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THE RENIN-ANGIOTENSIN SYSTEM AND ITS VASOACTIVE METABOLITE ANGIOTENSIN-(1-7) IN THE MECHANISM OF THE HEALING OF PREEXISTING GASTRIC ULCERS. THE INVOLVEMENT OF MAS RECEPTORS, NITRIC OXIDE, PROSTAGLANDINS AND PROINFLAMMATORY CYTOKINES

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The inhibition of angiotensin-converting enzyme (ACE) or the blockade of angiotensin (Ang) AT-1 receptors affords protection against acute gastric mucosal injury, but whether the major metabolite of renin-angiotensin system (RAS), Ang-(1-7), accelerates the healing process of preexisting gastric ulcers remains unknown. Previous studies documented that Ang-(1-7) acting *via* its own Mas receptor exerts vascular responses opposing those of Ang II. We studied the effects of the Ang-(1-7)/Mas receptor axis on the healing rate of acetic-acid-induced gastric ulcers with or without the blockade of Mas receptors by A 779 and compared it with the effects of activation and blockade of the AT-1 receptor by the treatment with Ang II and losartan, respectively, the inhibition of ACE by lisinopril, the NO/cNOS inhibition by L-NAME and inhibition of prostaglandin/COX system by indomethacin in the presence of Ang-(1-7). Additionally, *ex vivo* metabolism of Ang I in gastric tissue was assessed by LC/MS method. At day 9 after ulcer induction, the area of these ulcers and the accompanying changes in total gastric blood flow (GBF) were determined as were gastric mucosal blood flow (GMBF) at ulcer margin and gastric oxygen uptake (GVO₂). The gastric mucosal expression of mRNAs for constitutive nitric oxide synthase (cNOS), superoxide dismutase (SOD), and pro-inflammatory cytokines interleukin 1 β (IL-1 β) and tumor necrosis factor alpha (TNF- α) and plasma level of both cytokines were determined by RT-PCR and ELISA. The 9 days treatment with Ang II dose-dependently increased the area of gastric ulcers and this effect was accompanied by a significant fall in the GBF, GVO₂ and GMBF at ulcer margin. In contrast, treatment with Ang-(1-7) which produced a significant rise in the luminal content of NO significantly reduced the area of gastric ulcer and significantly increased the GBF, GVO₂ and the GMBF at ulcer margin. Similar GMBF changes and significant reduction the area of gastric ulcer was observed in rats with gastric ulcers treated with the agonist of Mas receptor, AVE 0991. These effects of Ang-(1-7) and AVE 0991 were eliminated by blockade of the Mas receptor with A779. Similarly to Ang-(1-7), treatment with losartan or lisinopril significantly reduced the area of gastric ulcers and the accompanying increase in the GMBF at ulcer margin and these effects were significantly attenuated by a concomitant administration of L-NAME and indomethacin. The rate of healing of ulcers was associated with a decrease in *ex vivo* Ang-(1-7) formation and this effect was attenuated by lisinopril. The treatment with Ang-(1-7) or AVE 0991 increased the expression of mRNA for cNOS and SOD and downregulated that of IL-1 β and TNF- α followed by the decrease in the plasma IL-1 β and TNF- α levels. We conclude that the Ang-(1-7)/Mas receptor system accelerates the healing of preexisting gastric ulcers *via* an increase in the gastric macro- and microcirculations, and an increase in gastric tissue oxygenation. These effects are mediated by PG and NO derived from overexpression of cNOS, an increase in the expression of antioxidizing enzyme SOD 2 and an anti-inflammatory action involving the inhibition of expression and release of pro-inflammatory cytokines IL-1 β and TNF- α . Our results seem to underlie the importance of the Ang-(1-7), AT-1 and Mas receptors in the regulation of local vascular and metabolic effects associated with mechanism of gastric ulcer healing.

Key words: *renin-angiotensin system, angiotensin-(1-7), gastric ulcer healing, Mas receptor, angiotensin II, gastric blood flow, gastric oxygen uptake, nitric oxide, superoxide dismutase, interleukin-1 β , tumor necrosis factor- α*

INTRODUCTION

The pathogenesis of chronic gastric ulcers is a complex process that results from an imbalance in the aggressive factors such as acid, pepsin bile acids, *Helicobacter pylori* and its

cytotoxins, non-steroidal anti-inflammatory drugs (NSAIDs), ethanol, gastrotoxins and defensive factors such as undisturbed microcirculation, alkaline secretion, prostaglandins (PG) and nitric oxide (NO) protecting the gastric mucosa (1-3). Under physiological conditions, the gastric mucosal integrity is

maintained by complex defense mechanisms. The pre-epithelial component appears to be one of major components of the gastric mucosal defense system against the detrimental action of the damaging factors. This includes the mucosal barrier that is created by the unstirred layer of mucus and bicarbonate, as well as phospholipids preventing the surface of the epithelial cells connected by the tight junctions from the damage by acidic environment in the stomach (2, 3). The generation and subsequent secretion of bicarbonates, phospholipids, PG, NO, trefoil peptides, and heat shock proteins, as well as the presence of tight junctions between the gastric surface epithelial cells constitute the second so called 'epithelial' component of the mucosal defense system (1-5). Another mechanism which may contribute to the gastric mucosal defense involves the rapid restitution of the epithelial cells regulated by growth factors (6, 7).

Gastric blood flow (GBF) is considered as the third 'post-epithelial' component of gastric epithelial defense (8). The major function of gastric microcirculation is to deliver oxygen, nutrients, and bicarbonate to the surface epithelial cells. Moreover, the gastric circulation is known to deliver systemic humoral factors including hormones released from gastric endocrine cells to the stomach and local electrolytes i.e. bicarbonate and mucus preserving both the integrity and secretory functions of the epithelial cells (9). The gastric circulation also plays a role in removing metabolic products, absorbed materials, and hydrogen back diffusing ions. The physiological and pathological relationships between GBF, gastric secretory functions, and gastric protection have been studied by numerous investigators (9-11). When the stomach experiences acute reductions in blood flow, tissue injury results from both the hypoxia induced during flow reduction and the damaging effects of reperfusion (12). Gastric ischemic injury is a consequence of a complicated sequence of cellular events that reflect the basal metabolic rate of the tissue, as well as the duration of blood flow reduction or its complete cessation. The GBF is affected by a variety of systemic, neural, humoral, and local factors, with particular deleterious effect exhibited by the gastric mucosal generation of reactive oxygen species (ROS) (8, 12-15).

The sympathetic nervous system releasing adrenergic neurotransmitters known to act mainly *via* adrenergic receptors seem to be of primary importance in maintaining the resting gastric arteriolar tone (14). In addition, other endogenous vasoactive substances such as circulating catecholamines, vasopressin, prostaglandins (PGs), thromboxane, and leukotrienes have all been implicated in the pathophysiology of gastric ischemic injury (16-19). Vasoconstriction of the gastric vasculature results in a gastric ischemia, which in turn produces a broad spectrum of alteration in the blood flow depending on the onset, duration, and cause of the vasoconstriction. The ischemic injury of the stomach results from the deprivation of tissue oxygen and nutrients necessary to maintain the cellular integrity. The mucosal tissue hypoxia resulting from gastric ischemia has been shown to cause a local acidosis, diminished acid-buffering capacity, decreased mucosal secretion of bicarbonate and mucus, and impaired rapid restitution (17, 19).

Among the factors that play an important role in the control of gastric macro- and microcirculation under both, the physiological and pathological conditions is Ang II, the major peptide of the renin-angiotensin system (RAS) (20). RAS is currently believed to control the homeostatic mechanisms involving the regulation of hemodynamic system, arterial pressure, and water and electrolyte balance (20, 21). The activation of circulating RAS and the rise in the plasma level of Ang II have been considered as a major factors in reducing the blood supply to the gastrointestinal tract, in particular the stomach, by increasing the local vascular tone and causing a potent vasoconstrictor effect (22-24). The gastric mucosal

ischemia induced by Ang II leads to the development of ischemia-reperfusion injury and potentially contributes to the stress-induced lesions of the rodent stomach (25-29). The effects of Ang II within the stomach are mediated by the interaction of this peptide with specific high-affinity receptors in the plasma membranes of its target cells. Previous studies revealed the existence of the angiotensin 1 receptor (AT-1), and angiotensin 2 receptor (AT-2) located within the stomach wall and their functional role in the regulation of GBF during physiological and pathological conditions (24, 26). The Ang II-induced vasoconstriction is mediated predominantly by the AT-1 receptor because treatment with AT-1 receptor antagonists can eliminate this effect (27-31). We and others have shown that administration of Ang II causes intestinal and gastric ischemia that causes hypoxia due to potent vasoconstriction (14, 24, 29-34). For many years, Ang II was considered to be exclusively a circulating hormone, however, recently Ang II as well as other components of RAS have been identified in many peripheral tissues, causing this concept to be revised (20-23). The subsequent discovery of locally synthesized RAS components within the stomach wall changed the concept of the physiological and/or possible pathological role of this local endocrine system in this organ. Thus, particular components of RAS including Ang II, renin, ACE, and chymase (an enzyme capable of Ang I conversion) are expressed in the gastric tissue (22, 23, 35). The Ang II receptors are localized in both gland structures and the surface epithelium as well as in the mesenchymal cells and blood vessels (25, 26). A subpopulation of endocrine cells in the antral mucosa also showed a marked expression of the AT-1 receptors. These findings indicate that Ang II is formed within the stomach wall to control its motor and secretory activity and takes a part in the regulation of both, the GBF and the gastric mucosal blood flow (GMBF) (36, 37). The relationship between RAS and the gastric vasculature has received new attention by the demonstration that a peptide metabolites of RAS can oppose and counteract the vasoconstrictor, proinflammatory, pro-oxidant and hypertrophic effects of Ang II in the gastric mucosa (36, 37). The primary products of RAS are Ang II and Ang-(1-7), Ang III, and Ang IV. Ang-(1-7) is an amino terminal heptapeptide that can be generated from Ang I and Ang II in the vasculature *via* the tissue peptidases including ACE2. The peptidase ACE2 is an important Ang-(1-7)-generating enzyme, which cleaves one residue from Ang I to generate angiotensin-(1-9) and a single residue from Ang II to generate Ang-(1-7) (36, 37). The physiological effects of Ang-(1-7) metabolite involve the G protein-coupled Mas receptor widely considered as an Ang-(1-7) receptor in the vascular bed (20-22). Interestingly, Ang-(1-7) can be generated by the vascular wall in abundant amounts (22, 23). Since ACE2 is present in endothelial cells, it plays a major role in the vascular formation of Ang-(1-7). The accumulated evidence indicates that Ang-(1-7)-induced vasodilation is endothelium-dependent and mediated by the mechanism(s) essentially involved in the release of NO, EDRF, and PGs from endothelial cells (38-41). Olszanecki *et al.* (42) reported that Ang-(1-7) is formed in the rat gastric wall mainly from Ang I. The detection of Ang-(1-7) in the stomach suggests that the gastric RAS is regulated independently of the peripheral RAS and that this system in the stomach wall might be of physiological importance. This raises the possibility of a therapeutic approach that would stimulate or amplify the ACE2/Ang-(1-7) - Mas axis in the stomach in turn providing protection against acute gastric mucosal injury *via* improvement of GBF as presented earlier (25, 34, 43). However, the hypothesis that Ang-(1-7) could affect the rate of healing of preexisting ulcers and accompanying changes in gastric tissue oxygenation, GBF and GMBF at the ulcer margin, all essential for the ulcer to heal, has not been studied before. Therefore, we

attempted to study whether selective AT-1 receptor antagonism, the inhibition of ACE, and the activation of the Mas receptors could affect the healing rate of experimental ulcers and gastric circulatory responses. The major purpose of our present study was to examine the involvement of the Ang-(1-7)/Mas receptor axis, the effects of the activation and blockade of the AT-1 receptors, and the inhibition of ACE on the healing rate of experimental preexisting gastric ulcers. We also determined the possible mechanisms of the ulcer healing related to the changes observed in the total gastric and mucosal blood flow and gastric oxygen uptake. The experimental design of our study included the mechanistic aspects by determination of the role played by NO, PGs, the antioxidizing enzyme superoxide dismutase 2 (SOD 2) and proinflammatory cytokines in the mediation of the effects of Ang-(1-7) on ulcer healing and evaluation of the concomitant general and local gastric inflammatory responses.

MATERIAL AND METHODS

Animals

Experiments were performed in 147 adult male Wistar rats weighing 300 – 350 grams. The animals were housed in cages with a wire mesh bottom, at normal room temperature, and a 12 h light-dark cycle with no limitation of food and drinking water.

The procedures conducted during the investigation conformed to the guidelines of the Committee for Research and Animal Ethics of the Jagiellonian University and run according principles of Helsinki Declaration. Rats were fasted for 24 hours before ulcer induction but were allowed free access to water.

