JOURNAL OF PHYSIOLOGY AND PHARMACOLOGY 2014, 65, 6, 809-822
www.jpp.krakow.pl

M.W. PAWLIK[1], S. KWIECIEN[1], R. PAJDO[1], A. PTAK-BELOWSKA[1], B. BRZOZOWSKI[2], G. KRZYSIEK-MACZKA[1], M. STRZALKA[1], S.J. KONTUREK[1], T. BRZOZOWSKI[1]

ESOPHAGOPROTECTIVE ACTIVITY OF ANGIOTENSIN-(1-7) IN EXPERIMENTAL MODEL OF ACUTE REFLUX ESOPHAGITIS. EVIDENCE FOR THE ROLE OF NITRIC OXIDE, SENSORY NERVES, HYPOXIA-INDUCIBLE FACTOR-1ALPHA AND PROINFLAMMATORY CYTOKINES

[1]Department of Physiology Jagiellonian University Medical College, Cracow, Poland;
[2]Gastroenterology and Hepatology Clinic, Jagiellonian University Medical College, Cracow, Poland

Gastroesophageal reflux disease (GERD) is a global disease rapidly increasing among world population. The pathogenesis of reflux esophagitis which is considered as the early stage of GERD is complex, resulting from an imbalance between aggressive factors damaging the esophagus and a number of the natural defense mechanisms. The esophageal mucosa is in a state of continuous exposure to potentially damaging endogenous and exogenous factors. Important aggressive components of gastric refluxate include acid and pepsin and also pancreatic enzymes and bile. Among aggressive factors of exogenous origin, cigarette smoking, non-steroidal anti-inflammatory drugs (NSAID), and steroids are of the utmost importance. The basic level of esophageal defense against acid-pepsin damage consists of the anti-reflux mechanisms such as the luminal acid clearance and removal of the esophageal contents and neutralization of luminal acidity. In addition the esophageal mucosal protection includes the presence of pre-epithelial, epithelial and post-epithelial cellular and functional components. Recently, the progress have been made in the understanding of role of the heptapeptide member of the renin-angiotensin system (RAS), angiotensin-(1-7) (Ang-(1-7)) in the control of gastrointestinal functions. It has been shown that all components of local RAS including Ang-(1-7) are detectable in the gastrointestinal wall including not only the stomach but also the esophagus. Previous studies revealed that Ang-(1-7), which is an important component of the RAS, exerts vasodilatory, anti-inflammatory and antioxidant activities in the stomach. Ang-(1-7) was recently implicated in gastroprotection, but its effects on esophageal mucosa in a rodent model of reflux esophagitis and in human subjects presenting GERD symptoms have not been explored. The present study was aimed to evaluate the possible protective effects of Ang-(1-7) and Mas-receptors upon esophageal mucosal damage in acute reflux esophagitis (RE) induced in anesthetized rats by ligating the pylorus and the limiting ridge (a transitional region between the forestomach and the corpus of stomach). Consequently, the total gastric reservoir to store gastric juice was greatly diminished, resulting in the reflux of this juice into the esophagus. Because Mas receptors are functionally linked to nitric oxide (NO) formation, we also studied involvement of endogenous NO in the mediation of protective and circulatory effects of exogenous Ang-(1-7). Moreover, an attempt was made to assess the possible role of sensory neurons in the modulation of the protective effects exerted by Ang-(1-7)/Mas receptor system. Six series of rats were pretreated 30 min before induction of RE with 1) vehicle (saline), 2) Ang-(1-7) (5-50 μg/kg i.p.), 3) A779 (50 μg/kg i.p.), the selective Mas receptor antagonist applied alone, 4) Ang-(1-7) (50 μg/kg i.p.) combined with A779, 5) L-NNA (20 mg/kg i.p.) administered alone, and 6) Ang-(1-7) (50 μg/kg i.p.) combined with L-NNA. In separate group of rats, capsaicin (total dosage of 125 mg/kg within three days) was administered s.c. 2 weeks before the induction of RE to induce functional ablation of sensory nerves. Rats with intact sensory nerves and those with capsaicin-induced sensory denervation received vehicle (saline) or Ang-(1-7) (50 μg/kg i.p.) to determine whether this vasoactive metabolite of angiotensin I could be also effective in rats with capsaicin-induced impairment of the synthesis and release of sensory neuropeptides such as CGRP. Four hours after induction of RE, the mucosal damage was graded with mucosal lesion index (LI) from 0 to 6, the esophageal microcirculatory blood flow (EBF) was determined by H_2-gas clearance technique and plasma level of pro-inflammatory cytokines interleukin-1β (IL-1β), and tumor necrosis factor-α (TNF-α) was determined by ELISA. The expression of proinflammatory factors including COX-2, cytokine IL-1β and hypoxia inducible factor 1alpha (Hif1α) was analyzed in the esophageal mucosal biopsies. In rats with RE, the esophageal LI was significantly elevated comparing its value observed in intact rats, and the EBF was significantly decreased as compared with intact mucosa. Pretreatment with Ang-(1-7) of control rats without esophagitis induced increase in EBF by about 25% without any macroscopic changes in the esophageal mucosa or in the plasma level of cytokines. In animals with RE, pretreatment with Ang-(1-7) significantly reduced gross and histological esophageal mucosal injury and significantly increased EBF in comparison to vehicle-pretreated animals. The observed gross and histologic esophagoprotective effect of Ang-(1-7) was totally abolished by A779 so in rats with combined treatment of A779 with Ang-(1-7), the LI was identical with this observed in control RE and the EBF was decreased in these animals by about 39%. Inhibition of NO synthase by L-NNA significantly reduced the LI and the rise in EBF caused by Ang-(1-7).

Similarly, the capsaicin denervation also significantly attenuated the vasodilatory and the esophagoprotective effects of Ang-(1-7). The expression of proinflammatory factors COX-2, Hif1α and IL-1β which was negligible in intact esophageal mucosa, was upregulated in esophageal mucosa of rats with RE. In contrast, the administration of Ang-(1-7) resulted in a downregulation of mRNA for COX-2, Hif1 and IL-1β in esophageal mucosa an this effect was abolished in A779-dependent manner. The Ang-(1-7) significantly decreased the RE-induced elevation of plasma levels of IL-1β and TNF-α, and this effect was also reversed by pretreatment with A779, and significantly attenuated by pretreatment with L-NNA and capsaicin-induced sensory denervation. The present study indicates that the protective effect of Ang-(1-7) observed in the esophageal mucosa during early acute stage of gastroesophageal reflux depends upon the enhancement of esophageal microcirculatory blood flow *via* the activation of Mas receptor possibly due to NO synthase/NO system activation, stimulation of sensory nerves, the inhibition of expression of pro-inflammatory factors including COX-2, Hif1α and IL-1β and release of proinflammatory cytokines IL-1β and TNF-α.

Key words: *angiotensin-(1-7), reflux esophagitis, esophageal blood flow, Mas receptor, nitric oxide, afferent sensory neurons, cyclooxygenase-2, hypoxia inducible factor 1α, interleukin-1β, tumor necrosis factor-α*

INTRODUCTION

Gastroesophageal reflux disease (GERD) is a multifactorial process and one of the most common diseases of mankind (1-3). This disease is the logical consequence of the failure of the physiological anti-reflux barrier to protect against frequent and abnormal load of gastroesophageal reflux of acid or alkaline content or both. The pathogenesis of GERD is complex, resulting from an imbalance between defensive factors protecting the esophagus such as anatomical antireflux barriers, esophageal acid clearance, the mucosal barrier, and aggressive factors from the stomach and duodenum (4-6). Important endogenous aggressive factors of gastric origin which affect esophageal mucosa during gastric reflux include gastric acid and pepsin (7, 8). The development of reflux esophagitis (RE) on a cellular level is due to hydrogen ion diffusion into the mucosa, leading to tissue acidification and necrotic damage. It has been demonstrated that acid alone induces minor injury of esophageal mucosa at pH of less than 3. However, acid combined with even small amounts of pepsin causes a potent damage of the mucosal barrier, resulting in increased hydrogen ion permeability, mucosal morphologic changes, and local hemorrhage (8-10). Thus, the ability of pepsin to induce esophageal mucosa injury is acid-dependent, with maximal damaging activity below pH 3 (5-8). Along with acid and pepsin, duodenal contents such as bile acids, trypsin, and hyperosmolality may be injurious to the esophageal mucosa during the presence of duodenogastric reflux (9, 10). Experimental studies demonstrate that bile acids induce their greatest injury in the presence of hydrochloric acid and pepsin (10). Among aggressive factors of exogenous origin, cigarette smoking, alcohol and anti-inflammatory non-steroidal drugs such as aspirin, naproxen, indomethacin, and ibuprofen have been identified as risk factors for the development of erosive esophagitis (11-14). Development of RE is sometimes potentiated by steroids. It has also been demonstrated that aspirin makes the esophageal mucosa more sensitive to the injurious action of acid and pepsin (15, 16).

The basic level of esophageal defense against acid damage consists of the natural anti-reflux mechanisms, which are created by special physiological properties of the gastroesophageal junction (1-4). The lower esophageal sphincter is considered the most important mechanical barrier preventing the backflow of gastric contents from the stomach into the esophagus. The evidence indicates that abnormalities in the lower esophageal sphincter, such as transient sphincter relaxations, contribute to the development of reflux of gastric content into the esophagus. The next level of esophageal luminal defense against reflux damage is primarily achieved by luminal acid clearance which is determined by esophageal peristalsis, removal of the esophageal contents, and neutralization of esophageal luminal acidity by bicarbonate secreted locally by submucosal glands or delivered with swallowed saliva (4, 5). The esophageal mucosal defense also includes a tissue resistance, which is determined by the presence of pre-epithelial, epithelial, and post-epithelial defensive mechanisms which consist of morphological and functional components (17, 18). Morphological components include cell layers of esophageal mucosa which create a relatively tight epithelium with high resistance to ionic movement. The pre-epithelial functional components of mucosal resistence include mucus and unstirred water layers with bicarbonate ion located on the surface of the esophageal epithelium to buffer and extrude hydrogen ions (4-6). However, the hydrogen ion buffering capacity of esophageal mucus is low. Although the buffer capacity of the pre-epithelial defense in the esophagus is limited, it still has significant protective importance. Epithelial (intraepithelial) defense against injury induced by acid are created by structural and functional barriers to hydrogen ion diffusion. Morphological barriers include the apical cell membrane and intercellular junctional complex. Functional defense components include buffering capacity of negatively charged proteins, bicarbonate ions, and H^+ extrusion processes (4-6, 16-18).

