF, with the extent 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 effect of perindopril on lesion number and the rise in GBF evoked by Ang-(1–7) or perindopril (Fig. 4).

Effect of COX-1/PG and COX-2/PG Suppression on Ang-(1–7)-Induced Gastroprotection against WRS-Induced Gastric Damage and Alteration in GBF. As shown in Fig. 5, the pretreatment with Ang-(1–7) (50 \( \mu g/kg \) i.p.) caused a similar decrease in the mean number of WRS-induced lesions compared with vehicle-control pretreated rats. This decrease in lesion number and an increase in GBF induced by AVE 0991 were completely reversed by the combination of its treatment with the combination of A-779 and AVE 0991 (P < 0.05). The combination of AVE 0991 and Ang-(1–7) significantly reduced the lesion number and increased GBF, as compared to the Ang-(1–7) group alone.

Table 2: Effect of pretreatment with vehicle (Veh), Ang II (40 \( \mu g/kg \) i.p.), and Ang-(1–7) (50 \( \mu g/kg \) i.p.) with or without combination with A-779 (50 \( \mu g/kg \) i.p.) on changes in GBF expressed in absolute values (ml/min per 100 g) in gastric mucosa of rats exposed to WRS.

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

Note: 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.

Materials and Methods: The pretreatment with vehicle-pretreated animals as indicated under Materials and Methods. Asterisk indicates a significant change (\( P < 0.05 \)) compared with the respective values in vehicle-controls.
gastric lesions accompanied by a significant rise 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 in lesion number by Ang-(1–7) (50 μg/kg i.p.) was significantly attenuated by pretreatment with indomethacin (5 mg/kg i.p.), rofecoxib (10 mg/kg i.g.), and SC-560 (5 mg/kg i.g.) (P < 0.05), and these effects were accompanied by 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 treated with INDO, SC, and ROFE in the presence of Ang-(1–7) but without combination with PGE2.

As shown in Fig. 6, the pretreatment with Ang-(1–7) and 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
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 i.p.) significantly decreased (P < 0.05) the plasma levels of IL-1β and TNF-α compared to the vehicle-control group and Ang II-pretreated group (Fig. 8).

Expression of cNOS, iNOS, and TNF-α mRNAs in Rats Treated with Ang-(1-7) with or without the Combination with Ang-(1-7)

Figure 8 (left panel) shows the expression of cNOS mRNA in gastric mucosa of vehicle-pretreated rats and those administered Ang-(1-7) with or without combination administration of A-779 and then exposed to WRS. Vehicle-pretreated rats exposed to WRS showed a signal for cNOS mRNA 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 compared with those pretreated with vehicle (Fig. 8, right panel). Ratio of cNOS mRNA over β-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 β-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).

A strong signal for 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 β-actin mRNA 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 when 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). 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).
Fig. 8. Determination of cNOS mRNA, IL-1β, and TNF-α 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 vehicle (Veh)-control gastric mucosa (lane 1), and in those pretreated with Ang-(1–7) (50 μg/kg i.g.) (lane 2), and A-779 (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 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.

Fig. 9. Determination of iNOS expression by RT-PCR (left panel) and the ratio of iNOS mRNA 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 iNOS expression in gastric mucosa was performed between values in 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.

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 cNOS and rise in luminal NO content. Blockade of Mas receptor by A-779 inhibited the Ang-(1–7)-induced protection in mice, while AVE 0991, the agonist of Ang-(1–7) receptors, mimicked the gastroprotective and hyperemic actions of Ang-(1–7). Our results provide the evidence that NO-NOS system and PG-COX pathways could be involved in the protective and hyperemic activities of this Ang I metabolite because this protection and an increase in GBF were reversed by the NOS activity inhibitor L-NNA, and by either nonselective or selective COX-1 and COX-2 inhibitors. We have demonstrated that these protective and hyperemic effects of Ang-(1–7), which disappeared in COX-1- and COX-2-treated animals, have been restored by PGE2 coadministered with this peptide in the presence of COX-1 and COX-2 inhibitors. The involvement of NO in gastroprotection and the hyperemic actions of Ang-(1–7) is further supported by the fact that expression of cNOS was upregulated while expression of iNOS, considered as proinflammatory marker, was downregulated in the gastric mucosa of Ang-(1–7)-pretreated rats. This gastroprotective and hyperemic effect of Ang-(1–7) was similar to those exhibited by perindopril, a long lasting ACE inhibitor. The protective and hyperemic effects of Ang-(1–7) were lost in rats with capsaicin denervation consistent with the notion that this peptide may trigger the sensory afferent endings to release vasodilatory and protective CGRP. Indeed, the pretreatment with CGRP coadministered with Ang-(1–7) enhanced the protective activity of this Ang I metabolite, resulting in gastric hyperemia but also counteracted the capsaicin-induced gastric impairment and the accompanying fall in the gastric GBF observed in...
Ang-(1–7)-treated rats with deactivated sensory nerves. These findings indicate that sensory neuropeptide CGRP can cooperate with PG and NO in the mechanism of Ang-(1–7)-induced gastroprotection and gastric hyperemia against WRS-induced gastric lesions (Fig. 10).