Procedure of chronic gastric ulcer induction and experimental groups

Gastric ulcers were produced using our modified acetic acid methods originally proposed by Okabe *et al.* (44) and then modified by our group as described before (45, 46). Briefly, the animals were anesthetized with isofluran and put on operating table. After midline abdominal incision the stomach was exposed and a round plastic mold (6 mm in diameter) was placed tightly on the anterior serosal surface of the stomach at the antroxyntic border. The volume of 75 μ l of 100% acetic acid were poured into the mold and allowed to remain on the gastric wall for 25 s. Our previous studies documented that these ulcers became chronic within 2 – 3 days and healed completely within 2 – 3 weeks (18, 45, 46). After the application of acetic acid, the surface of the stomach was gently washed with saline and the abdominal cavity was closed by sutures. The treatment of these animals with gastric ulcers has started next day when animals fully recovered from anesthesia.

The following treatment groups (series A-D) of animals with chronic gastric ulcers were employed. Rats of series A were treated daily with: 1) vehicle (0.5 ml of 0.9% NaCl once daily, i.p.); 2) Ang II applied in graded doses ranging from 12.5 μ g/kg-d up to 100 μ g/kg-d, i.p.; 3) Ang-(1-7) applied in graded doses ranging from 1.0 μ g/kg-d up to 50.0 μ g/kg-d, i.p.; the effects of each applied dose of Ang II and Ang-(1-7) were studied in a separate subgroup of animals; 4) AVE 0991 (1 mg/kg-d, i.p.), the agonist of Mas receptors; 5) A 779 (10 mg/kg-d, i.p.), the antagonist of Mas receptors; 6) Ang-(1-7) (50 μ g/kg-d, i.p.) combined with A 779 (10 mg/kg-d, i.p.); 7) AVE 0991 (1 mg/kg-d, i.p.) combined with A 779 (10 mg/kg-d, i.p.); 8) losartan (1 mg/kg-d, i.p.), the antagonist of AT-1 receptors, and 9) lisinopril (5 mg/kg-d, i.p.), the inhibitor of ACE.

The involvement of NO and PG in ulcer healing activity of Ang-(1-7), the AT₁ receptor or ACE inhibitor was determined in

series B and C of rats with gastric ulcers treated with or without L-NAME (5 mg/kg-d, i.p.), an inhibitor of NO synthase activity, and indomethacin (5 mg/kg-d, i.p.), the non-selective inhibitor of cyclooxygenase (COX)-1 activity. In this series B and C of rats with gastric ulcers, L-NAME and indomethacin were administered alone or co-administered with: 1) vehicle (saline), 2) losartan (1 mg/kg-d, i.p.), 3) lisinopril (5 mg/kg-d, i.p.), and 4) Ang-(1-7) (50 μ g/kg-d, i.p.). On day 9 after ulcer induction, the animals were anesthetized, and measurements of GBF and GMBF at ulcer margin and GVO₂ in the gastric microcirculation were performed as described elsewhere (8, 13, 16, 34). The venous blood sample was taken for determination of the plasma levels of IL-1 β and TNF- α as reported previously (13, 18). The area of gastric ulcers was measured by planimetry, and the gastric mucosal biopsy samples were taken for the assessment of the expression of mRNAs for IL-1 β , TNF- α , constitutive nitric oxide synthase (cNOS) (13, 16, 18, 45), as well as for *ex vivo* measurements of Ang I metabolism.

Determination of the gastric blood flow at ulcer margin, the gastric oxygen uptake and the area of gastric ulcers

Before each experiment the rats were anesthetized with sodium pentobarbital (10 mg/kg, i.p., Vetbutal, Polfa Pulawy, Poland) then placed on a heated table that maintained body temperature at 37°C as it was monitored by a rectal thermostat and regulator (model 74, Yellow Springs Inc.). After induction of general anesthesia the jugular vein was exposed. Then a plastic tube of 0.8 mm in external diameter was filled with saline, inserted to administer 0.9% NaCl (2 ml/h) and supplemental doses of anesthetics. The trachea was exposed and incised for placing a tube, which enabled spontaneous ventilation. Then the left carotid artery was exposed and cannulated with a saline-filled catheter connected to a pressure transducer so that mean arterial blood pressure (MAP) values could be monitored continuously. As the next step, a midline laparotomy was performed to expose the stomach. GMBF was determined at the ulcer margin, in the ulcer crater, and in the contra-lateral intact mucosa using H₂-gas clearance technique (13, 16, 18). Values of flow were expressed in ml/min/100 g of gastric tissue and as a percentage of the control blood flow measured in each corresponding area of the stomach such as the ulcer margin or the base of the ulcer. The area of acetic acid-induced gastric ulcers expressed in mm² was determined by computerized planimetry (Morphomat, Carl Zeiss, FRG) by the person who was blinded to which experimental group the animals belonged to (45, 46).

For gastric oxygen uptake determination (47, 48), a small midline laparotomy was performed. Then the splanchnic artery and vein were identified and exposed. All non-gastric branches of the splanchnic artery were ligated. A polyethylene catheter was inserted in a retrograde fashion into a branch of the gastric vein. This catheter was used to obtain venous blood samples from the stomach for the determination of the oxygen content. An ultrasonic blood flow probe (RS1) was placed on the splanchnic artery trunk, and the GBF was determined with the use of a directional ultrasonic Doppler flowmeter (T206 Transonic systems, Ithaca, N.Y.). The recorded data was expressed in ml/min. GVO₂ was calculated from samples of whole blood obtained from the aorta and gastric vein. The oxygen content in the blood samples was measured by an AVOXimeter 1000E (AVOX Systems, Inc., Texas, USA), which is a fast, multi-wavelength spectrophotometer (47, 48). The results were expressed in O₂ ml per 100 ml of the whole blood. GVO₂ in ml O₂/min was calculated by multiplying GBF times the arterial-venous oxygen content difference (AV Δ O₂) (33, 48, 49). At the termination of GMBF, GBF, and GVO₂

measurements, the stomach was removed, rinsed with saline, and pinned open for macroscopic examination.

Determination of gastric luminal nitric oxide content

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 Lab, Michigan, USA. This method is based on the Griess reaction and the generation of chromophore absorbed at 595 nm, according to the original procedure (50, 51). For this purpose, the gastric contents were extracted just before the removal of the stomach following an i.g. injection of 1 ml of saline to wash out the luminal content. After centrifugation for 10 min at 3000 rpm, the samples were mixed with Griess reagent from the commercially available kit as described in detail elsewhere (52).

Determination of plasma IL-1 β and TNF- α level

At the termination of the experiments, immediately after GBF and GVO₂ measurements, a venous blood sample was withdrawn from the *vena cava* into EDTA-containing vials and used to determine the plasma levels of IL-1 β and TNF- α by a solid phase ELISA sandwich (Biosource International Inc. Camarillo, CA, USA), according to the manufacturer's instruction. Briefly, each sample (50 μ l) was incubated with biotinylated antibodies specific for rat IL-1 β and TNF- α , which was washed three times with an assay buffer and finally conjugated with streptavidin peroxidase to form a complex with stabilized chromogen as described in our previous study (53).

Reverse-transcriptase-polymerase chain reaction (RT-PCR) for detection of messenger RNA (mRNA) for cNOS, SOD, IL-1 β and TNF- α in rats with acetic acid-induced gastric ulcer with or without Ang-(1-7) and AVE 0991 alone or combined with a Mas receptor antagonist A779

The stomachs were removed from intact rats and from those treated with vehicle (control), Ang-(1-7) or AVE 0991 or with or without treatment with Mas receptor blocker A779 for the determination of cNOS, SOD, IL-1 β , and TNF- α expression by RT-PCR using specific primers. Samples of the gastric oxyntic mucosa (about 200 mg) were scraped off on ice using a glass slide and then immediately snapped frozen in liquid nitrogen and stored at minus 80°C. The RNA was isolated from the gastric mucosa according to Chomczynski and Sacchi (54) using a rapid guanidinium isothiocyanate/phenol chloroform single-step extraction kit from Stratagene (Stratagene GmbH, Heidelberg, Germany).

The first strand cDNA was synthesized from cellular RNA (5 μ g) using 200 U Strata Script™ reverse transcriptase and oligo (dT) primers (Stratagene GmbH, Heidelberg, Germany). The primers for the β -actin, cNOS, SOD 2, IL-1 β and TNF- α were synthesized by Biometra (Gottingen, Germany). The cNOS primer sequences were designed according to the published cDNA sequence for primer sequences were as follows: up-stream, 5'TAC GGA GCA GCA AAT CCA C; downstream, 3'CAG GCT GCA GTC CTT TGA TC. The expected product length was 840 bp. The SOD primer sequences were as follows: up-stream, CAG CCT TGT GTA TTG TCT CC; downstream, GCT TCT CTC GTC TCC TTG CT. The expected length of the product was 240 bp. The IL-1 β primer sequences were as follows: up-stream, 5'GCT ACC TAT GTC TTG CCC GT; downstream, 3'GAC CAT TGC TGT TTC CTA GG. The expected length product was 543 bp. The TNF- α primer sequences were as follows: up-stream, 5'TAC TGA ACT TCG GGG TGA TTG GTC C; downstream, 3'CAG CCT TGT CCC

TTG AAG AGA ACC. The expected length of the product was 295 bp. Concomitantly, amplification of control rat β -actin was performed on the same samples to verify the RNA integrity. The DNA amplification was carried out under the following conditions: denaturation at 94°C for 1 min, annealing at 60°C for 45 s, and extension at 72°C for 45 s. Each PCR-product (8 μ l) was electrophoresed on 1.5% agarose gel stained with ethidium bromide, and then visualized under UV light. The location of the predicted PCR product was confirmed by using a 100-base pair ladder (Gibco BRL/Life Technologies, Eggenstein, Germany) as a standard marker. A comparison between the different treatment groups was made by determining the cNOS- and SOD-, IL-1 β -, TNF- α / β -actin ratio of the immunoreactive area by densitometry.

Ex vivo assessment of Ang I metabolism

Five days after induction of ulceration, rats were administered fraxiparine (2850 IU, i.p.) and anaesthetized with 50 mg of thiopentone (50 mg/ml, i.p.). Fragments of stomach wall (whole-wall fragments from margin of ulceration or whole-wall from corresponding areas of healthy rats) were washed with cold, standard Krebs-Henseleit solution and cleaned of thrombi and tissue remnants. Tissue incubation was done as described previously (55, 56). Briefly, tissue fragments were incubated for 30 minutes at 37°C in Eppendorf tubes in 550 μ l of Krebs-Henseleit solution and continuously bubbled with 95%O₂/5%CO₂. Samples of 50 μ l of buffer were removed to provide information on background production of angiotensin metabolites. Then, Ang I was added to a final concentration of 1 μ M. Samples of 50 μ l of buffer were removed after another 15 min of incubation. Each sample was promptly frozen at -70°C until further analysis. Tissue pieces were dried overnight at 60°C to allow estimation of angiotensin metabolite production per mg of dry tissue.

Sample preparation

Samples were purified and concentrated using Ultra-Micro Spin C-18 column (Harvard Apparatus, USA). Briefly, 200 μ l of sample were applied on columns and centrifuged (2 min, 1000 \times g). Then columns were washed with 300 μ l of 0.1% TFA by centrifugation as above. Finally, angiotensin peptides were eluted by centrifugation with 300 μ l of 0.1% TFA in 40% acetonitrile. Then the samples were lyophilized overnight, and the dry remnants were reconstituted in 0.1% TFA for LC-MS analysis. Samples for calibration curves of each examined peptides (mixture of standards) were prepared in the same mode as above (56).

Measurement of angiotensin peptides concentration by LC-MS method

Separation of angiotensin peptides was performed on a reversed-phase, high-performance, liquid chromatography (HPLC) system (Ultimate 3000, Dionex, USA) as described previously (42). The column used for separation was Acclaim PepMap 100 C18 column (150 mm \times 300 μ m ID, 5 μ m particle size) with a guard column (5 mm \times 1 mm; 5 μ m particle size) working at a flow rate of 5 μ l/min (Dionex, USA). Mass spectrometric detection was performed using an LCQ ion-trap mass spectrometer (Finnigan, San Jose, USA), equipped liquid-junction ESI ion source (ion spray voltage 2.5 kV, capillary voltage -5 V, capillary temperature 200°C). For angiotensin peptide detection, selected ion-monitoring (SIM) mode was used, set at 450.32 Da for Ang-(1-7); 466.38 Da for Ang III; 523.95 Da for Ang II; 592.39 Da for Ang-(1-9); 648.95 Da for Ang I; 665.27 Da for Ang-(1-5); 775.27 Da for Ang IV. Acquired

data were analyzed by Xcalibur Software, version 2.0. Concentrations of angiotensin peptides were calculated using the standard calibration curves, constructed by linear regression analysis plotting of peak area versus peptide concentration. Calibration curves were prepared for each examined peptide in a concentration range of 2.5 – 250 ng/ml.

Statistical analysis

Results are expressed as means \pm S.E.M. Statistical analysis was done using an analysis of variance and the two-way ANOVA test with Tukey *post hoc* test, where appropriate. Differences of $P < 0.05$ were considered significant.