Esophageal post-epithelial defense mechanisms are mainly determined by blood supply to the mucosa and additionally by the local tissue acid-base status (19, 20). Esophageal blood flow is the main post-epithelial defense mechanism which critically determines the ability of the esophageal mucosa to resist injury induced by noxious factors (acid, pepsin) by interacting with epithelial factors to protect against acid injury. Circulating blood delivers oxygen and nutrients for metabolic activity and humoral factors responsible for control of the mucosal microcirculation. In response to luminal acid, mucosal blood flow delivers more bicarbonate to the intercellular space and removes hydrogen ion, CO_2, and other metabolites, thereby maintaining normal mucosal acid-base balance (19, 20).

Numerous investigators have studied the physiological and pathological relationships among esophageal blood flow, esophageal function, and protection of the esophageal mucosa (21-24). When the esophagus experiences acute reductions in blood flow, tissue injury results from both the hypoxia induced by reduced blood flow and the deleterious effects of reperfusion resulting in the enhanced generation of reactive oxygen species (ROS). 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 interruption or

reduction. Like gastric blood flow, esophageal blood flow is affected by a variety of systemic neurohormonal regulatory mechanisms and local factors (20, 21, 25). Previous studies documented that, under physiological conditions, blood flow to the esophageal mucosa increases in response to an increase in mucosal secretory activity and to the stress induced by luminal acid (21-24). The most important protective role of esophageal mucosal hyperemia, which takes place during reflux of acidic gastric contents into the esophagus, is related to the alkalinizing of epithelial surface due to the increased bicarbonate delivery to the mucosa, an increase in mucosal-surface bicarbonate ion concentration, thus normalizing of the acid-base balance in mucosal tissue (24, 26). The mechanism of esophageal mucosal hyperemia due to acid stress is complex and includes activation of mucosal acid sensors, activation of capsaicin-sensitive afferent neurones of the esophagus, and locally released sensory neurotransmitters such as calcitonin gene-related peptide (CGRP), substance P (SP), vasoactive intestinal peptide (VIP). Nitric oxide also serves as an endothelial mediator of mucosal hyperemia (9, 16, 19, 27, 28). Recently, it has been shown that capsaicin-sensitive sensory nerves are involved in maintaining the integrity of the esophageal mucosa (27-29). The rise in mucosal microcirculatory blood flow evoked by the presence of hydrogen ion in the rat esophagus could be inhibited by pretreatment with a neurotoxic dose of capsaicin so as to induce degeneration of capsaicin-sensory neurons (9, 18, 19, 29).

Among the hormonal regulatory mechanisms which play an important role in the control of esophageal function and the esophageal macro- and microcirculations in physiological and pathological conditions is the renin-angiotensin system (RAS) (30, 31). The RAS is presently considered to control the homeostatic mechanisms that regulate hemodynamics, arterial pressure, and water and electrolyte balance (31, 32). RAS functions as both a circulating endocrine system and a tissue (local) paracrine/autocrine system. The main circulating peptide of RAS, namely the angiotensin II system (Ang II) acts on the AT-1 receptor and thus plays an important role in blood pressure control (33). Activation of circulating RAS and the rise in the plasma level of Ang II have been proven as one of the major factors reducing blood supply to the gastrointestinal tract, including the esophagus, by increasing local vascular tone and potent vasoconstriction (33-35). The many different effects of Ang II in the esophagus are mediated by the interaction of the peptide with specific, high-affinity receptors in the plasma membranes of its target cells. The existence of angiotensin-1 (AT_1) and angiotensin-2 receptors (AT_2) located in the esophageal wall and their possible role in the control of the function of esophagus under physiological and pathological conditions were recently reported (32-35). For long period of time, Ang II was considered to be exclusively a circulating hormone, but since Ang II as well as other peptide components of the RAS have been identified in a large number of tissues (31, 32), the concept of the existence of the general or circulating RAS system has been recently revised (32, 35, 36). The subsequent discovery of locally identified components of the RAS in the walls of gastrointestinal (GI) tract including esophagus and stomach (34-37) changed the concept of the physiological role of this system in these organs. Thus, the esophagus constitutively express all the components of a local RAS (25, 34, 36, 38). The receptors of the principal mediator of RAS, Ang II, are localized in both gland structures and the surface epithelium as well as in mesenchymal cells and blood vessels (38, 39). These findings indicate that Ang II is formed within the wall of the esophagus to control its motor and secretory activity and to regulate total and mucosal esophageal blood flow. The relationship between RAS and the esophageal vasculature received a new impetus from studies which showed that the some of peptide metabolites of Ang I and/or Ang II within the RAS were shown to function to oppose the vasoconstrictory, pro-inflammatory, and trophic effects of Ang II in the GI tract, including its circulatory system (25, 38, 39). The primary products of the RAS are Ang II, angiotensin-(1-7) (Ang-(1-7)), angiotensin III (Ang III), and angiotensin IV (Ang IV) (36-39).

Ang-(1-7) is an amino terminal heptapeptide that can be generated in the vasculature from Ang I and Ang II; *via* the activity of tissue peptidases including angiotensin-converting enzyme 2 (ACE 2) and neutral endopeptidase (NEP). ACE 2 is an important Ang-(1-7)-generating enzyme which cleaves on residue from Ang I to generate angiotensin-(1-9) which can be metabolized into Ang-(1-7) by ACE as well as the conversion of Ang II into Ang-(1-7) which acts *via* the G protein-coupled receptor-Mas receptor (31, 38, 39). Since ACE2 is present in the endothelial cells, it is supposed to play a major role in the vascular formation of Ang-(1-7). This metabolite Ang-(1-7)-induced vasodilation is endothelium-dependent and possibly mediated by the release of nitric oxide (NO), endothelium-derived relaxing factor (EDRF), and prostaglandins from endothelial cells (37-39). The detection of Ang-(1-7) in the esophagus confirmed that the esophageal RAS is regulated independently of the peripheral RAS, suggesting that this Ang 1 metabolite may play an important role in the mechanism of esophageal defense. Based on the above findings it can be hypothesized that amplification or stimulation of the ACE2 - Ang-(1-7)-Mas axis in the esophagus might be of therapeutic importance. Doing so Ang-(1-7) could provide protection against the development of acid-induced esophageal mucosal injury and increase in the rate of the healing of pre-existing mucosal injury by increasing esophageal mucosal blood flow.

In this study using surgically-induced reflux esophagitis, we examined the involvement of Ang-(1-7)-Mas receptor axis in the formation of esophageal mucosal injury induced by acute acid RE in rats treated with exogenous Ang-(1-7) without or with a Mas receptor inhibitor. Because the vasodilatory effect of Ang-(1-7) is endothelium-dependent and mediated by the endothelial generation of NO, prostaglandins, and EDRF (25, 39), the additional aim of the present study was to examine involvement of NO in the mechanism of Ang-(1-7) - Mas-axis-induced esophageal protective and microcirculatory responses. Primary chemosensitive afferents and sensory neuropeptides released from these neurons are involved in the regulation of esophageal blood flow during acid and pepsin stimulation of the esophageal mucosa (20). In the present study we also determined whether sensory innervation plays a role in the mediation of local circulatory and protective effects of Ang-(1-7) and Mas receptor. RE induced by acid and pepsin is always associated with the appearance of local mucosal cell injury as well as with local and general inflammatory responses (39-42). The range of local inflammation is related to the degree, duration, and extent of esophageal wall injury. The observed inflammation is triggered by the local release of cytokines and a rise in the blood level of these compounds. Therefore, we attempted to assess the correlation between the accompanying changes in the esophageal blood flow (EBF), macroscopic and microscopic features of mucosal injury, the plasma level of major proinflammatory cytokines such as interleukin-1beta (IL-1β) and tumor necrosis factor alpha (TNF-α) and the alterations in esophageal mucosal mRNA expression for proinflammatory markers such as COX-2, IL-1β and hypoxia-inducible factor 1 alpha (HIF1α).

MATERIAL AND METHODS

The experimental procedures of this study were approved by the Institutional Animal Care and Use Committee of Jagiellonian University Medical College in Cracow. All trials were followed

in accordance with statements of the Helsinki Declaration regarding handling of experimental animals.

Experiments were performed on 127 male Wistar rats weighting 280-340 grams. The animals were housed in cages with wire mesh bottoms, at normal room temperature, in a 12 h light-dark cycle, and with unlimited food and drinking water. The rats were fasted for 24 hours before the experiment but were allowed free access to water.

Development of acute reflux esophagitis and experimental groups

The experimental model of RE employed in our present study has been previously described in detail by Omura *et al*. (43) and then modified in our laboratory as described before (16, 44). Briefly, under ether anesthesia a midline laparotomy was performed to expose the stomach, and then both the pylorus and the transitional junction between the fore-stomach and the corpus were ligated with 2-0 silk thread. After the surgical preparation was completed, EBF was measured to confirm that esophageal blood vessels were not ligated, and then the abdominal cavity was temporarily closed and rats were allowed to recover from anesthesia and they remained in awake state to the end of experimentation.

In a separate series of experiments, we examined the time-course relationship between changes in EBF and the degree of esophageal mucosal lesion, and the relationship between changes in EBF and the duration of development of acute esophagitis were also investigated. In these series of experiments, animals were treated with vehicle (saline) only. The rats were then anesthetized with pentobarbital (50 mg/kg, i.p.) a second time at 2 h, 4 h, 8 h, 12 h, and 24 h after onset of RE induction. Then, the abdominal cavity was reopen, and EBF was measured. After EBF determination was completed, the animals were killed with an overdose of pentobarbital and the esophagus with the proximal portion of the stomach was gently dissected, removed, and pinned open for assessment of macroscopic lesions.