Since stress causes gastric damage of poorly recognized mechanism and etiology, and RAS has been implicated in the pathogenesis of gastric mucosal integrity (Brzozowski et al., 2012) and stress ulcerogenesis (Ender et al., 1993; Kwiecien et al., 2007; Konturek et al., 2011), we determined the effect of vasoactive Ang-(1–7) against stress-induced gastric lesions and compared it with that of Ang II. In clear contrast to Ang-(1–7), the pretreatment with Ang II failed to exert gastroprotection and exacerbated the WRS-induced gastric lesions accompanied by the fall in the GBF. Moreover, Ang-(1–7) markedly decreased the expression and release of proinflammatory cytokines IL-1β and TNF-α (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; Breggio et al., 2010; Garg et al., 2012). Breggio et al. (2009) observed that AT1 blockade led to increased adrenal corticosterone, in TNF-α and intercellular adhesion molecule 1 (ICAM-1) expression, and neutrophil infiltration in stressed animals. However, the blockade of AT1 receptors does not influence gastroprotective action of dexamethasone released during stress (Filaro et al., 1998; Cleeland et al., 2008). Similarly, AT1-receptor antagonists dose-dependently attenuated gastric ulcers in rodents (Merai et al., 2009; Morsy et al., 2009) and counteracted the effects of ischemia and inflammation in the reduction of mucosal neutrophil infiltration and expression of gastric intercellular adhesion molecule 1 and TNF-α (Szlachcic et al., 2013). 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 this hypothesis requires further studies.

Our results show that WRS increased the expression and plasma levels of TNF-α and IL-1β, and that the plasma level of these proinflammatory cytokines was significantly reduced by Ang II, suggesting that the antagonists Ang-(1–7) and Ang-(7–9), known as a potent vasoactive (5,7)-aggravated WRS-induced gastric damage due to its proinflammatory action. This is corroborative with the observation that high levels of circulating Ang-(1–7) in rats pretreated with Ang II-induced the metabolic stress induced gastric hyperemia via decrease in the proinflammatory profile adipose tissue cytokines (Santos et al., 2012). Ang-(1–7) decreases body weight, increased HDL cholesterol, and decreases plasma level of TNF-α and IL-1β in abdominal fat of overweight rats (Santos et al., 2012). Moreover, Clarini et al. (2001) reported a direct binding of Ang-(1–7) to the Ang receptor 1, resulting in downregulation of these receptors. In keeping with these findings, we observed decreased expression and plasma level of IL-1β and TNF-α in Ang-(1–7)-treated rats with deactivated sensory nerves.

Ang-(1–7) acts via specific Mas receptor and stimulates body weight, increased HDL cholesterol, and decreased tissue cytokines (Santos et al., 2012). Ang-(1–7) decreased plasma levels of TNF-α and IL-1β (Neves et al., 2000; Olszanecki et al., 2009), but this hypothesis requires further studies.

In our study, the gastroprotection and increase of gastric mucosal integrity was achieved by the specific Mas receptor agonist Ang-(1–7) (Szlachcic et al., 2013). Interestingly, the antagonist A-779 has been shown to inhibit most of the physiologic effects of Mas receptor (Tani et al., 2008). 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 was reduced by Ang-(1–7), suggesting that NO derived from COX-2 and IL-1β in abdominal fat of obese rats (Santos et al., 2012). Furthermore, Ang-(1–7) activates an endogenous inhibitor of ACE, enhanced the vasodilatory effect 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 observed with Ang-(1–7). l-NNA reduced the gastroprotective and hyperemic activity of perindopril, suggesting that this contribution and rise in the GBF caused by ACE inhibitor might be also mediated by NO. Finally, the luminal content of NO and gastric mucosal expression of mRNA for cNOS were both increased by Ang-(1–7), suggesting that NO derived from cNOS pathway contributes to the beneficial effect of Ang-(1–7) against stress ulcerogenesis. In contrast, the mRNA expression of iNOS was downregulated in these rats, which is consistent with the notion that Ang-(1–7) inhibits WRS lesions due to its potent anti-inflammatory activity.

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

**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 mucus protective mechanisms due to an activation of NONOS and COX/PG systems, sensory neuropeptides such as CGRP released from sensory nerves, and the potent inhibition of proinflammatory cytokines and gastric mucosal inflammation.
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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 gastroprotective action against various gastric damaging factors, including stress and Helicobacter pylori lipopolysaccharide (LPS). In Sprague-Dawley rats, COX and COX-2 inhibitors restored stress-induced gastric lesions induced by endogenous prostaglandins and sensory nerves. Regional PG production in the stomach was increased after COX blockade (Konturek et al., 2002). GNE evoked by Ang-(1–7) might be a potent vasodilator and protect factor in the stomach, which can cooperate with Ang-(1–7) in this protection.

In summary, Ang II and Ang-(1–7) have opposite actions against stress ulcerogenesis, because Ang II is enhanced stress ulcerogenesis but Ang-(1–7) afforded protection against stress ulcerogenesis, because Ang II enhanced stress ulcerogenesis but Ang-(1–7) prevented gastric ulcers during cold-restraint stress. Angiotensin II AT1 receptor blockade prevents gastric ulcers during cold-restraint stress.

References


Dr. Tomasz Brzozowski, Chair, Department of Physiology, Jagiellonian University Medical College, 16 Grzegorzecka Street, 30-387 Krakow, Poland. E-mail: mpbrzozo@cyf-kr.edu.pl