RESULTS

The effects of treatment with Ang II on the alterations in area of gastric ulcer and the gastric mucosal blood flow at ulcer margin

The effects of vehicle (saline) or ANG II applied i.p. in the graded doses ranging from 12.5 $\mu\text{g}/\text{kg-d}$ up to 100 $\mu\text{g}/\text{kg-d}$ on the ulcer area and GBF at ulcer margin determined at 9th day after ulcer induction are shown in Fig. 1. The initial ulcer area at day 0 averaged 28.5 mm² whereas in the control vehicle-treated rats, the mean value of ulcer area on day 9th after ulcer induction was 12.5 \pm 2.8 mm², while the GBF at ulcer margin was decreased to a value of 68 \pm 3% as compared to the value determined in intact rats on the initial day (day 0). Ang II applied at a dose of 12.5 $\mu\text{g}/\text{kg-d}$, i.p. failed to significantly influence the gastric ulcer area

and the GBF when compared with the respective values in control animals treated with vehicle. However, at higher daily doses of Ang II ranging from 25 $\mu\text{g}/\text{kg}$ up to 100 $\mu\text{g}/\text{kg}$ dose-dependently raised ulcer area ($P < 0.05$) and significantly decreased ($P < 0.05$) the GBF at ulcer margin (Fig. 1).

Ex vivo Ang I conversion in rats with gastric ulcer before and after treatment with lisinopril

Fig. 2 shows the mass spectrophotometry analysis of various angiotensins in the rat gastric mucosa. The incubation of Ang I for 15 min with rat stomach wall resulted in production of high amount of Ang-(1-7) (Fig. 2). The metabolism of Ang I in stomach wall resulted also in formation of significant amounts of other metabolites of Ang I, namely Ang-(1-9) and Ang-(1-5), however, their concentrations were lower than those of Ang-(1-7) (Fig. 2). Importantly, the generation of Ang-(1-7) was almost 7-fold more intensive than production of Ang II (Fig. 2).

Interestingly, in gastric mucosal tissue excised from margin of ulcers, the generation of Ang-(1-7), but not that of Ang II or Ang-(1-9), was significantly decreased as compared to healthy (vehicle-control) stomach wall (Fig. 3). The treatment with lisinopril reversed the decrease of Ang-(1-7) content observed in rats with gastric ulcers ($P < 0.05$) (Fig. 3). Lisinopril failed to significantly influence the gastric concentration of Ang II and Ang-(1-9) in both control non-ulcerated and ulcerated gastric mucosa (Fig. 3). These results with endogenous Ang-(1-7), the most abundant metabolite of Ang I which has been identified in the stomach wall, prompted us to further examine the effect of 9 days administration of exogenous Ang-(1-7) on the time course of ulcer healing in rats with gastric ulcers.

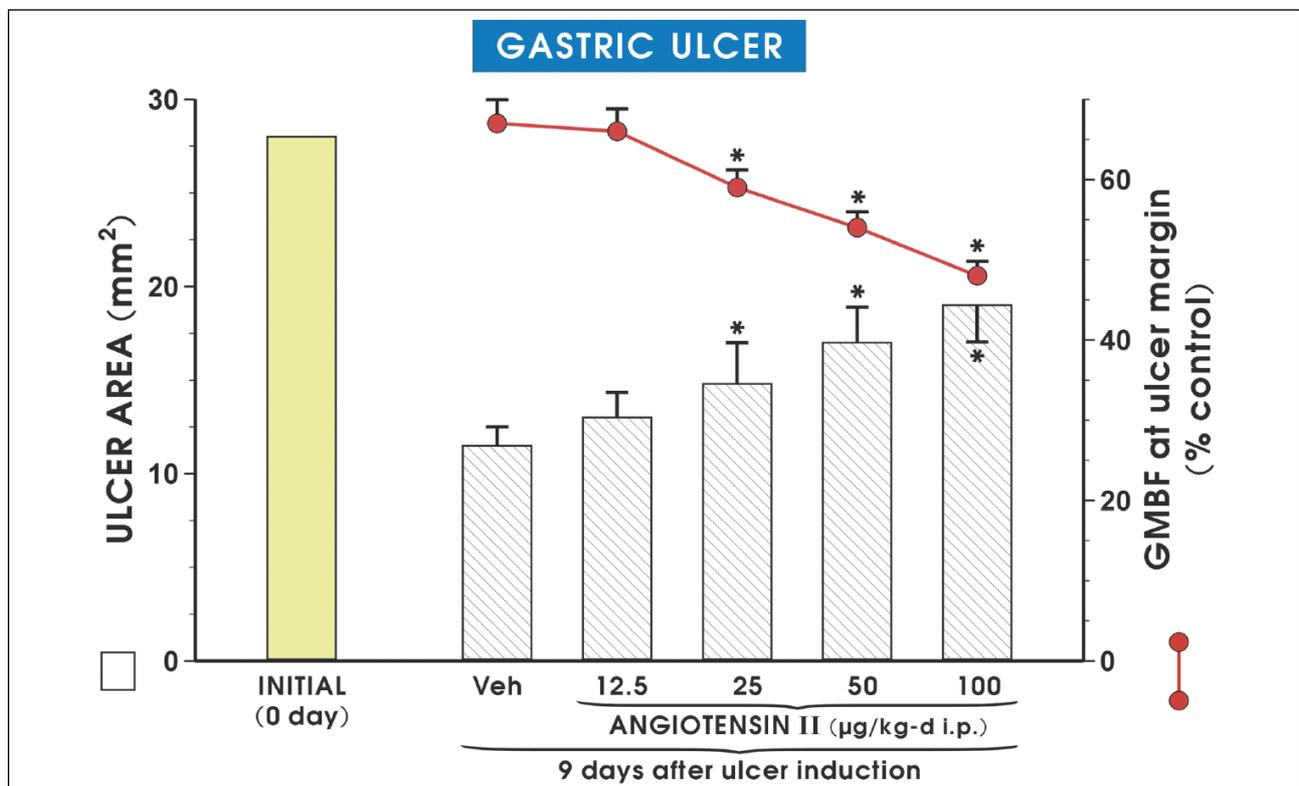


Fig. 1. Gastric ulcer area and alterations in gastric mucosal blood flow (GMBF) at ulcer margin in the gastric mucosa of rats at 9th day after induction of gastric ulcer without and with intraperitoneal (i.p.) treatment with vehicle or angiotensin II (Ang II) administered at graded doses starting from 12.5 $\mu\text{g}/\text{kg}$ up to 100.0 $\mu\text{g}/\text{kg}$ per day. Results are mean \pm S.E.M. of 6 – 8 rats. Asterisk indicates significant change ($P < 0.02$) as compared to the value recorded in vehicle-saline treated animals.

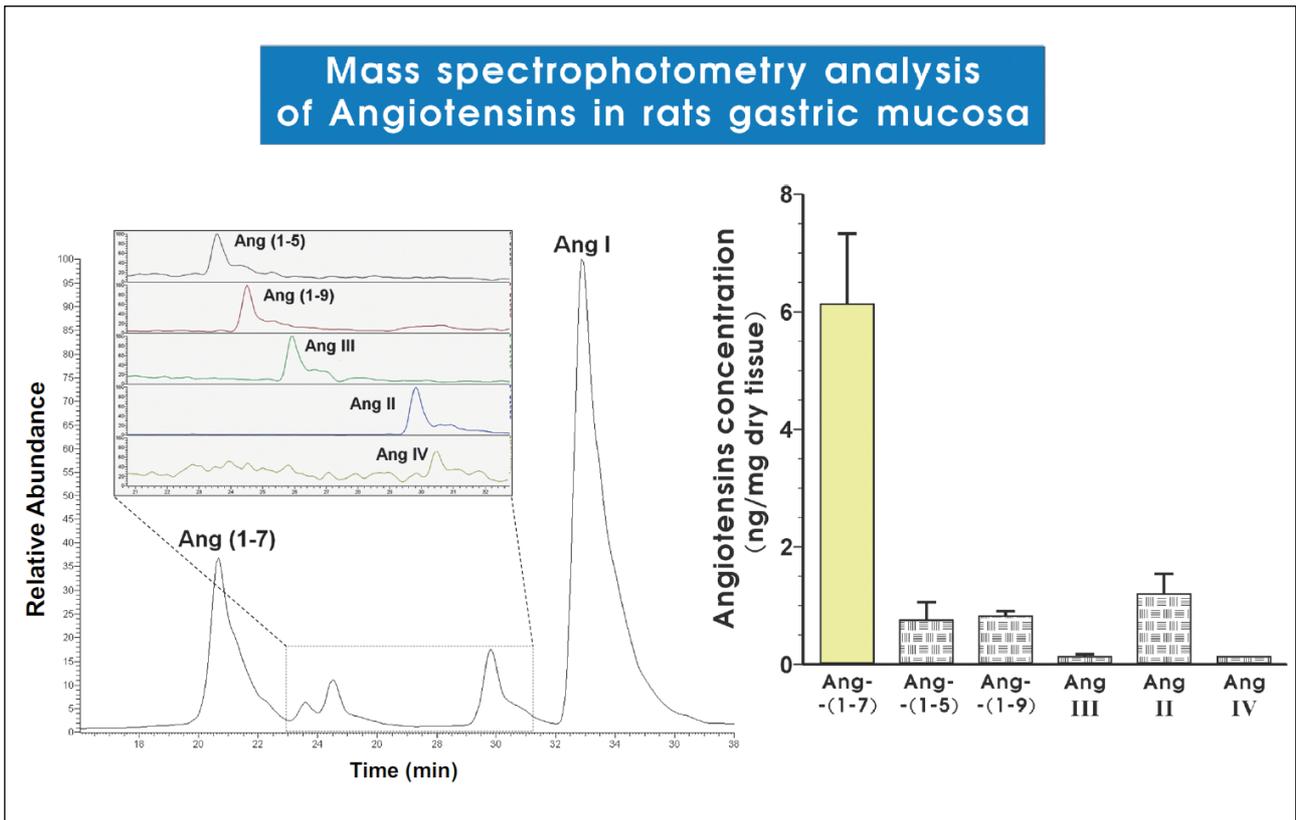


Fig. 2. The mass chromatogram of products of Ang I conversion by stomach wall of control rat with magnification of low-abundant metabolites (presented as extracted base peak, left panel) and the quantitative data regarding metabolism of Ang I in organ bath of stomach wall of control rat (right panel).

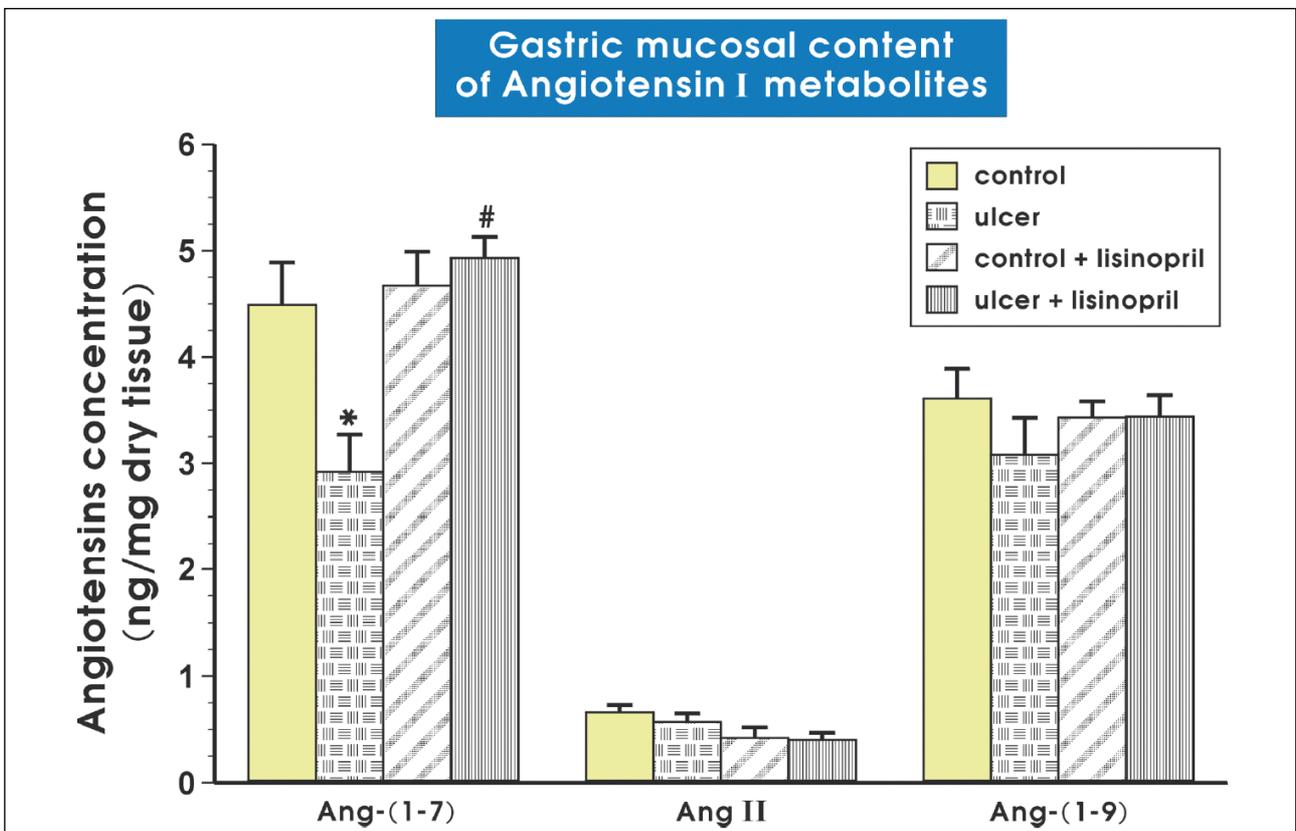


Fig. 3. The formation of Ang I metabolites Ang-(1-7), Ang II and Ang-(1-9) by control (saline) and ulcerative stomach wall in rats non-treated (control) and treated with lisinopril (5 mg/kg-d, i.p.) for 5 days. * $P < 0.05$ vs. control; # $P < 0.05$ vs. non-treated.