After these series of experiments were completed, we selected the duration time of acute esophagitis based on the time of reflux being 4 hours from the time of pylorus ligation. With this particular time of 4 hours, the esophageal lesions were fully developed and reproducible (16). Animals with RE were randomized into six major experimental groups of 7-9 rats per group that received one of the following single pretreatments: 1) vehicle (0.5 ml of 0.9% NaCl, i.p.), 2) Ang-(1-7) applied in the graded doses (5.0, 10.0, 25.0 and 50.0 μg/kg, i.p.); the effects of each applied dose of Ang-(1-7) being studied in a separate subgroup of animals, 3) the Mas receptor antagonist - A779 (10 mg/kg, i.p.) applied alone; 4) Ang-(1-7) (50 μg/kg, i.p.) combined with A 779 (10 mg/kg, i.p.) 5) the inhibitor of nitric oxide synthase - L-NNA (Sigma) (20 mg/kg, i.p.) applied alone, and 6) L-NNA (20 mg/kg, i.p.) combined with Ang-(1-7) (50 μg/kg, i.p.). The involvement of sensory afferent nerves in the esophagoprotective effects of Ang-(1-7) was tested in animals with capsaicin-induced sensory denervation (series 6 and 7). In this experimental groups, rats were chronically pretreated with capsaicin (Sigma Co., St. Louis, MO, USA) injected s.c. for 3 consecutive days at a dose of 25, 50, or 50 mg/kg at 2 weeks prior to RE induction. The effectiveness of the capsaicin treatment was confirmed by ocular sensitivity tests (9, 44). In these series of rats with capsaicin denervation vehicle (saline) or Ang-(1-7) was administered 30 min before the induction of RE as in control rats with intact sensory nerves.

Measurement of esophageal mucosal blood flow and assessment of esophageal injury

Upon the termination of gastric acid reflux in most experiments at the standard time of 4 hours after the ligation as described above, the animals were anesthetized with pentobarbital, their abdomens were opened by midline incision, and the abdominal part of the esophagus was exposed for measurement of EBF by means of the H_2-gas clearance technique as described previously (16, 44). For this purpose a double electrode of an electrolytic regional blood flowmeter (Biotechnical Science, Model RBF-2, Osaka, Japan) was inserted from the serosa into the esophageal mucosa and positioned at the post-epithelial layer. The measurements of the washout of hydrogen gas were performed in three areas of the mucosa, and the mean value of the EBF was calculated from the washout curve and expressed in ml/min/100g. The changes in EBF were expressed as percentage changes of those recorded in the control vehicle-treated animals (44).

After the termination of the EBF evaluation, the animals were euthanized by pentobarbital overdose, and the esophagus was removed. Immediately after its removal, it was opened longitudinally, rinsed with saline, and pinned open for macroscopic examination. The lesion index score was calculated (macroscopic degree of injury 0-6) after gross inspection of the esophagus under a dissecting microscope (9, 16, 44) by a researcher blind to the experimental grouping. The total area (mm^2) of lesions that had developed in the esophagus was determined under a dissecting microscope (10×) with a square grid, and graded with the lesion index (LI), as follows: 0) no visible lesions, 1) a few erosions and bleedings, 2) total area of lesions 15 mm^2, 3) total area of lesions 30 mm^2, 4) total area of lesions 40 mm^2, 5) total area 45 mm^2, 6) perforation. For the histological assessment, the biopsy of the esophageal mucosa involving the esophageal damage was taken from vehicle-control rats and those with and without Ang-(1-7) applied alone or combined with A 779, the antagonist of Mas receptor. The mucosal strips were then fixed in 10% formalin for the histological evaluation. The degree of epithelial loss was graded by a person blinded to thetreatment according to a previously described method (10) with histological index score as follows: 0) lack or minor superficial erosions without signs or mucosal regeneration, 1) moderate damage to esophageal mucosa and submucosa associated with inflammation with a small regenerative hyperplastic epithelial changes (basal hyperplasia, mitosis, balloon cells, akantosis, parakeratosis) 2) mild mucosal and submucosal damage to esophageal mucosa with clearly developed inflammation as manifested by polymorphonuclear leukocytes and lymphocytes infiltration, and 3) severe damage with a significant intensity and extension accompanied by a heavy inflammatory reaction and distinct regeneration (basal hyperplasia and mitosis).

Expression of, cyclooxygenase-2, interleukin-1β and hypoxia-inducible factor 1α transcripts in the rat esophageal mucosa determined by reverse transcriptase-polymerase chain reaction

The mRNA expression for cycloxygenase-2 (COX-2), interleukin-1β (IL-1β) and hypoxia-inducible factor 1α (Hif1α) was determined by reverse transcriptase-polymerase chain reaction (RT-PCR) in the unchanged mucosa of esophageus in intact rats or those with RE. The biopsy samples of esophageal mucosa weighing about 200 mg the mucosa was scraped off using a slide glass and immediately snap frozen in liquid nitrogen, and stored at -80°C until analysis. The total RNA was extracted from the mucosal samples by a guanidium isothiocyanate/phenol chloroform method using a kit from Stratagene (Heidelberg, Germany) according to methods described by Chomczynski and Sacchi (45). The concentration of RNA in RNase-free Tris EDTA buffer was measured at absorption of 260 nm wavelengths by spectrophotometry. Five

μg of total cellular RNA single-stranded cDNA was generated using StrataScript reverse transcriptase and oligo(dT) primers (Stratagene). The polymerase chain reaction mixture was amplified in a DNA thermal cycler (Perkin-Elmer-Cetus, Norwalk, CT). The nucleotide sequences of the primers used in PCR were as follows:

β-actin (size PCR product 764 bp), forward: 5'-TTG TAA CCA ACT GGG ACG ATA TGG-3', reverse: 5'-GAT CTT GAT CTT CAT GGT GCT AGG-3';

COX-2 (size PCR product 201 bp), forward: forward: 5'-ACA ACA TTC CCT TCC TTC-3'; reverse: 5'-CCT TAT TTC CTT TCA CAC C-3';

IL-1β (size PCR product 543 bp), forward: 5'-GCT ACC TAT GTC TTG CCC GT-3', reverse: 5'-GAC CAT TGC TGT TTC CTA GG-3'; Hif1α (size of PCR product 510 bp), forward: 5'-TCT GGA CTC TCG CCT CTG-3', reverse 5'-GCT GCC CTT CTG ACT CTG-3'.

PCR products were separated by electrophoresis in 2% agarose gel containing 0.5 μg/mL ethidium bromide and then visualized under UV light as described previously (25, 44). The signal intensity of expression of mRNAs for IL-1β, TNF-α and Hif1α was analyzed by densitometry (Gel-Pro Analyzer, Fotodyne Incorporated, Hartland, WI, USA) (44).

Determination of plasma proinflammatory cytokines

At the termination of experiments immediately after EBF measurements, a venous blood sample was withdrawn from the vena cava into EDTA-containing vials and used for the determination of plasma IL-1β and TNF-α by a solid phase sandwich ELISA (Biosource International Inc. Camarillo, CA, USA) according to the manufacturer's instructions. Briefly each sample (50 μl) was incubated with biotinylated antibodies specific for rat IL-1β and TNF-α, was washed three times with assay buffer, and finally conjugated with streptavidin peroxidase to form a complex with stabilized chromogen as described in our previous studies (16, 25, 44).

Statistical analysis

Results are expressed as mean ± S.E.M. Statistical comparisons of two groups were performed with Student's T-test, where appropriate. Comparison involving more than two groups was performed by ANOVA with Tukey *post-hoc* test. A difference with a $P < 0.05$ was considered statistically significant.

RESULTS

Time sequence of changes in esophageal injury and esophageal blood flow during development of reflux esophagitis

Fig. 1 shows the time-related development of esophageal injury induced by RE as determined by the macroscopically evaluated area of damage expressed as macroscopic lesion index and histologic lesion score as well as the alterations observed in EBF during various periods of RE. The determinations of both examined parameters were performed in intact animals and in rats 2, 4, 8, 12, and 24 hours after the onset of RE. In the intact animals esophageal mucosa did not show any macroscopic alterations, and mean EBF averaged 72.3 ± 3.5 ml/min/100 g tissue, which was considered as the control value (100%). As shown in *Fig. 1*, the significant mucosal injury appeared 2 hours after the onset of reflux of gastric juice. The observed mucosal injury was characterized by the appearance of local mucosal edema and a small number of focal hemorrhagic erosions. Mucosal injury expressed by lesion index was low with an average value of 1.2 ± 0.2 and EBF was significantly decreased by about 25.0 ± 5.0% ($P<0.05$) at 2 hours after the initiation of reflux of gastric juice in comparison to intact animals. Four hours after induction of RE, further increase in esophageal mucosal lesions were observed which increased the lesion index to a mean value of about 3.5 ± 0.5 and decreased EBF by 39.0 ± 3.6% compared to the value recorded in intact animals. Prolongation of the duration of gastric reflux exposure of esophageal mucosa to 8, 12 and 24 h tended to increase the LI of esophageal injury and to diminish the EBF but this increase in LI and fall in the EBF at each time interval after the beginning of reflux failed to reach statistical significance as compared with the value of LI recorded at 4 h after the induction of gastric reflux (*Fig. 1*). With the prolongation of the time of RE, the observed degree of increase of the mucosal lesion index and the degree of decrease of EBF were not significantly different than the values observed after 4 hours of RE. Based on the above

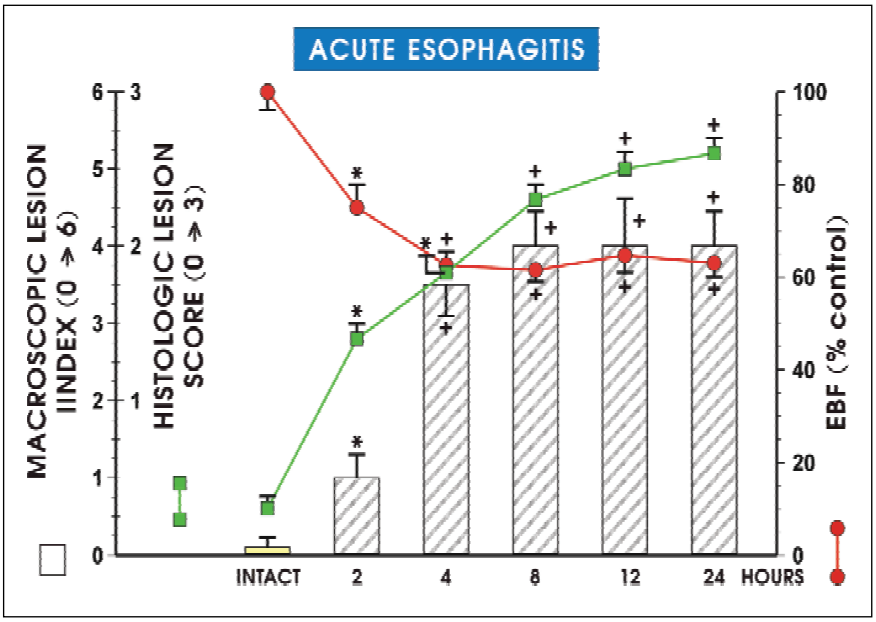

Fig. 1. The macroscopic esophageal mucosal lesion index, the histologic lesion score and the alterations in the esophageal blood flow (EBF) at 2, 4, 8, 12 and 24 hours after induction of experimental acute reflux esophagitis. Mean ± S.E.M. of 6 to 9 rats. The asterisk indicates significant change ($P<0.02$) as compared to intact rats. Cross indicates significant difference as compared to the values of lesion index and histologic lesion score and EBF at 2 hours after the initiation of acute gastric acid reflux.

findings and our previous studies (16, 44), we decided to employ the 4 hours duration of RE as a standard time to assess the mucosal injury in rats with or without Ang-(1-7) administration.