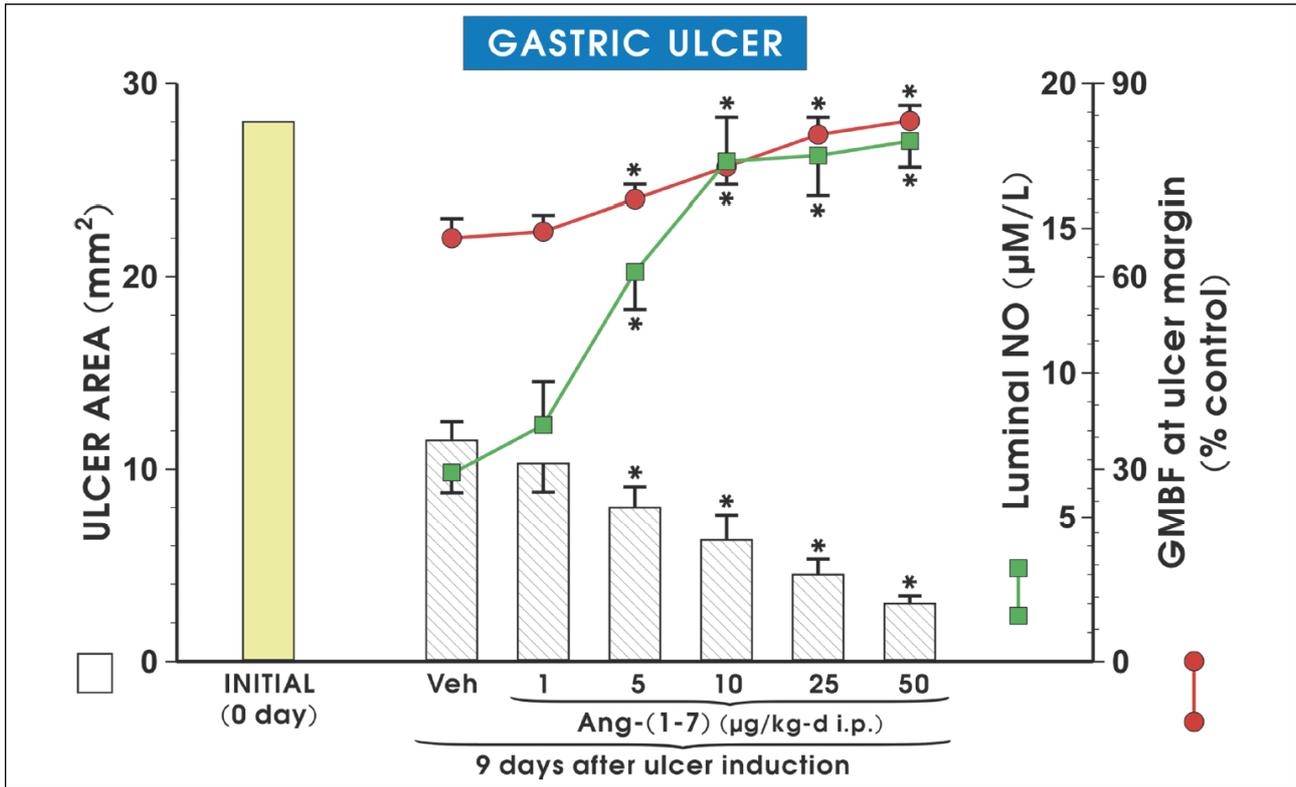


Fig. 4. Gastric ulcer area, the alterations in the gastric mucosal blood flow (GMBF) and gastric luminal content of NO in rats at 9th day after induction of gastric ulcer without and with intraperitoneal (i.p.) treatment with vehicle or Ang-(1-7) administered at graded doses starting from 1.0 µg/kg-day up to 50 µg/kg-day. Results are mean ± S.E.M. of 6 – 8 rats. Asterisk indicates significant change (P < 0.02) as compared to the value recorded in vehicle-saline pretreated animals.

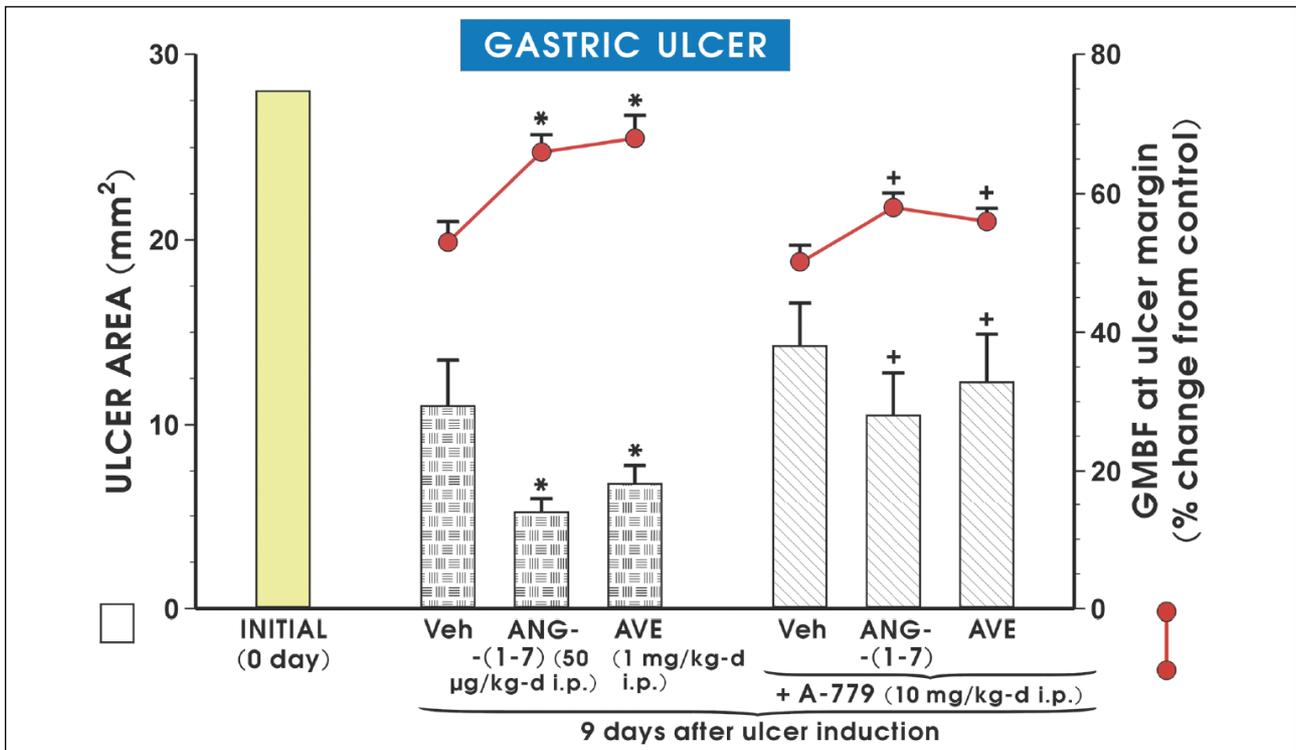


Fig. 5. Effect of the treatment with vehicle (saline), Ang-(1-7) (50 µg/kg-d, i.p.) and AVE (1 mg/kg-d, i.p.) on the area of gastric ulcer at 9th day upon ulcer induction and the accompanying changes in the GMBF in rats without and with the administration of A779 (1 mg/kg-d, i.p.). The results are mean ± S.E.M. of 6 – 8 rats. Asterisk indicates a significant change (P < 0.05) from the corresponding value in vehicle (saline) treated animals. Cross denotes a statistically significant difference (P < 0.05) from the corresponding values in rats without A779 treatment.

Effects of treatment with Ang-(1-7) on gastric ulcer area, the gastric mucosal blood flow at ulcer margin and gastric luminal content of nitric oxide

Fig. 4 shows the data on the administration of Ang-(1-7) applied i.p. in graded doses ranging from 1.0 µg/kg-d up to 50 µg/kg-d on the healing rate of the gastric ulcer as manifested by the reduction of the ulcer area, the accompanying changes in the GMBF at ulcer margin and the content of NO released to the gastric lumen. Treatment with the lowest applied dose of Ang-(1-7) (1.0 µg/kg-d, i.p.) was without any significant influence on the mean value of the ulcer area and neither affected the GBF at ulcer margin nor the gastric luminal content of NO (Fig. 4). Ang-(1-7) applied i.p. in higher doses starting from 5 µg/kg-d up to 50 µg/kg-d, significantly decreased ($P < 0.02$) in the area of acetic acid ulcers, and this effect was accompanied by a significant increase in the GBF at ulcer margin ($P < 0.02$). As shown in Fig. 4, the gastric luminal release of NO was significantly raised ($P < 0.05$) by treatment with Ang-(1-7) starting from the dose of 5 µg/kg-d, i.p. up to 50 µg/kg-d, i.p., as compared to the luminal NO concentration measured in vehicle-treated animals.

Effect of treatment with Ang (1-7) and AVE, the agonist of Mas receptor with or without co-treatment with A 779, an antagonist of Mas receptor, on the area of gastric ulcer and gastric mucosal blood flow at ulcer margin

As shown in Fig. 5, the treatment with Ang-(1-7) applied in a high dose (50 µg/kg-d, i.p.), significantly decreased the area of the acetic-acid gastric ulcer ($P < 0.05$) and significantly raised the GBF ($P < 0.05$) as compared with respective values obtained in the vehicle-treated rats. Treatment with AVE (1 mg/kg-d, i.p.), a Mas-receptor agonist, resulted in a similar significant attenuation in the ulcer area ($P < 0.05$) and similarly raised the GBF at ulcer margin ($P < 0.05$) as observed in rats treated with Ang-(1-7). Fig. 5 also shows the effect of the daily treatment with A 779 (10 mg/kg-d, i.p.) on the mean area of gastric ulcer and accompanying changes in the GBF at ulcer margin. Treatment with A779 was without any effect on the mean ulcer area and GBF at ulcer margin in comparison with the vehicle-only treated animals. However, when A 779 was combined with Ang-(1-7) or AVE, this agent significantly abolished the reduction in the mean ulcer area and the accompanying rise in the GMBF at ulcer margin ($P < 0.05$) evoked by both Ang-(1-7) and AVE 0991 (Fig. 5).