Effect of pretreatment with Ang-(1-7) alone and Ang-(1-7) combined with antagonist of Mas receptor; compound A779 on the macroscopic and microscopic esophageal mucosal injury and esophageal blood flow during development of reflux esophagitis

Fig. 2 shows the data effect of the administration of Ang-(1-7) applied i.p. in graded doses ranging from 5 µg/kg up to 50 µg/kg on the macroscopic mucosal mean lesion index and accompanying changes in the EBF in the esophageal mucosa of rats with reflux esophagitis. In this set of experiments The mean value of EBF in the intact esophagus without reflux reached the value of 68.7 ± 6.7 ml/min/100g of tissue, and this value was considered as the control value. The lesion index at 4 hours of duration of the time of reflux was similar to that presented in *Fig. 1*. The pretreatment with Ang-(1-7) administered i.p. in graded doses starting from 5 µg/kg up to 50 µg/kg induced a dose-dependent decrease in the lesion index which was accompanied by a progressive rise in EBF ($P < 0.05$). As shown in *Fig. 3* the macroscopic lesion index of esophageal mucosa and EBF in rats pretreated with vehicle and exposed to 4 hours of RE served as control for comparison of both measurements with the treatment with Ang-(1-7). Treatment with Ang-(1-7) (50 µg/kg i.p.) significantly decreased the index of mucosal lesions and significantly increased the EBF as compared to those in vehicle-treated animals ($P<0.05$). *Fig. 3* also shows the effect of treatment with Mas receptor antagonist A779 (10 mg/kg, i.p.) on the esophageal mucosa lesion index and accompanying changes in EBF ($P < 0.05$). Pretreatment with A779 alone was without any significant effect on the mean values of lesion index and EBF in comparison with vehicle-alone treated animals. However, pretreatment with A779 combined with Ang-(1-7) abolished the decrease in the mean esophageal lesion index and the accompanying rise in EBF ($P < 0.05$) evoked by Ang-(1-7) alone in rats with RE (*Fig. 3*).

As shown in *Fig. 4A-4D* (upper panel), the intact esophagus failed to exhibit the macroscopic injury but in vehicle(saline)-

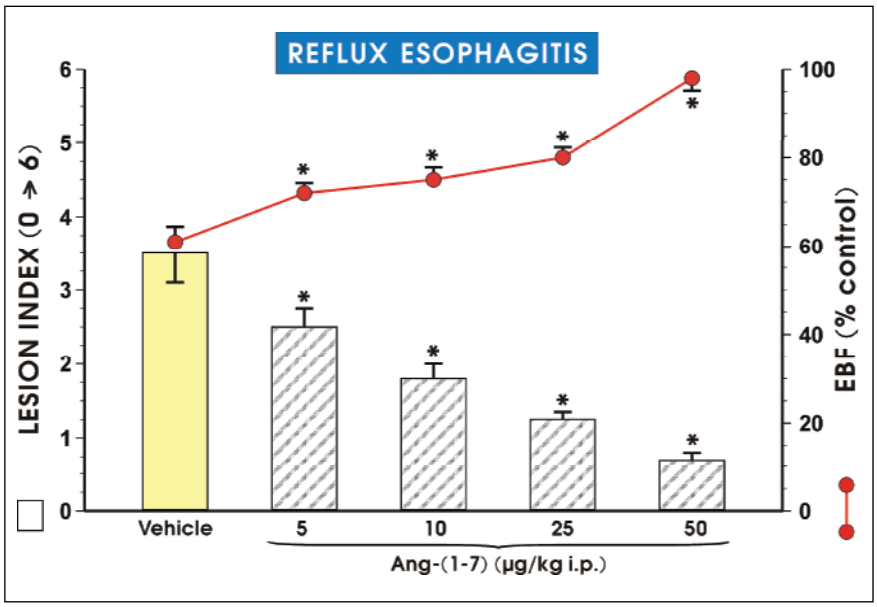

Fig. 2. Esophageal mucosal lesion index and esophageal blood flow (EBF) in rats at 4 h after induction of acute esophagitis with intraperitoneal (i.p.) pretreatment with vehicle or angiotensin-(1-7) (Ang-(1-7)) administered at graded doses starting from 5.0 µg/kg up to 50.0 µg/kg. Results are mean ± S.E.M. of 6-9 rats. Asterisk indicates significant change ($P<0.02$) as compared to the value recorded in vehicle-saline pretreated animals.

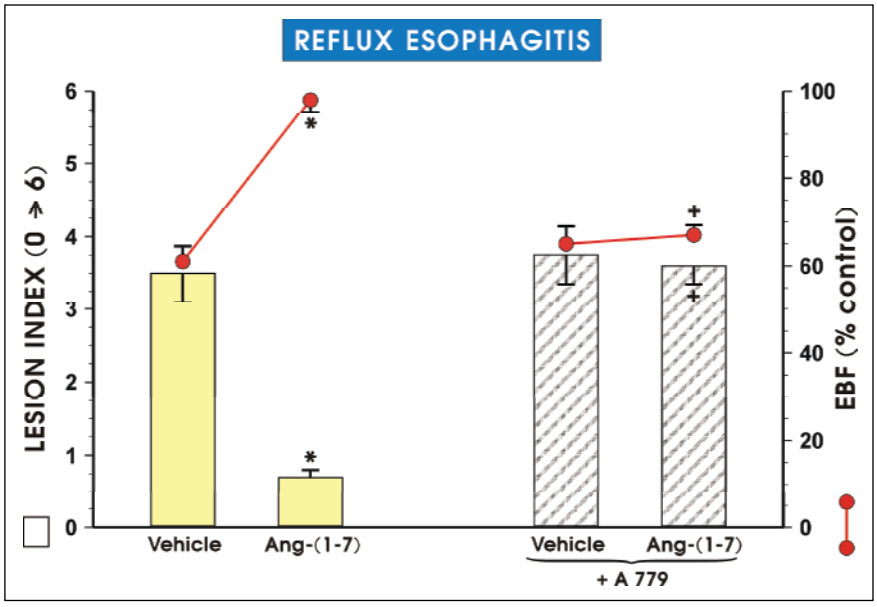

Fig. 3. Esophageal mucosal lesion index and esophageal blood flow (EBF) in acute reflux esophagitis with intraperitoneal (i.p.) pretreatment with vehicle-saline or Ang-(1-7) (50 µg/kg i.p.) in rats without and with prior administration of A779 (10 mg/kg i.p.). Results are mean ± S.E.M. of 7-9 rats. Asterisk indicates a significant ($P<0.02$) difference from the corresponding value in vehicle treated animals. Cross denotes significant ($P<0.02$) difference between the effects of vehicle-saline and Ang-(1-7) as compared to the value recorded in rats without the combined treatment with A779.

treated rats exposed to 4 h of RE, the reflux of gastric content caused severe mucosal damage (*Fig. 4B* vs. *Fig. 4A*). The extent of these lesions was significantly smaller in Ang-(1-7)-pretreated esophageal mucosa than in those pretreated with vehicle (*Fig. 4C* vs. *Fig. 4B*). When A779 was combined with Ang-(1-7), the protective effect of Ang-(1-7) against the RE was completely lost (*Fig. 4D* vs. *Fig. 4B*) and the macroscopic appearance of esophageal mucosa was similar as observed in vehicle-pretreated esophageal mucosa exposed to RE presented in *Fig. 4B*.

As shown in *Fig. 4A-4D* (lower panel), the histological assessment of esophageal mucosa stained with H&E documented no microscopic signs of mucosal damage in intact animals (*Fig. 4A*, lower panel). The normal appearance of esophageal structure of thin epithelial layer with squamous cells and only few inflammatory cells observed in submucosal layers. In contrast, the 4 h exposure of esophageal mucosa to gastric refluxate in vehicle-pretreated rat resulted in the mucosal damage, neutrophil infiltration and signs of hyperemia of epithelial layers and edema