Fig. 6. A, B and C presents the representative gross macroscopic appearance of gastric ulcer in rats treated with vehicle (saline), Ang-(1-7) (50 µg/kg-d, i.p.) or Ang-(1-7) (50 µg/kg-d, i.p.) combined with A 779 (10 mg/kg-d, i.p.). As presented in Fig. 6A, a typical gastric acetic acid ulcer with distinct ulcer crater was observed in the rat treated with vehicle (control) at day 9 upon ulcer induction. In contrast, the area and the depth of gastric ulcer was reduced in rat treated with Ang-(1-7) (Fig. 6B) as compared with those observed in vehicle-control rat (Fig. 6A). Furthermore, the

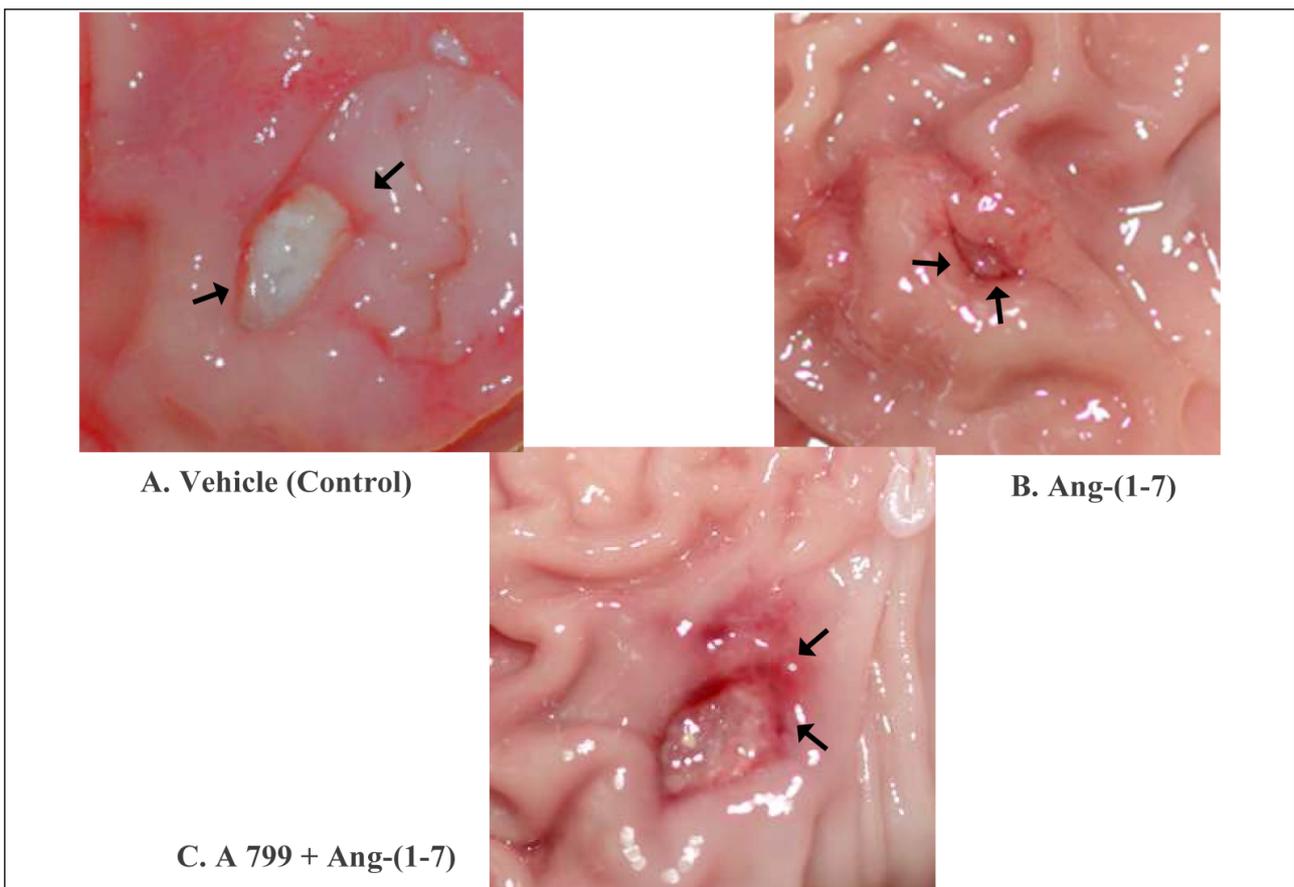


Fig. 6. The macroscopic appearance of the gastric ulcer at day 9th upon ulcer induction in rat treated with vehicle (saline, control) (A), Ang-(1-7) (50 µg/kg-d, i.p.) (B) or Ang-(1-7) (50 µg/kg, i.p.) combined with A 779, the Mas receptor antagonist. Note, the presence of the typical gastric ulcer with deep ulcer crater (A) with the advanced healing phase as reflected by the decrease in the size of gastric ulcer in rat treated with Ang-(1-7) (B). The majority of Ang-(1-7)-treated rats have demonstrated the distinguishable ulcer margin reflecting the regeneration of the mucosa around the ulcer bed (B). When Ang-(1-7) was combined with A 779 (10 mg/kg-d, i.p.) the ulcer healing was markedly delayed as evidenced by an increase of the ulcer size and deep ulcer crater (C).

margin of gastric ulcer much pronounced in Ang-(1-7)-treated rat comparing with vehicle-control animal (Fig. 6B vs. Fig. 6A). When Ang-(1-7) was administered together with A 799, the ulcer size and the depth of the ulcer crater were much greater than those observed in rat treated with Ang-(1-7) alone (Fig. 6C vs. Fig. 6B).

The effect of treatment with losartan, the AT1 receptor antagonist, lisinopril, the ACE inhibitor, Ang-(1-7), and the combination of A 779, the Mas receptor antagonist, with Ang-(1-7) on the gastric blood flow and gastric oxygen uptake

As shown in Figs. 7 and 8, the treatment with the AT1 receptor antagonist losartan (1 mg/kg-d, i.p.) resulted in a significant decrease in the mean ulcer area and this reduction was followed by a significant rise in the GBF by about 43% and GVO₂ by about 40% ($P < 0.05$). The treatment with an ACE inhibitor, lisinopril (5 mg/kg-d, i.p.), also significantly reduced ($P < 0.05$) the ulcer area and produced an increase in the GBF and GVO₂ (Figs. 7 and 8). Similar ulcer-healing, circulatory, and metabolic effects in the gastric mucosa were observed after treatment with Ang-(1-7) (50 µg/kg-d, i.p.) (Figs. 7 and 8). The above mentioned increases in ulcer-healing, circulatory, and metabolic effects of Ang-(1-7) in the gastric mucosa were abolished by concurrent treatment with A779 combined with Ang-(1-7) (Figs. 7 and 8).

The effect of treatment with a losartan, lisinopril and Ang-(1-7) on the area of gastric ulcers and the alterations in the gastric mucosal blood flow at ulcer margin in rats before and after suppression of nitric oxide-synthase with L-NAME

The results with the effect of L-NAME (5 mg/kg-d, i.p.), the inhibitor of NO-synthase activity, on the healing rate of gastric ulcers and accompanying changes in the GBF at ulcer margin induced by losartan (1 mg/kg-d, i.p.), lisinopril (5 mg/kg-d, i.p.) and Ang-(1-7) (50 µg/kg-d, i.p.) are presented in Fig. 10. Treatment with losartan, lisinopril, or Ang-(1-7) significantly reduced the area of gastric ulcers ($P < 0.05$) and significantly increased the GMBF at ulcer margin ($P < 0.05$) (Fig. 10). In contrast, the 9 days of administration of L-NAME alone significantly reduced the area of gastric acetic-acid-induced gastric ulcers in vehicle (saline) treated rats. When L-NAME was administered concomitantly with losartan, lisinopril or Ang-(1-7), the gastric ulcer area was significantly increased, and the GMBF at ulcer margin was significantly attenuated ($P < 0.05$) as compared to those obtained in rats without concomitant treatment with L-NAME.

The effect of suppression of PG generation by indomethacin on losartan, lisinopril and Ang-(1-7)-induced reduction of ulcer area and the alterations in the gastric mucosal blood flow at ulcer margin

As shown in Fig. 10, treatment with losartan, lisinopril or Ang-(1-7) applied in the doses indicated in Figs. 8 and 9, resulted in a similar reduction in the area of gastric ulcers and a similar increase in the GMBF at ulcer margin when compared with those in Figs. 8 and 9. In contrast, the treatment with indomethacin (5 mg/kg-d, i.p.), which by itself almost completely suppressed the mucosal generation of PG (not shown), produced a significant increase in the ulcer area ($P < 0.05$) and a marked fall in the GMBF at ulcer margin ($P < 0.05$)

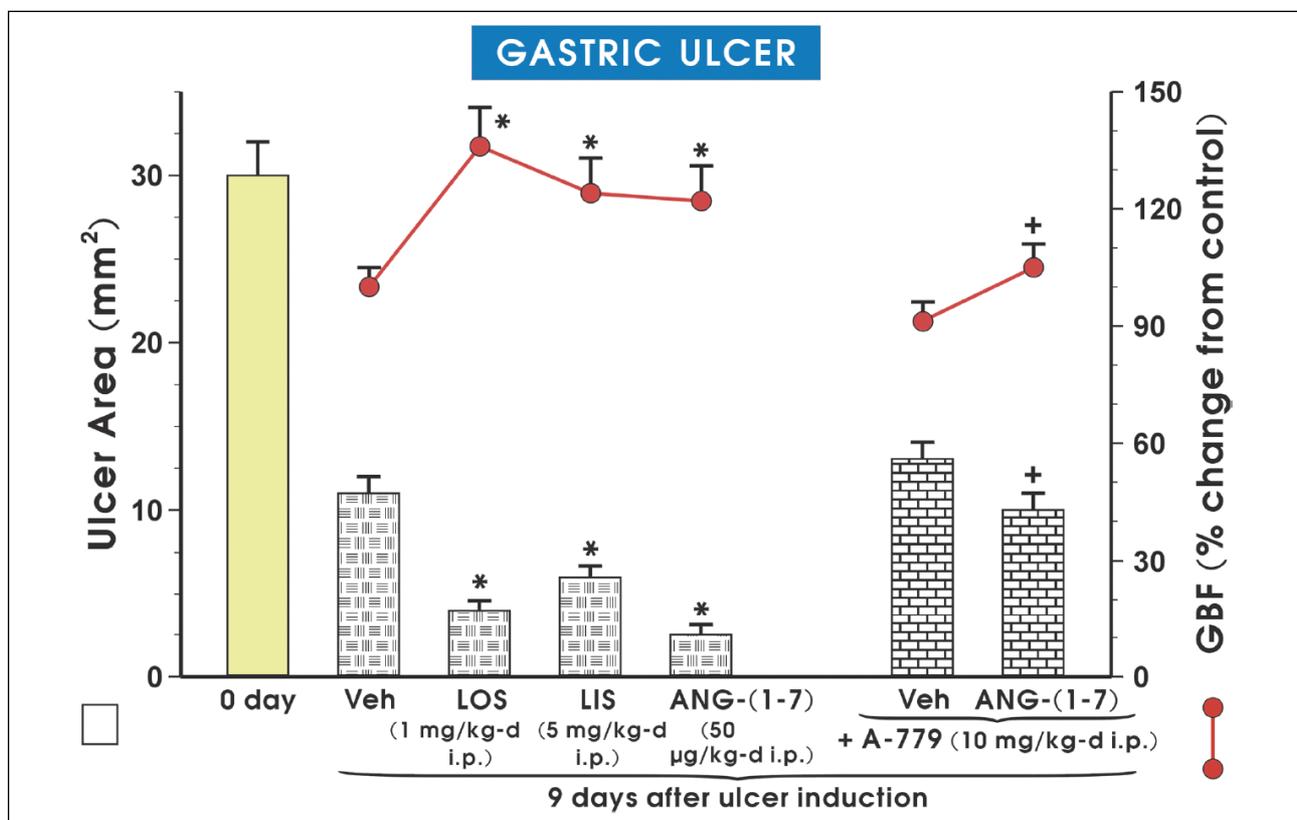


Fig. 7. Effect of the treatment with vehicle (saline), losartan (LOS), lisinopril (LIS) and Ang-(1-7) alone and Ang-(1-7) combined with A779, on the area of gastric ulcers at 9th day upon ulcer induction and accompanying changes in the total gastric blood flow (GBF). The results are mean \pm S.E.M. of 6 – 8 rats. Asterisk indicates a significant change ($P < 0.05$) from the corresponding value in vehicle (saline) treated animals. Cross denotes significant difference ($P < 0.05$) from the effects of Ang-(1-7) applied alone.

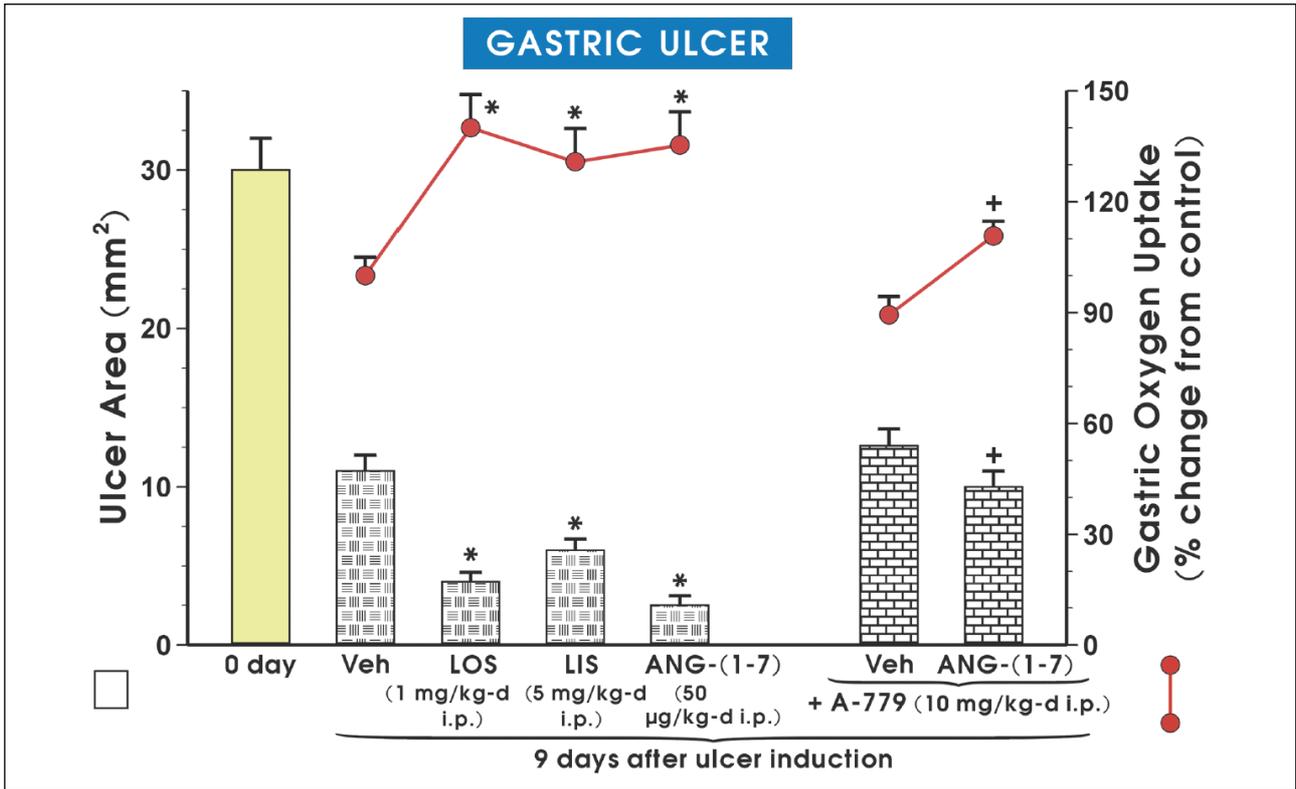


Fig. 8. Effect of the treatment with vehicle (saline), losartan (LOS), lisinopril (LIS) and Ang-(1-7) and Ang-(1-7) combined with A779 on the area of gastric ulcers at day 9th after ulcer induction and accompanying changes in the gastric oxygen uptake (GVO₂). The results are mean ± S.E.M. of 6 – 8 rats. Asterisk indicates a significant change (P < 0.05) from the corresponding value in vehicle (saline) treated animals. Cross denotes significant difference (P < 0.05) between the effects of Ang-(1-7) alone and after combined treatment with A779.

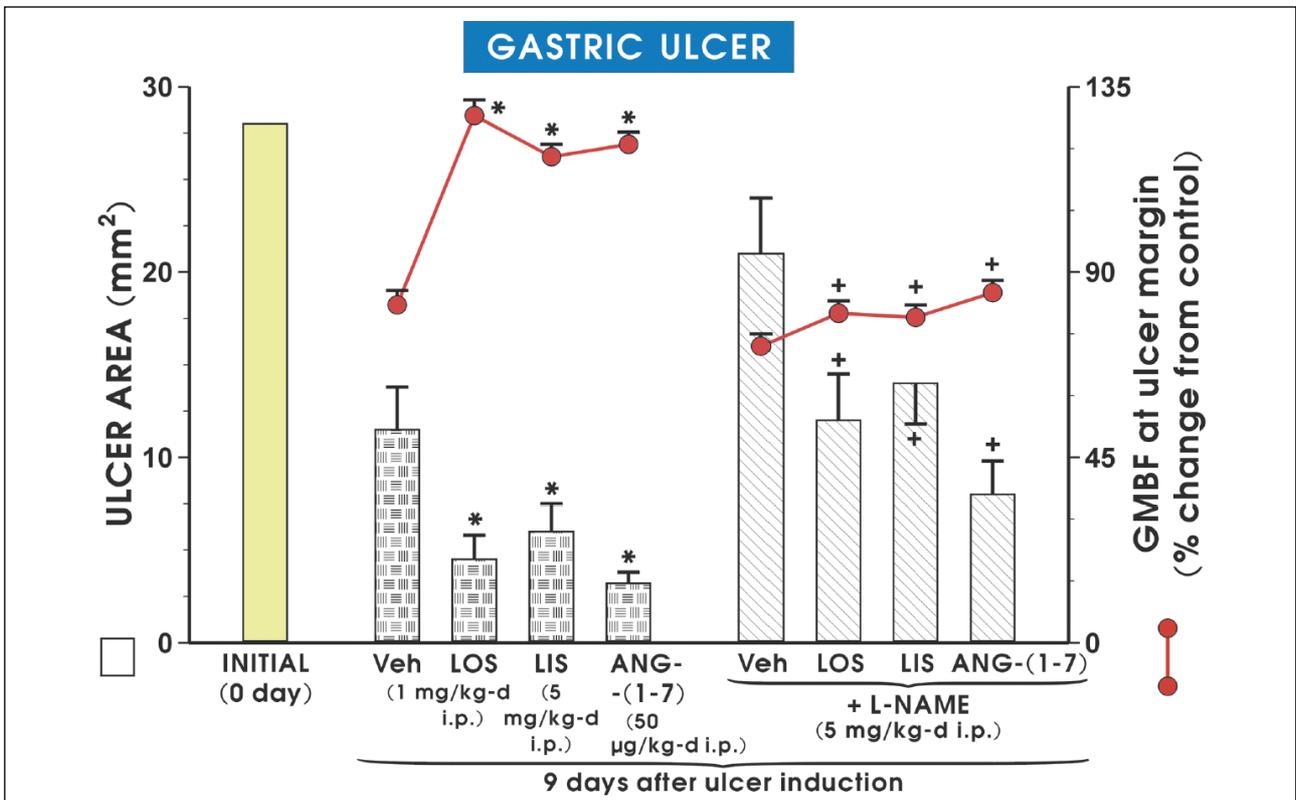


Fig. 9. Effect of the treatment with vehicle (saline), losartan (LOS), lisinopril (LIS) and Ang-(1-7) on the area of gastric ulcer at 9th day upon ulcer induction and accompanying changes in GMBF in rats with or without the treatment with L-NAME (5 mg/kg-d, i.p.). The results are mean ± S.E.M. of 6 – 8 rats. Asterisk indicates a significant change (P < 0.05) from the corresponding value in vehicle (saline) treated animals. Cross denotes a statistically significant difference (P < 0.05) from the corresponding values in rats without L-NAME treatment.

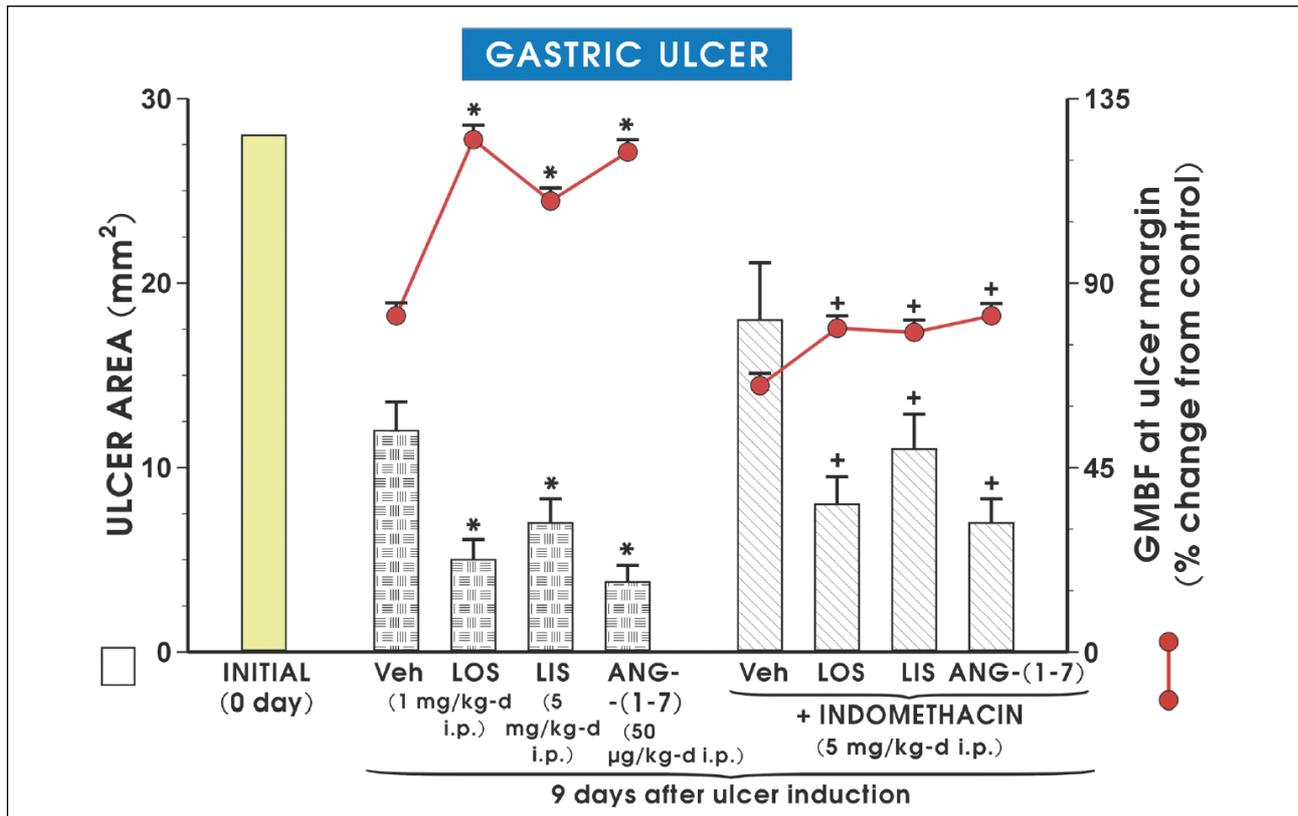


Fig. 10. Effect of treatment with vehicle (saline), losartan (LOS), lisinopril (LIS) and Ang-(1-7) on the area of gastric ulcer at 9th day upon ulcer induction and accompanying changes in gastric mucosal blood flow (GMBF) at ulcer margin with or without combined treatment with indomethacin (5 mg/kg-d, i.p.). The results are mean \pm S.E.M. of 6–8 rats. Asterisk indicates a significant change ($P < 0.05$) from the corresponding value in vehicle (saline)-treated animals. Cross denotes a statistically significant difference ($P < 0.05$) from the corresponding values in rats without indomethacin treatment.

as compared with those recorded in animals treated with vehicle (Fig. 10). Such treatment with indomethacin significantly reduced the decrease in ulcer area ($P < 0.05$) and the accompanying rise in the GMBF at ulcer margin induced by losartan, lisinopril, and Ang-(1-7) ($P < 0.05$) (Fig. 10).

Mucosal expression of cNOS, SOD, TNF- α and IL-1 β mRNAs in the rats with acetic acid-induced ulcers treated with Ang-(1-7) and AVE 0991 with and without treatment with the Mas receptor antagonist A 779

Fig. 11 (left panel) displays the expression of cNOS and SOD mRNAs in the mucosa of intact rats and vehicle-treated control rats and those treated with Ang-(1-7) or AVE 0991 alone or those concomitantly treated with Ang-(1-7) or AVE 0991 in combination with A 779. The weak signal for cNOS mRNA was detected in the vehicle-treated rats, but strong signal for cNOS mRNA expression was observed in gastric mucosa of rats treated with Ang-(1-7) and AVE 0991 (Fig. 11, left panel). In Ang-(1-7)- and AVE 0991-treated rats in which A-779 was co-administered, the signal for cNOS mRNA was significantly inhibited. The ratio of cNOS mRNA to β -actin confirmed that mRNA for cNOS was significantly increased in rats treated with Ang (1-7) or AVE 0991 ($P < 0.05$) and this effect of Ang-(1-7) and AVE 0991 was abolished by concurrent treatment with A 779 (Fig. 11, right panel). The signal for SOD mRNA was detected in intact rats (Fig. 11, left panel). In contrast, the SOD mRNA expression was significantly reduced ($P < 0.05$) in gastric mucosa with ulcer when compared with intact animals (Fig 11, left panel).

Treatment with Ang-(1-7) and AVE 0991 significantly increased the signal for SOD mRNA as reflected by the significant increase in the ratio of SOD mRNA to β -actin ($P < 0.05$) as compared with respective ratio of SOD mRNA to β -actin in vehicle-control gastric mucosa (Fig. 11, right panel). When A779 was co-administered with Ang-(1-7) or AVE 0991, the ratio of SOD mRNA over β -actin was significantly decreased ($P < 0.05$) compared with the respective value of Ang-(1-7) or AVE 0991-alone treated rats (Fig. 11, right panel).