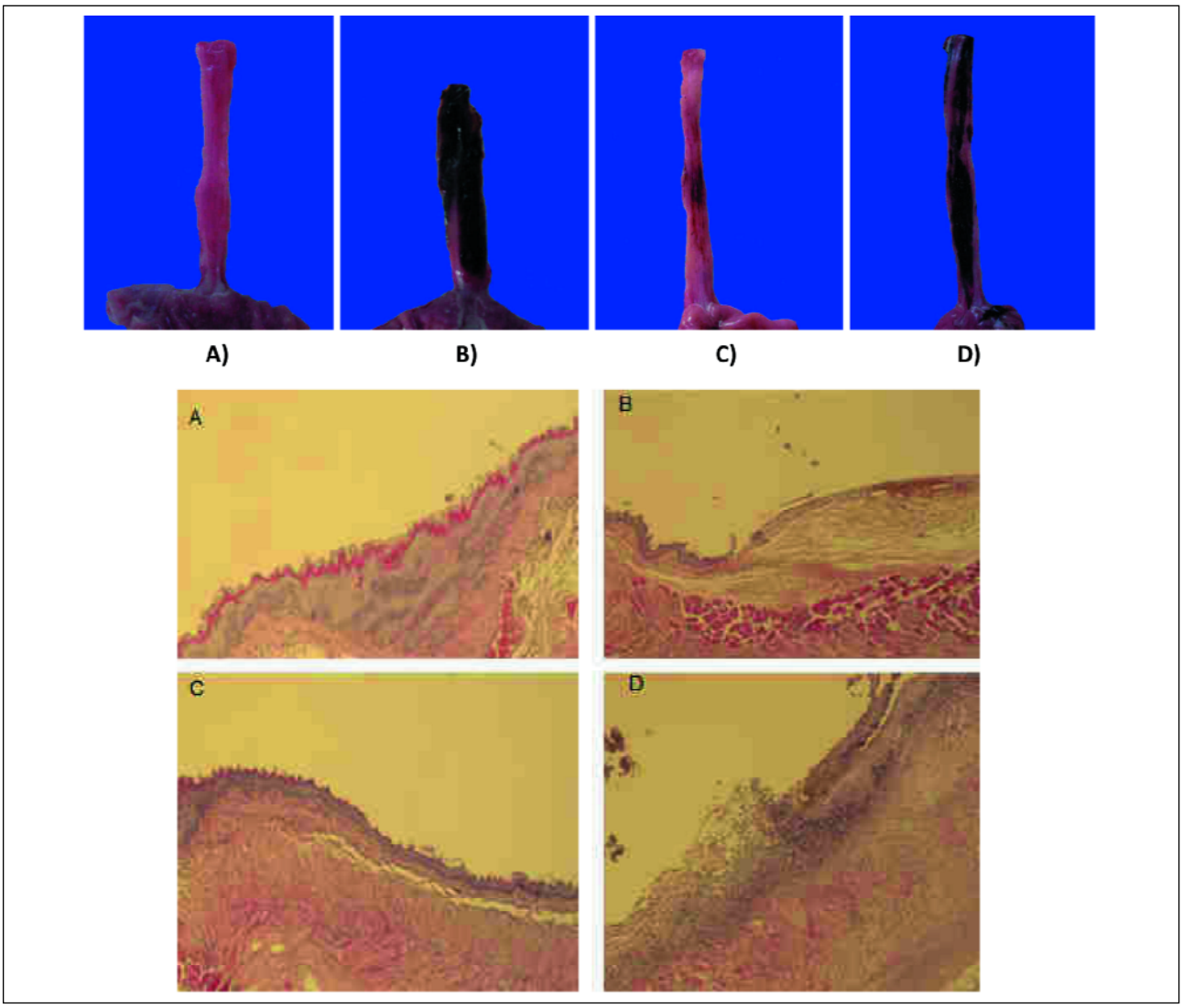

Fig. 4A-D. Macroscopic (upper panel) and microscopic (lower panel) appearance of esophageal mucosa in intact rats (*A*) and those pretreated i.p. 30 min before initiation of reflux esophagitis with vehicle-saline (*B*), Ang-(1-7) (50 μg/kg i.p.) (*C*), Ang-(1-7) combined with A779 (10 mg/kg, i.p.) (*D*). Note, the formation of esophageal lesions in vehicle-control rat exposed to gastric reflux compared with normal appearance of the intact esophageal mucosa (*B* versus *A*, upper panel). This damage of esophageal mucosa induced by gastric acid reflux is significantly diminished by Ang-(1-7) (*B* versus *C*, upper panel). In contrast, pretreatment with A779 abolished the protective effect of Ang-(1-7) and exacerbated the esophageal mucosal damage (*C* versus *D*, upper panel). *Fig. 4A-4D* (lower panel) shows the microscopic appearance of the esophageal mucosa and submucosa in intact rat(*A*, lower p anel) and those pretreated i.g. 30 min before the initiation of reflux esophagitis with vehicle (*B*), Ang-(1-7) (50 μg/kg i.p.) with or without A779 (10 mg/kg i.p.) (*C* and *D*, lower panel).

The normal appearance of the esophageal mucosa with the thin epithelial layer and squamous cells and few inflammatory cells is shown (*A*, lower panel). In contrast, the mucosal thickening, edema, mucosal damage, basal hyperplasia and leukocyte (neutrophil and eosinophils) infiltration are seen in vehicle-pretreated rats exposed to RE (*B*, lower panel). In Ang-(1-7)-pretreated rat, the mucosal damage and neutrophil infiltration were less pronounced, however, the signs for the focal mucosal thickening and edema were still observed reflecting uncompleted protective response observed by histology (*C*, lower panel). In contrast to the protective effect of Ang-(1-7), the co-administration of A779 with Ang-(1-7) reversed the Ang-(1-7)-induced partial preservation of the mucosal architecture (*D* vs. *C*, lower panel).

in mucosa and submucosa (*Fig. 5B*, lower panel). In esophagus of rat pretreated with Ang-(1-7) (Fig. *5C*, lower panel), the esophageal mucosal damage, neutrophil infiltration and edema of both, mucosa and submucosa were markedly reduced as compared with those observed in vehicle-pretreated rats with RE. In contrast, in rats pretreated with combination of A779 and Ang-(1-7), the reduction of histologic damage caused by treatment with Ang-(1-7) alone was reversed by concurrent treatment with A779 (*Fig. 5D* vs. *Fig. 5C*, lower panel) and the extent of histological damage, neutrophil infiltration and edema of epithelial and subepithelial tissues were similar to those noticed in vehicle (control) esophageal mucosa with RE.

Effect of inhibition of nitric oxide synthase and sensory denervation on the Ang-(1-7)-induced attenuation of esophageal injury and alterations in esophageal blood flow

Fig. 5 shows the macroscopic lesion index of esophageal injury and the alterations in EBF following RE in rats pretreated with vehicle or Ang-(1-7) alone and vehicle or Ang-(1-7) combined with L-NNA pretreatment. Treatment with Ang-(1-7) (50 µg/kg i.p.) significantly decreased the index of mucosal lesions and significantly increased the EBF ($P<0.05$) when compared with those in the vehicle-pretreated animals. In contrast, pretreatment of animals with L-NNA (20 mg/kg i.p.) tended to potentiate the esophageal mucosal injury over those observed in control rats pretreated with vehicle (*Fig. 5*). The mucosal injury was accompanied by a further significant decrease in EBF, reaching 53 ± 2% of the value recorded in vehicle-pretreated animals ($P<0.05$). Whereas, when L-NNA was combined with Ang-(1-7), it abolished the esophageal mucosal protective and circulatory effects of Ang-(1-7) alone (*Fig. 5*).

Fig. 6 shows the effects of vehicle and Ang-(1-7) alone on the esophageal mucosal LI and EBF in rats with RE before and after capsaicin-induced functional sensory denervation of these animals. Vehicle-treated only and Ang-(1-7) alone pretreated animals with RE exhibited esophageal mucosal injury and EBF

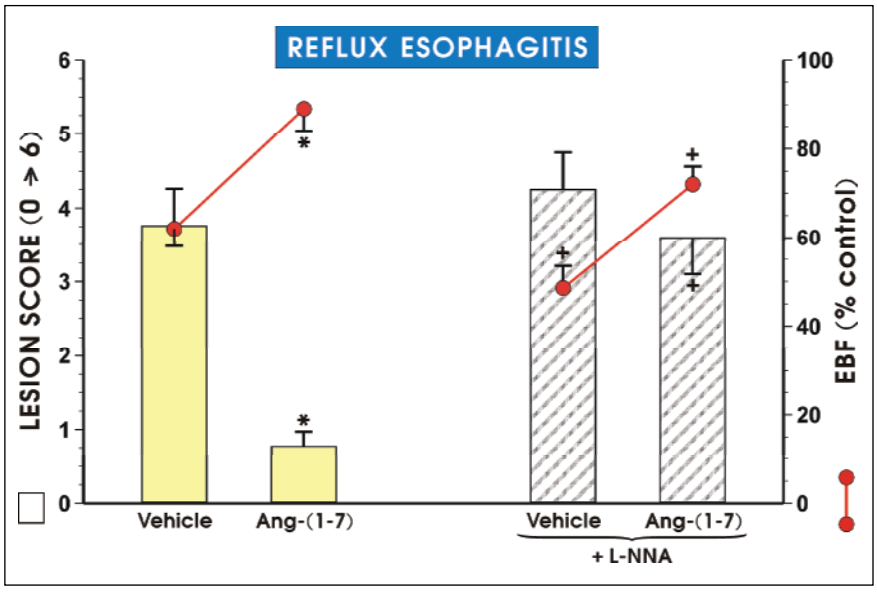

Fig. 5. Esophageal mucosal lesion index and esophageal blood flow (EBF) in rats with acute reflux esophagitis treated with Ang-(1-7) (50 µg/kg, i.p) without or with combination with L-NNA (20 mg/kg, i.p.). Results are mean ± S.E.M. of 6-9 rats. Asterisk indicates significant change ($P<0.05$) as compared to the value recorded in vehicle-saline pretreated rats. Cross indicates a significant change ($P<0.05$) as compared to the value recorded in animals without L-NNA pretreatment.

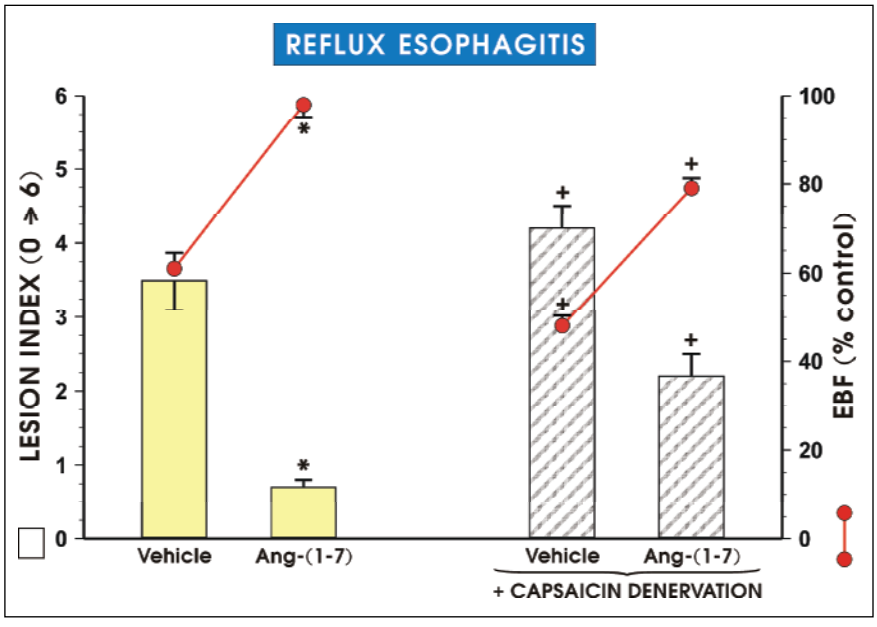

Fig. 6. Esophageal mucosal lesion index and esophageal blood flow (EBF) with intraperitoneal (i.p.) pretreatment with vehicle-saline or Ang-(1-7) (50 µg/kg i.p.) in rats pretreated with capsaicin. Results are mean ± S.E.M. of 6-9 rats. Asterisk indicates significant change ($P<0.02$) from the corresponding value in vehicle-saline treated animals. Cross denotes significant ($P<0.02$) difference between the effects of vehicle-saline and Ang-(1-7) after sensory denervation with capsaicin.