Fig. 12 (left panel) shows the expression of mRNA for IL-1 β , TNF- α , in the gastric mucosa from ulcer margin of rats with gastric ulcer pretreated with vehicle (saline), Ang-(1-7), AVE 0991 and Ang-(1-7) or AVE 0991 combined with A 779. The strong signals for IL-1 β and TNF- α mRNAs were detected in the gastric mucosa of vehicle-treated rats compared with intact control rats (Fig. 12, left panel). In Ang-(1-7) or AVE 0991-treated rats, the signals for mRNAs expression of IL-1 β - and TNF- α were significantly inhibited (Fig. 13, left panel). The ratios of IL-1 β mRNA to β -actin and of TNF- α to β -actin confirmed that mRNAs for IL-1 β and TNF- α were significantly decreased ($P < 0.05$) in rats treated with Ang-(1-7) and AVE 0991 when compared to vehicle-treated animals. The combined treatment with A 779 and Ang-(1-7) or AVE 0991 significantly reduced the Ang-(1-7) or AVE 0991-induced attenuation of the mRNAs expression for IL-1 β and mRNA in rats with gastric ulcer (Fig. 12, right panel). The ratio of IL-1 β or TNF- α mRNA to β -actin mRNA confirmed that the decrease in the expression of mRNAs for IL-1 β and TNF- α induced by Ang-(1-7) and AVE 0991 was reversed by treatment with A 779 (Fig. 12, right panel).

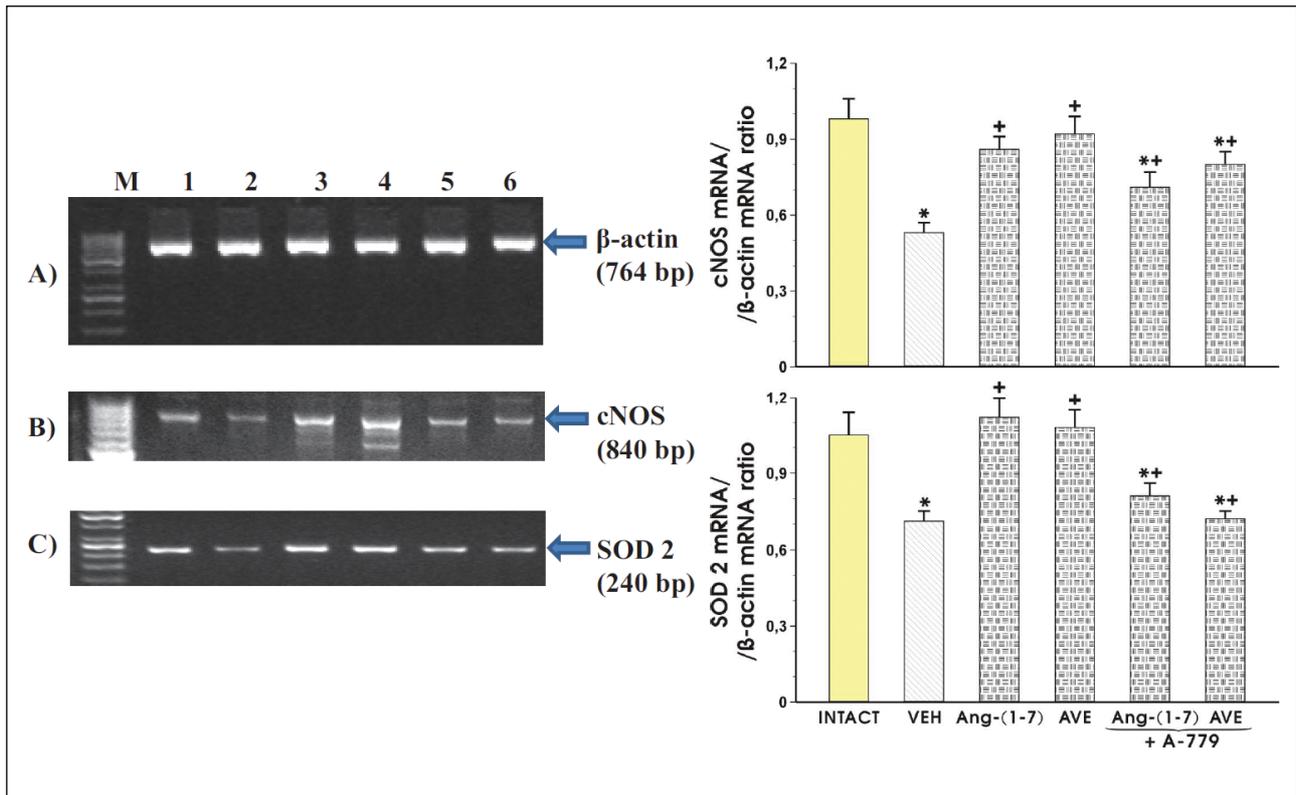


Fig. 11. Determination of β -actin, cNOS and SOD mRNAs (left panel) by RT-PCR and the ratio of cNOS and SOD mRNA over β -actin mRNA (right panel) in the intact gastric mucosa (lane 1), in vehicle (saline) treated animals (lane 2) and in animals treated with Ang-(1-7) (50 μ g/kg-d, i.p.) alone (lane 3) and after treatment with AVE 0991 (1 mg/kg-d, i.p.) alone (lane 4) and in those treated with Ang-(1-7) (lane 5) and AVE 0991 (lane 6) combined with A-779 (10 μ g/kg-d, i.p.). M is a DNA size marker. Mean \pm S.E.M. of 4 – 6 rats. Asterisk indicates a significant change ($P < 0.05$) as compared with intact gastric mucosa. Cross indicates a significant change ($P < 0.05$) as compared with vehicle (saline) treated gastric mucosa. Asterisk and cross indicate a significant difference ($P < 0.05$) as compared with Ang-(1-7) or AVE 0991 administered alone.

The plasma levels of IL-1 β and TNF- α in rats with gastric ulcer treated with vehicle, Ang-(1-7), AVE 0991 alone and Ang-(1-7), AVE 0991 combined with A 779

The results with the effect of Ang-(1-7) and AVE 0991 applied alone in the doses that reduced the area of gastric ulcers, or administered in combination with A 779, on the plasma levels of IL-1 β and TNF- α are presented in Table 1. The plasma levels of IL-1 β and TNF- α reached significantly higher values on the 9th day after ulcer induction in vehicle-treated rats when compared to those values recorded in the intact animals. The control concentration of IL-1 β and TNF- α in the intact animals was 11 ± 0.8 pg/ml and 0.6 ± 0.03 pg/ml, and a significant increase ($P < 0.05$) in the plasma IL-1 β and TNF- α levels was observed, reaching the values of 68 ± 4.5 pg/ml and 8.5 ± 1.2 pg/ml, respectively, in vehicle pretreated rats with gastric ulcer on the 9th day after its induction. Both Ang-(1-7) and AVE 0991, similar to the treatment with losartan and lisinopril, significantly reduced the plasma IL-1 β and TNF- α levels compared to control animals ($P < 0.05$) (Table 1). When A 779 was concurrently administered together with Ang-(1-7) and AVE 0991, the significant increases in the plasma levels of IL-1 β and TNF- α ($P < 0.05$) were observed as compared with plasma cytokine levels recorded in animals treated with Ang-(1-7) or AVE 0991 alone (Table 1).

DISCUSSION

Our present study demonstrated for the first time that RAS product Ang-(1-7) is involved in the mechanism of healing of

preexisting gastric ulcers. The mechanism of action of RAS in the gastric mucosal integrity and ulcer healing process is complex and appears to depend on the activation of AT-1, AT-2, and/or Mas receptors by particular components of this system including Ang I, Ang II or Ang-(1-7) (23-25, 36, 37, 57). Herein we have demonstrated that the agonist of Mas receptor, Ang-(1-7) have accelerated the ulcer healing causing an increase in the gastric microcirculatory responses, and that these actions can be mediated by the COX-PG and NOS-NO systems, the activation of ROS scavenging system, and an anti-inflammatory mechanisms. Previous studies have shown that the alteration in gastric microcirculation reflected by the increase in GMBF seems to act as the key factor involved in the process of healing of gastric ulceration (3, 9, 14, 18). In our present study, the treatment with Ang-(1-7) significantly increased the GMBF at the ulcer margin when compared to the non-ulcerated gastric mucosa. This treatment with Ang-(1-7) was found to accelerate the healing process of gastric ulcerations, possibly by an improvement in gastric blood flow, especially perfusion within the gastric ulcer margin the crucial place for the ulcer to heal, with newly formed microvessels due to angiogenesis (7, 45, 46).

As shown previously, the healing process of experimentally induced gastric ulceration could be affected by various endogenous and exogenous factors including PG and growth factors (3, 4, 15, 17-19). The GMBF and gastric oxygen uptake have been previously shown to act as the one of important factors contributing to the maintenance of the gastric mucosa restitution and mucosal repair processes (1, 5, 8, 13). The two most important structures of the gastric microcirculation, which

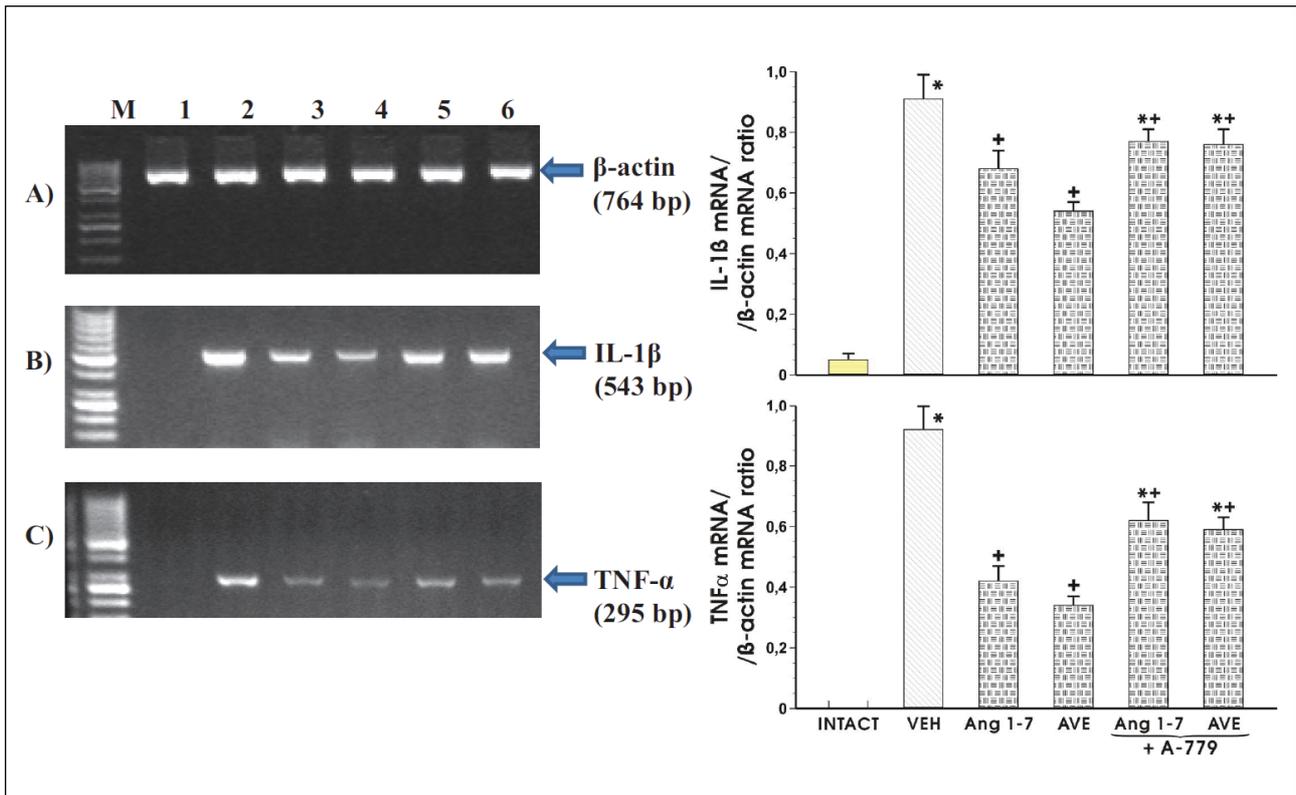


Fig. 12. Determination of β -actin, IL- β and TNF- α (left panel) by RT-PCR and the ratio of IL-1 β mRNA and TNF- α mRNA over β -actin mRNA (right panel) in the intact gastric mucosa (lane 1) in vehicle (saline) treated animals (lane 2) and in animals treated with Ang-(1-7) (50 μ g/kg-d, i.p.) alone (lane 3) and after treatment with AVE 0991 (1 mg/kg-d, i.p.) (lane 4) and in those treated with Ang-(1-7) (lane 5) and AVE 0991 (lane 6) combined with A-779 (10 μ g/kg-d, i.p.). M is DNA size marker. Mean \pm S.E.M. of 4 – 6 rats. Asterisk indicates a significant change ($P < 0.05$) as compared with intact gastric mucosa. Cross indicates a significant change ($P < 0.05$) as compared with vehicle (saline). Cross and asterisk indicate a significant change ($P < 0.05$) as compared with values obtained in Ang-(1-7) or AVE 0991 alone treated rats.