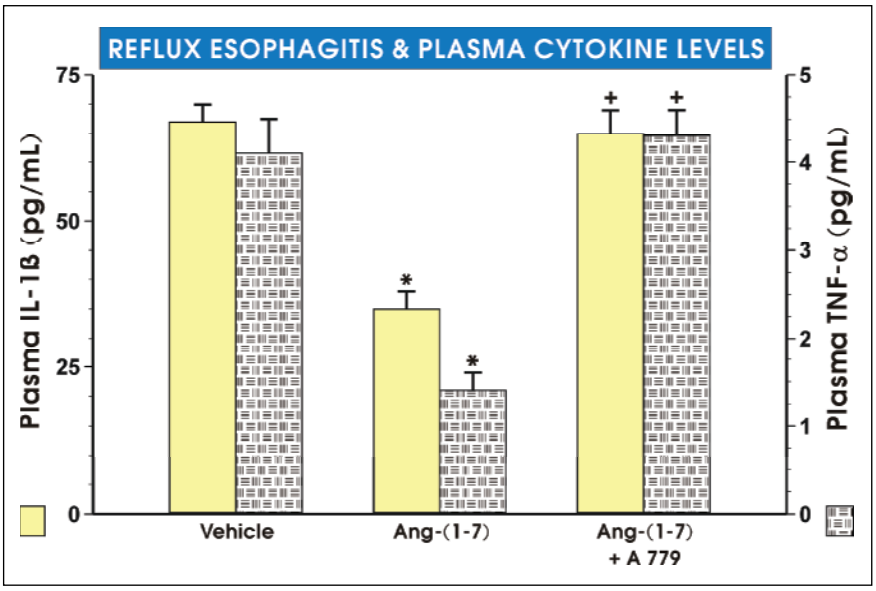

Fig. 7. Plasma level of interleukin-1 (IL-1β) and tumor necrosis factor-α (TNF-α) in animals with acute esophagitis without or with intraperitoneal (i.p.) pretreatment with vehicle-saline, Ang-(1-7) (50 µg/kg i.p.) before and after pretreatment with A779 (10 mg/kg i.p.). Results are mean ± S.E.M. of 6-9 rats. Asterisk indicates significant change ($P<0.05$) as compared to the vehicle-saline treated animals. Cross indicates significant change ($P<0.05$) as compared to the Ang-(1-7) alone treated animals.

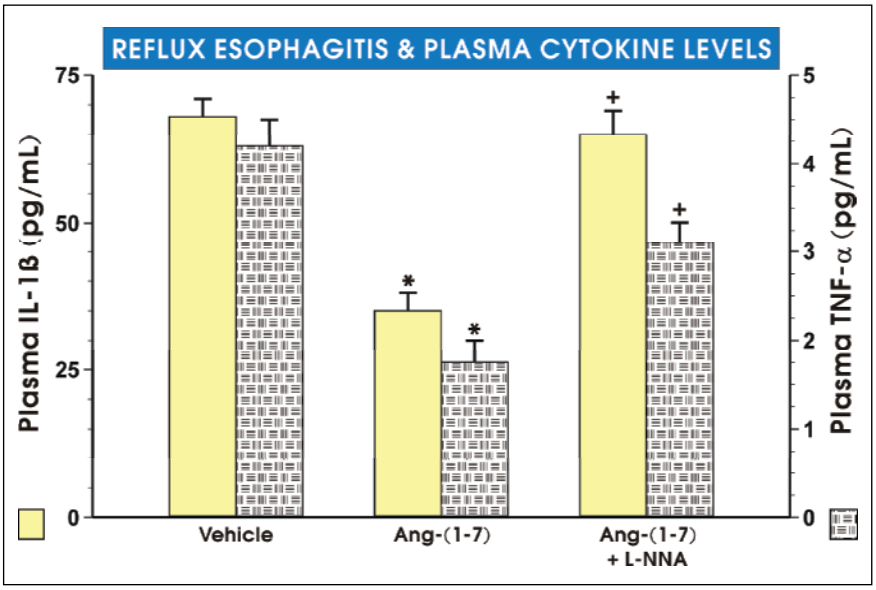

Fig. 8. Plasma level of IL-1β and TNF-α in vehicle-saline treated rats with reflux esophagitis without or with intraperitoneal (i.p.) pretreatment with vehicle-saline, Ang 1-7 (50 µg/kg i.p.) before and after preadministration of L-NNA (20 mg/kg, i.p.). Results are mean ± S.E.M. of 6-9 rats. Asterisk indicates significant ($P<0.02$) change as compared to the vehicle-saline treated animals. Cross indicates significant ($P<0.02$) change as compared to the Ang-(1-7) alone treated animals.

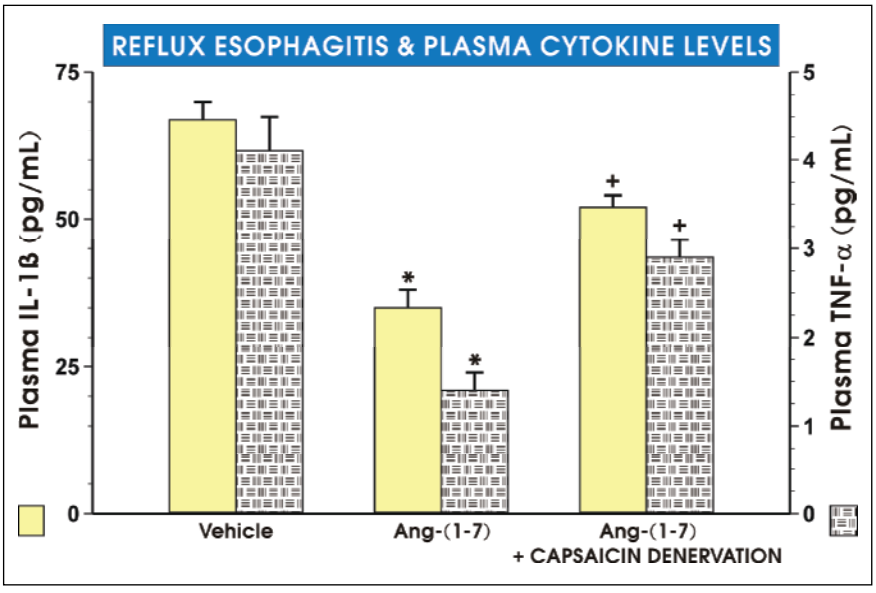

Fig. 9. Plasma level of IL-1β and TNF-α in vehicle-saline treated rats with reflux esophagitis, Ang-(1-7) (50 µg/kg, i.p.) alone and Ang-(1-7) (50 µg/kg, i.p.) after capsaicin denervation. Results are mean S.E.M. of 6-9 rats. Asterisk indicates significant ($P<0.02$) difference as compared to the vehicle-saline pretreated rats. Cross indicates significant ($P<0.02$) change as compared to Ang-(1-7) pretreated animals.

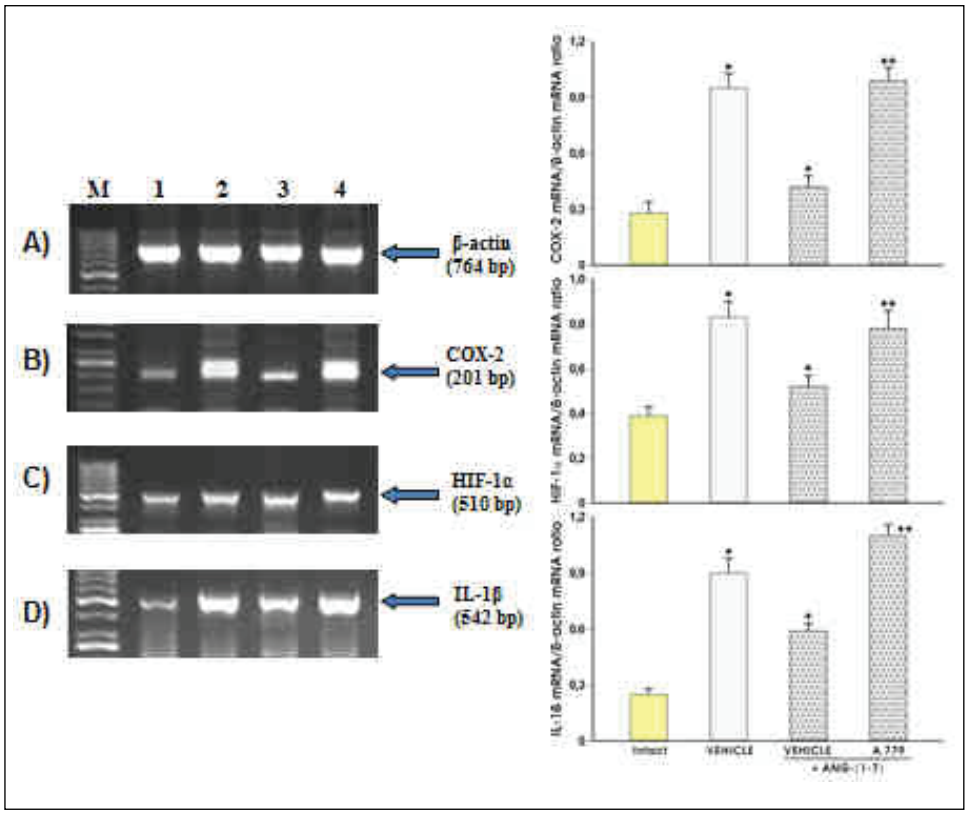

Fig. 10. Determination of expression of mRNAs for β-actin (*A*), COX-2 (*B*), Hif1-α (*C*) and IL-1β (*D*) by RT-PCR (left panel) and the ratio of COX-2 , Hif1-α and IL-1β mRNAs over β-actin mRNA (right panel) in the intact esophageal mucosa (lane 1) and in those pretreated with vehicle (saline i.g.) (lane 2), Ang-(1-7) (50 μg/kg i.p.) (lane 3) and A779 (10 mg/kg i.p.) combined with Ang-(1-7) (lane 4) and exposed to reflux esophagitis (RE) for 4 hours; M - DNA size marker. Mean ± S.E.M. of 4-6 rats. Asterisk indicates a significant change ($P<0.05$) as compared with vehicle (control) esophageal mucosa. Cross indicates a significant change ($P<0.05$) as compared with vehicle treatment in rats with RE. Double crosses indicate a significant change ($P<0.05$) as compared to the value obtained in rats pretreated with Ang-(1-7) alone.

responses that were comparable to those of the same groups presented in *Figs. 3* and *4*. Sensory denervation significantly aggravated the degree of mucosal injury (LI averaging about 4.2) and the significant reduction in EBF (49.5 ± 2.3% control value) in vehicle-treated animals with RE ($P<0.05$) in comparison with sensory intact rats with RE. Sensory denervation also significantly attenuated the effects of Ang-(1-7) on esophageal mucosa injury and EBF ($P<0.05$). The mucosal lesion index was significantly increased in capsaicin-denervated animals treated with Ang-(1-7) in comparison to its value after pretreatment with Ang-(1-7) alone ($P<0.05$), and the degree of increase in EBF was significantly lower ($P<0.05$) as compared with respective values in rats with intact sensory nerves exposed to RE (*Fig. 5*).

*Plasma levels of IL-1β and TNF-*a *in the rats with esophagitis treated with Ang-(1-7) alone and Ang-(1-7) combined with Mas receptor antagonist, inhibition of nitric oxide synthase activity and after sensory denervation*

As shown in *Figs. 7*, *8*, and *9*, the plasma IL-1β and TNF-α reached significantly higher values after 4 hours of RE in vehicle-treated rats as compared to those recorded in intact control animals. The concentration of IL-1β in intact rats was 15 ± 2 pg/ml and significant rise in IL-1β level was observed in rats with RE (68 ± 4 pg/ml). Pretreatment of animals with Ang-(1-7) (50 μg/kg i.p), prior to induction of RE, led to a significant decrease in plasma IL-1β level, as compared to that measured in the 4 hours of RE rats pretreated with vehicle ($P<0.05$). The treatment with A779 combined with Ang-(1-7) restored the plasma IL-1β level to that observed in vehicle-pretreated rats exposed to RE. The plasma level of TNF-α in intact animals was negligible but at 4 hours of RE the significant increase in the plasma levels of this cytokine was observed ($P<0.05$). In rats pretreated with Ang-(1-7) (50 μg/kg, i.p), the plasma level of TNF-α was significantly decreased ($P<0.05$) below the level observed in vehicle control animals. In animals pretreated with the combination of A779 and Ang-(1-7), the plasma concentration of TNF-α was significantly increased to the level observed in vehicle-pretreated animals ($P<0.05$).

In rats pretreated with L-NNA, plasma levels of IL-1β and TNF-α significantly rose above the values observed in Ang-(1-7)-pretreated animals with RE ($P<0.05$). Co-treatment with Ang-(1-7) and L-NNA significantly increased the plasma level of the two cytokines in comparison to treatment with Ang-(1-7) alone (*Fig. 8*). Capsaicin-induced sensory denervation significantly inhibited ($P<0.05$) the anti-inflammatory effect of Ang-(1-7) estimated on the basis of changes of the plasma levels of IL-1β and TNF-α in rats with RE (*Fig. 9*).

Expression of proinflammatory markers cyclooxygenase-2 and interleukin-1β, Hif1α by reverse-transcriptase polymerase chain reaction in gastric mucosa of rats with or without Ang-(1-7) alone or Ang-(1-7) combined with A779

Fig. 10 presents the RT-PCR expression of COX-2, IL-1β and Hif1α mRNAs in the gastric mucosa of intact rats and those pretreated with vehicle (saline), Ang-(1-7) or A779, the inhibitor of Mas receptor combined with Ang-(1-7) administered i.p. before the onset of RE. The expression of β-actin mRNA was well preserved in the mucosal biopsy samples taken both from intact rats and those treated with vehicle or Ang-(1-7) with or without the concomitant treatment with A779 and then subjected to RE (*Fig. 10*, left panel). The signal for COX-2, IL-1β and Hif1α mRNA was weakly detectable in the intact gastric mucosa but strong signal of mRNAs for COX-1, IL-1β and Hif1α were recorded in vehicle pretreated animals exposed to RE (*Fig. 10* left panel, lane 1). Semi-quantitative analysis of the ratio of COX-2/β-actin mRNA, Hif1α/β-actin mRNA or IL-1β/β-actin RNA revealed that the expression of mRNAs for COX-2, Hif1α and IL-1β was significantly increased in vehicle-pretreated animals exposed to RE ($P<0.05$) as compared with those recorded in intact

gastric mucosa. Signals for the expression of COX-2, Hif1α and IL-1β mRNAs were less intense in animals pretreated with Ang-(1-7) as compared with the respective signals recorded in vehicle-pretreated rats with RE. Indeed, the value of ratio of COX-2, Hif1α or IL-1β mRNAs over β-actin was significantly lower ($P < 0.05$) than that recorded for all proinflammatory markers in vehicle-control RE rats. Signal of the expression of COX-2, Hif1α or IL-1β mRNAs over β-actin was much stronger in RE animals treated with combination of A779 and Ang-(1-7) as compared with Ang-(1-7) alone. The analysis of ratio of COX-2, Hif1α and IL-1β over β-actin had indicated that the expression of mRNAs for COX-2, Hif1α and IL-1β was significantly increased in RE pretreated with A779 combined with Ang-(1-7) ($P<0.05$) comparing to that in rats with RE pretreated with Ang-(1-7) alone.

DISCUSSION

Many advances have been made in the gastrointestinal field in the last several decades. Among them is the progress accumulated to date in the understanding of clinical symptoms, diagnosis, and treatment of GERD (1-3). However, basic mechanisms of this disease which is presently one of the most important gastrointestinal disorders of our population have not yet been fully explained. The results of experimental and clinical studies performed thus far clearly indicate that the mechanisms of pathological changes and clinical symptoms of RE are multi-factorial. The imbalance between defensive and offensive factors is proposed as a major mechanism in the development of RE (4-7). The maintenance of the integrity of the epithelial barrier of the esophageal mucosa against deleterious factors involves several mechanisms such as anti-reflux mechanisms, luminal acid clearance, and the presence of esophageal mucosal pre-epithelial, epithelial, and post-epithelial components affording protection of esophageal structure (6-9). The esophageal mucosa is almost continuously exposed to aggressive, potentially damaging components of gastric refluxate such as acid and pepsin and also pancreatic enzymes and bile.

The major purpose of the present study was to characterize the early esophageal morphological, microcirculatory, and systemic inflammatory responses in an experimental rodent model of acute reflux esophagitis. In particular, we evaluated the effects of exogenous Ang-(1-7), one of the major metabolite of RAS and the involvement of Mas receptor on the esophageal mucosal injury, mucosal microcirculatory changes, and general inflammatory responses estimated from plasma levels of pro-inflammatory cytokines IL-1β and TNF-α in the early stage of reflux esophagitis. We also looked for the possible contribution of NO and sensory neurons in the esophagoprotective effects of Ang-(1-7)-Mas axis, the alterations in EBF, and the gene expression of inflammatory markers COX-2, Il-1β and HIF-1α as well as the release of IL-1β and TNF-α in rats with RE with and without pretreatment with Ang1(1-7). The experimental model of gastroesophageal reflux applied in this study seems to be especially suitable for examination of the early pathophysiological manifestations such as the development of acute esophageal injury caused by gastric acidic reflux. In this experimental model, gastric acid and pepsin are the main factors causing reflux esophagitis. The method of esophageal lesion induction employed in our present study requires only transient superficial anesthesia of the animals at the beginning of experimentation compared with the method when acid and bile (9) were used to perfuse the esophagus. Therefore, we think our present method with a reflux induced by endogenous gastric acid most likely mimics the natural mechanism of acute RE in humans.

In the present study, we confirmed that this method of inducing RE caused visible structural damage to the esophageal mucosa and that this damage could be quantified into a lesion index from macroscopic and microscopic determinations (10, 16). The average values of lesion index in the present study are comparable with those obtained in our previous study in which esophageal mucosal lesions were induced by RE (16, 30, 44) or by perfusion of the esophagus with acid-pepsin and bile (9). The characteristics of the esophageal mucosal microcirculatory response and changes in inflammatory response, which we observed after acute reflux esophagitis, are consistent with previous reports that acute gastric reflux caused a marked ischemia to esophageal mucosal in rats and evoked a systemic inflammatory response (16, 30, 43).

The circulating RAS is a well-recognized hormonal system which plays an important role in control of the cardiovascular system and extracellular fluid volume (31, 32). More recently, it has been recognized that RAS is present locally in most organs including the gastrointestinal tract (34, 35, 44). The components of local RAS are present in the stomach, small intestine, and colon (37, 39). In the esophagus, ACE, AT_1 and AT_2 receptors have been found in the microcirculation, superficial stratified epithelium, and longitudinal muscle (34, 35). In this study, we have demonstrated the important protective and microcirculatory effects of exogenous Ang-(1-7) in experimental RE. Results of our present study revealed that Ang-(1-7), while causing an increase in mucosal microcirculatory blood flow, also dose-dependently prevented the formation of esophageal mucosal lesions and afforded protection against damage to the mucosa exposed to gastric refluxate. This protection was provided possibly by a vasodilating the esophageal microvasculature and thus yielding improved tissue oxygenation, increased delivery of metabolic substrates, and more rapid removal of metabolic waste products (20, 21, 24). Our present findings suggest that these circulatory and consequently protective effects of Ang-(1-7) in the esophageal mucosa are mediated by Mas receptors as demonstrated by the ability of the specific Mas receptor blocker, A779, to inhibit the protective and hyperemic effects of Ang-(1-7). The Mas receptor is constitutively expressed on endothelial cells, and the expression of mRNA for Mas receptor has been identified in several organs including the esophagus (34). In the present study we found that selective blockade of Mas receptors with A779 was without any effect on the mucosal lesion index or EBF in vehicle-treated animals. This may suggest that endogenous Ang-(1-7) is not generated locally in the esophageal vascular endothelium under physiological conditions, or alternatively the generation of esophageal Ang-(1-7) is decreased or even totally abolished in case of mucosal damage caused by gastric refluxate. In contrast, Ang-(1-7) is generated in the gastric mucosa of rats under resting conditions and during the process of gastric ulcer healing the mucosal content of this peptide in gastric mucosa is significantly increased (25, 45). The present study shows that Ang-(1-7) which acts *via* its specific G-protein-coupled receptor, Mas, is one of the most attractive peptide of the RAS which participates in the protection of esophageal mucosa against damage induced by gastric contents in the early stage of RE. The mechanism of action of Ang-(1-7) in the protection against RE is complex and appears to depend on the activation of Mas receptor since the Mas antagonist, A779, blocked all the protective effects of Ang-(1-7). It is well established that ACE2, which is present in endothelial cells, plays a major role in Ang-(1-7) generation in the systemic vasculature, including the gastrointestinal circulation (36, 37). The mechanism of vasodilatatory action of Ang-(1-7)-Mas axis is endothelium-dependent since the endothelial cells are the major target for its actions, and activation of this axis stimulates the generation and release of endothelial vasodilators such as NO, endothelium-derived hyperpolarizing factor (EDHF), prostaglandins and bradykinin (38, 39). It should also be mentioned that Ang-(1-7)-Mas receptor system was reported to act as a biological antagonist of the AT_1 receptor and to inhibit the

pressor effect of Ang II mediated by stimulation of AT_1 receptor (38). The above mentioned interaction between Ang-(1-7)-induced activation of Mas receptor counteracting the stimulation of AT_1 receptor may be, at least in part, responsible for the esophageal vasodilatory effect of Ang-(1-7) observed in our study. Ang-(1-7) also has a very low affinity for AT_2 receptors, even though in some tissues, this mechanism may be also explanatory in understanding of in the circulatory effects of this peptide. However, the existence of such an interaction has not been shown in the esophagus so far. Our earlier study demonstrated that inhibition of the NOS/NO system and the deactivation of sensory nerves render the esophageal mucosa highly susceptible to the damage done by exogenously administered acid-pepsin, bile solution (9, 16), or aspirin (15).