Table 1. Plasma levels of interleukin-1 beta (IL-1 β), tumor necrosis factor alpha (TNF- α) in intact rats and animals with acetic acid-induced gastric ulcer without or with intraperitoneal (i.p.) treatment with vehicle (saline), Ang-(1-7) (50 μ g/kg-d, i.p.), AVE 0991 (1 mg/kg-d, i.p.), losartan (1 mg/kg-d, i.p.), lisinopril (5 mg/kg-d, i.p.) and Ang-(1-7) or AVE 0991 combined with A 779 (10 mg/kg-d, i.p.). Asterisk indicates a significant change ($P < 0.05$) as compared with intact gastric mucosa. Cross indicates a significant change ($P < 0.05$) as compared with vehicle (saline). Cross and asterisk indicate a significant change ($P < 0.05$) as compared with values obtained in group without A779 but treated with Ang-(1-7) or AVE 0991 alone.

Type of test	Plasma IL-1 β (pg/ml)	Plasma TNF- α (pg/ml)
Intact	11 \pm 0.8	0.6 \pm 0.03
Vehicle (saline)	68 \pm 4.5 *	8.5 \pm 1.2 *
Losartan (1 mg/kg-d i.p.)	33 \pm 2.1 +	4.3 \pm 0.8 +
Lisinopril (5 mg/kg-d i.p.)	36 \pm 2.4 +	3.8 \pm 0.6 +
Ang-(1-7) (50 μ g/kg-d i.p.)	28 \pm 1.9 +	2.9 \pm 0.4 +
AVE (1 mg/kg-d i.p.)	26 \pm 1.7 +	2.4 \pm 0.6 +
A 779 (10 mg/kg-d i.p.) + Ang-(1-7) (50 μ g/kg-d i.p.)	65 \pm 3.8 *+	7.8 \pm 1.3 *+
A 779 (10 mg/kg-d i.p.) + AVE (1 mg/kg-d i.p.)	71 \pm 4.2 *+	8.7 \pm 1.5 *+

regulate the total blood flow to the stomach and capillary blood flow, respectively, are the arteriolar smooth muscle and the muscle of pre-capillary sphincters known to play the role in control the density of the perfused capillary bed, which is an essential determinant of tissue oxygen extraction and uptake (12, 14). Moreover, our previous studies have demonstrated that the mesenteric circulation, the oxygen delivery and the changes in blood flow depend upon the density of the perfused capillary bed (33, 34, 49). Herein we provided an experimental evidence that the administration of Ang II, possibly acting *via* the stimulation of the AT-1 receptors caused a potent vasoconstriction in the gastric mucosa at ulcer margin, resulting in a decrease in GMBF which led to a dose-dependent inhibition of the healing rate of the gastric ulcer. The observed vasoconstrictor effects of Ang II in the gastric mucosa have been previously reported by us and others (21, 22, 24-26, 31, 34) but none of these investigations have considered the effect of Ang II on healing of gastric ulcers.

The major purpose of our present study was to assess whether endogenous components of RAS can contribute to the mechanism of healing of gastric ulcer, with the special focus to the participation of Ang-(1-7), the endogenous activator of Mas receptors in comparison with the activity of AT-1 receptor antagonist and ACE antagonist (58-60) during the time-course of ulcer healing. We found that the administration of losartan, an AT-1 receptor antagonist, by itself increased the GBF, GMBF at ulcer margin, and GVO₂ during the course of ulcer healing. These vascular and oxygen uptake effects were paralleled by an acceleration in the rate of healing of the gastric ulcer compared to the vehicle-treated control animals. Moreover, in our chronic ulcer model, the inhibition of ACE by treatment with lisinopril caused similar gastric circulatory, metabolic, and ulcer healing effects as those observed after a blockade of the AT-1 receptors. These gastric circulatory and ulcer healing observations strongly suggest that endogenously activated RAS plays an important role in modulating the magnitude of tonic vasoconstriction present in gastric the macro- and microvasculature. This supports the notion that endogenous vasoactive RAS metabolites can be critical for a vascular tone that might influence the healing rate of gastric ulcers. Therefore, the results of our study confirm that the peripheral AT-1 receptors and the level of circulating Ang II restrict the magnitude of the GBF, GMBF, and the respective changes in GVO₂. Moreover, the treatment with lisinopril that inhibited of ACE, influenced negatively the Ang I conversion to Ang II resulting in an enhancement in the GMBF at ulcer margin, GBF and GVO₂. The inhibition of ACE in our study also accelerated the rate of healing of the gastric ulcer and did so in a way similar to the blockade of the AT-1 receptors and the treatment with Ang-(1-7). The observed increases in GBF and gastric oxygen consumption appear to be secondary to the vascular microcirculatory effect rather than as a result of an increase in gastric tissue metabolic activity *per se*, since the degree of increased total GBF and gastric oxygen uptake were not significantly different. The observed gastric circulatory and secondary gastric tissue oxygenation changes induced by the blockade of the AT-1 receptors could also be due, at least in part, to increased plasma levels of Ang II and the stimulation of the AT-2 receptors localized in the gastric vasculature. This assumption is based on the fact that AT-2 receptors have been shown to counterbalance the vascular effects mediated by the stimulation of AT-1 receptors (26, 27, 38). The abundance of Ang II could rise the level of Ang I, which can be further converted to Ang-(1-7) resulting in a subsequent stimulation of the Mas receptor and gastric macro- and micro-vasodilatation (26, 39, 40). Similarly, ACE inhibition is known to cause an increased generation of Ang-(1-7) by inhibiting the conversion of Ang I to Ang II and by increasing the level of Ang I, which is the substrate for conversion to Ang-(1-7) by endopeptidases. The elevated level of

Ang-(1-7) observed after inhibition of ACE could be part of the inhibition of degradation of Ang-(1-7) to angiotensin-(1-5) by ACE (38, 57, 58).

The accumulated evidence indicates that endogenous Ang-(1-7) possesses important biological activity in the stomach wall where this peptide is generated as the main product of Ang I conversion (42). Indeed, our *ex vivo* LC/MS determination not only confirm Ang-(1-7) to be the major Ang I conversion product in the stomach wall, but also clearly show an impairment of Ang-(1-7) formation in gastric ulceration. Moreover, in our hands the beneficial action of lisinopril was associated with the increased production of gastric Ang-(1-7) suggesting that lisinopril-induced acceleration of gastric ulcer healing may be mediated by this Ang I metabolite.

In turn, our present study demonstrates that exogenous Ang-(1-7) exhibited a potent stimulatory influence on the healing rate of gastric ulcers and evoked a concomitant total-gastric and mucosal hyperemia as well as an increase in gastric tissue oxygenation. In keeping with these observations, the treatment of rats with preexisting gastric ulcers with Ang-(1-7) Mas receptor agonist, AVE 0991 (61), have resulted in a similar ulcer-healing and hyperemic activities as the treatment with Ang-(1-7). It is of interest that ulcer healing and the mucosal microcirculatory effects of Ang-(1-7) and AVE 0991 were significantly inhibited by the combined treatment with A779, the selective inhibitor of the Mas receptor. Moreover, the treatment of animals with A779 alone reduced the ulcer healing rate, GBF, and oxygen uptake, suggesting that, at least, in rats with gastric ulcers, the endogenous Ang-(1-7)/Mas receptor system is involved in the control of the healing process of gastric ulcers *via* an improvement of circulatory and metabolic mechanisms.

Previous studies documented that Ang-(1-7) can be formed in the vascular endothelium, which is also a major target for its actions (37, 62). This suggests that the observed gastric vasodilatory and hyperemic actions of Ang-(1-7) can be endothelium-dependent (12, 13, 25, 26). Ang-(1-7) stimulates release of NO (26, 28, 29) and PGs, namely prostacyclin (PGI₂) (26, 30), and this potentiated the vasodilatory actions of bradykinin (60). These endothelium-dependent vasodilatory effects of Ang-(1-7) are mediated by the Mas receptor, since antagonists of the Mas receptor attenuate Ang-(1-7)-induced vasodilatation (37, 62-64).

Because the mechanism of healing of gastric ulcer includes NO that could be released from vascular endothelium, gastric epithelial cells, or sensory nerves (4), we tested the hypothesis that the ulcer-healing effects of Ang-(1-7) are mediated by NO originating in the stomach wall. Firstly, we documented that indeed the exogenous Ang-(1-7) increased the healing rate of gastric ulcers and the gastric mucosal hyperemia. These effects were accompanied by an enhancement in the gastric luminal level of NO, possibly due to an increase in the expression of cNOS in the gastric mucosa. This increase in cNOS mRNA expression observed in rats treated with Ang-(1-7) or AVE 0991 was greatly attenuated in those treated with the combination of Ang-(1-7) or AVE 0991 and Mas receptor antagonist A 779. Secondly, the Ang-(1-7)-induced acceleration of ulcer healing and the accompanying gastric hyperemic response were inhibited by treatment with L-NAME, which acts as a potent NO-synthase inhibitor. These observations support the notion that the Ang-(1-7)- induced acceleration of ulcer healing and accompanying hyperemia are mediated by the activation of the Mas receptor, and endogenous NO derived from cNOS. The SOD family of enzymes has been proposed to play an essential role in cellular defense mechanism for managing radical superoxide level to turn it into less toxic hydrogen peroxide and therefore, somehow protecting cells from damaging effects of ROS (65, 66). Thus, the downregulation of this SOD defense system can increase cytotoxic levels of ROS ultimately leading

to tissue damage associated with mucosal injury (66-68) and would account for the prolongation of ulcer healing observed in our study. Indeed, we observed that the expression of SOD 2 was downregulated in the gastric mucosa at the ulcer margin of vehicle-control rats but this effect was reversed by the treatment with Ang-(1-7) or AVE 0991. This suggests that beneficial effect of Ang-(1-7) on ulcer healing observed in our study can depend, at least in part, upon amelioration of the ROS generation in inflamed tissue at ulcer margin and the restoration of the radical scavenging activity of SOD. We studied the implication of PG in the mechanism of the healing process of gastric ulcers induced by Ang-(1-7) because these arachidonate metabolites are considered as the classic mediators of gastroprotection and healing of gastric mucosal injury and gastric ulcers (2-4, 7, 34, 69, 70). This is why we tested whether the suppression of cyclooxygenase (COX) by indomethacin, a non-selective COX-1 and COX-2 inhibitor (10), could influence the ulcer healing and hyperemic activity of Ang-(1-7). Indomethacin significantly attenuated ulcer healing and the hyperemic effects of Ang-(1-7) indicating that endogenous PG, possibly derived from both COX-1 and COX-2 pathways, are involved in these beneficial effects of this Ang I metabolite on gastric ulcer healing. However, this notion should be further explored and confirmed in properly designed ulcer healing studies concerning the effect of Ang-(1-7) in the presence of selective COX-1 and COX-2 inhibitors.

Activated inflammatory cells in the gastric mucosa liberate important inflammatory mediators including a variety of cytokines that stimulate protective mucosal responses by recruiting and activating effector cells, altering microvascular vasoactive responses and vascular permeability as well as changing the permeability and secretion of epithelial cells. The proinflammatory cytokines IL-1 β and TNF- α besides causing the enhanced micro vascular permeability and edema during gastric inflammation can also trigger expression of COX-2 derived PG. Interestingly, both COX-1 and COX-2 are expressed at the edge of gastric ulcers and COX-2 derived PG in addition to COX-1 generated PGs have been also implicated in the mechanism of ulcer healing in experimental animals and humans (8, 10, 71-73).

In the present study we also observed the mRNA expression of proinflammatory cytokines IL-1 β and TNF- α had increased in the gastric mucosa of vehicle-treated animals with gastric ulcer and we found that their plasma levels were concomitantly increased in the vehicle-control rats with gastric ulcer. It is of interest to note that in the present study gastric ulcer healing and the hyperemic actions of Ang-(1-7) and Mas receptor agonist AVE 0991 were accompanied by a profound attenuation of the gastric mucosal expression of both pro-inflammatory cytokines IL-1 β and TNF- α . Furthermore, the plasma level of IL-1 β and TNF- α were also markedly diminished in animals pretreated with Ang-(1-7), AVE 0991, losartan and lisinopril. These anti-inflammatory effects of Ang-(1-7) and AVE 0991 as manifested by downregulation of mRNA expression for IL-1 β and TNF- α and their activity were markedly increased by the concurrent treatment of animals with the Mas receptor antagonist A779. This supports the notion that the Mas receptor appears also to be involved in the anti-inflammatory effect of Ang-(1-7) in gastric mucosa with ulcers. However, the precise mechanisms involved in the observed anti-inflammatory effects of Ang-(1-7) in the gastric ulcer remain largely unknown (74, 75). The observed beneficial anti-inflammatory effect of Ang-(1-7) in the process of healing of the gastric ulcer can be attributed to the Mas-receptor-mediated potentiation of the generation of NO and PG including mainly PGI₂ and PGE₂. This is supported by the fact that the treatment with A 799 reverses the Ang-(1-7)-induced beneficial effect on proinflammatory cytokines and the pleiotropic action of Ang-(1-7) in other systems of the body (76, 77) and that

endogenous NO and PG pathways activated by Ang-(1-7) may exert the anti-inflammatory action in the gastric mucosa by inhibiting the local generation of IL-1 β and TNF- α as documented in our study by Ang-(1-7)-induced downregulation of these cytokines and their blood concentrations.

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