The maintenance of integrity of the epithelial barrier of esophageal mucosa against aggressive factors involves several mechanisms including the mucosal microcirculation which is partially responsible for maintaining normal mucosal acid-base balance (3). Esophageal mucosal hyperemia takes place in response to activation of its normal secretory activity and the presence of luminal acid (19, 20). It is well established that general or local mucosal ischemia increases the vulnerability of the gastrointestinal and esophageal mucosa to the development of mucosal erosions, ulcerations, the activation of inflammation, and serious ulcer complications (21). These mucosal-destructive effects of ischemia and hypoxia are known to inhibit an important protective factor such as NO generation. The mechanisms through which NO protects mucosa are complex and include mucosal hyperemia, stimulation of mucus and bicarbonate secretion, and prevention of disruption of the gastrointestinal mucosal barrier (9, 16, 20, 21). Among these mechanisms, maintaining the mucosal microcirculation is thought to be the most important for the protective effects of NO in the gastrointestinal tract (35-38). In our study, the pretreatment with Ang-(1-7) exerted a dose-dependent protection against the mucosal injury, and this effect was accompanied by a parallel increase in EBF. These responses to Ang-(1-7) could be explained by ability of this peptide to stimulate endothelial NOS and potentiation of esophageal, mainly endothelial, NO generation. NO is a potent vasodilator in the gastrointestinal tract including the esophageal microcirculation, and it is involved in the mediation of vascular autoregulatory responses including functional and reactive hyperemia (20-22). In line with such well-recognized circulatory actions of NO in the esophagus, we documented that blocking Mas receptors eliminated the protective effects of Ang-(1-7) and potentiated vasoconstriction which is known to induce a local ischemia and as a final consequence, tissue hypoxia predisposing the esophageal mucosa to mucosal lesions due to the presence of gastric refluxate in esophageal lumen. These damaging effects of ischemia on esophageal mucosa are exacerbated by the presence of hydrogen ion and pepsin which are able by themselves to induce mucosal damage. In response to blockade of Mas receptors, the apparent reduction in microcirculatory blood flow occurs also as a result of the adherence of neutrophils to vascular endothelium in gastrointestinal microvasculature via an increase in expression of adhesion molecules on endothelial cells (35, 40).

The maintenance of integrity of the epithelial barrier of gastrointestinal tract and the protection of esophageal mucosa against aggressive factors may also involve a paracrine and neuronal mechanisms (21, 26, 27) mediated by capsaicin-sensitive sensory nerves. Esophageal primary sensory fibers are carried in the vagus and the spinal nerves. These unmyelinated nerves contain and release a variety of well-recognized peptides, such as substance P, vasoactive intestinal polypeptide (VIP) and CGRP (27). Such peripheral peptide-containing fibers have been shown to participate in the transmission of sensory impulses from esophageal mucosa (26-28). Therefore, it was of interest to determine whether or not peptidergic afferent neurons are involved in the mechanism of Ang-(1-7) - Mas-axis-induced protection of esophageal mucosa exposed to RE. Previous studies have presented evidence to support a physiological role for capsaicin-sensitive neurons as modulators of esophageal mucosal blood flow and mediators of reactive mucosal hyperemia due to stimulation of mucosal acid-sensing sensory endings of esophageal mucosa (21, 29). The implication of afferent sensory neurons in the mechanism of the mucosal protection induced by Ang-(1-7) is based on our previous observation that sensory neurons and sensory mediators are involved in the mechanism of mucosal protection (9, 16). In the present study, we confirmed our previous finding that ablation of capsaicin-sensitive sensory neurons augmented detrimental effects of gastric acid refluxate in acute RE, partly due to impairment of microcirculation in esophageal mucosa (30). Furthermore, we showed that ablation of sensory neurons by capsaicin attenuated protective and vasodilatory effects of Ang-(1-7), indicating that activation of sensory neurons and their vasoactive mediators are involved, at least in part, in protective action of this peptide in RE.

Damage of esophageal mucosal cells due to intracellular acidification that cannot be buffered by epithelial protective mechanisms results in the loss of osmoregulation and cell swelling. Cell membranes rupture and the release of inflammatory mediators by damaged cells facilitates the development of inflammation (40-42). We confirmed our own and others previous findings that the local generation and release of proinflammatory cytokines from injured esophageal mucosa contribute to the development of esophagitis (16, 46). This is supported by the observation that a significant increase in the gene expression and release of IL-1β was recorded in rats with RE. One of the important finding of our present study is the observation that pretreatment of RE animals with Ang-(1-7) markedly diminished the plasma level of not only IL-1β but also another proinflammatory cytokine TNF-α in acute phase of experimental RE. A similar, general anti-inflammatory effect of Ang-(1-7) was observed earlier under various inflammatory conditions (47-49) and also during the healing of chronic gastric ulcers (46, 47). The anti-inflammatory effect of Ang-(1-7) observed in the present study by an downregulation of IL-1β expression and its release as well as the apparent fall inTNF-α concentration in esophageal mucosa were reversed by the concurrent treatment of animals with the Mas receptor antagonist A779, supporting the notion that Mas receptors are involved in the mediation of observed anti-inflammatory effects of Ang-(1-7).

We demonstrated herein that besides the downregulation of expression of IL-1β, the expression of another proinflammatory factors such as COX-2 and HIF1α was markedly abrogated in Ang-(1-7)-pretreated animals. Previous studies revealed that hypoxia resulting from the ischemia to gastrointestinal tissues triggers activation of genes for HIF1α and growth factors (e.g., VEGF, EGF, HGF, IGF-1, their receptors) (48, 49). The inhibition of Hif1α expression was proposed to act as a sensitive marker of the acceleration of ulcer healing by appetite peptides ghrelin, orexin-A, leptin and obestatin (50). The role of appetite hormones in the mucosal defense of esophagus should be further investigated but adipocytokines including leptin and adiponectin and their receptors were implicated in the initial step of development of Barrett's esophagus (BE) (51). The mechanisms by which adiponectins influences the esophageal integrity remains unexplained but the high expression of adiponectin receptors in cancer tissue may promote the anti-carcinogenic effect of adiponectin (51).

Recent studies revealed that HIF1α protein co-associated with novel protein cytoglobin (Cygb) were significantly increased along with the enhanced expression of VEGF in early and late phase of ulcer healing in rats (52). Importantly, in their study (52), both Cygb and HIF1α were abundantly colocalized at the ulcer margin before angiogenesis development. In contrast, we demonstrate for

the first time, a rapid expression of HIF1α mRNA in the esophageal mucosa exposed to RE, which has been downregulated by the treatment with Ang-(1-7). Moreover, this downregulation of HIF1α expression by Ang-(1-7) was completely reversed in rats concomitantly treated with A779 revealing another mechanism involving overexpression of HIF1α, by which the antagonism of Mas receptor leads to reversal of beneficial protective effect this peptide. Interestingly, these potent anti-inflammatory effects of Ang-(1-7) were also significantly attenuated by pretreatment of animals with L-NNA and almost completely abolished by the functional ablation of sensory neurons with capsaicin. This indicates that Mas-receptor-mediated increases in NO generation are responsible, at least in part, for the observed anti-inflammatory action of Ang-(1-7) in RE induced by gastric reflux. The attenuation of Ang-(1-7)-induced anti-inflammatory response observed in rats with ablation of sensory neurons could also be attributed to the decreased generation of NO due to cessation of release of sensory peptides and elimination of anti-inflammatory response which is brought about by CGRP (9, 16, 27).

In summary, the present studies confirmed previous findings that, in the early stage of experimental acute reflux esophagitis, a significant esophageal mucosa injury develops, and the observed macroscopic and microscopic mucosal esophageal damage is accompanied by the fall in EBF and overexpression of proinflammatory markers COX-2, Hif-1α and IL-1β followed by a rise in plasma levels of proinflammatory cytokines IL-1β and TNF-α. Furthermore, we have demonstrated an esophagoprotective effect of Ang-(1-7) in experimental model of RE as reflected by this peptide dose-dependent protection against the damage of the esophageal mucosa exposed to gastric refluxate, possibly mediated by Ang-(1-7)-induced vasodilation of the esophageal microcirculation. Our present findings suggest that these protective, circulatory and anti-inflammatory effects of Ang-(1-7) in RE are mediated by Mas receptors as demonstrated by the ability of the specific Mas receptor antagonist, A779, to block completely the above-mentioned protective, anti-inflammatory, and local circulatory effects of Ang-(1-7). Moreover, we also found that Ang-(1-7) - Mas-axis activation-induced protective, circulatory, and anti-inflammatory effects in RE are, at least in part, mediated by NO and neuropeptides released from sensory afferent neurons. Thus, it is concluded that Ang-(1-7) exhibits many beneficial effects in GI-tract including protection against damage induced by reflux esophagitis. Consequently, these novel experimental findings confer Ang-(1-7) as the promising therapeutic approach for treating GERD in humans.

Acknowledgements: This study received financing from National Research Center in Poland (grant No. NN 40 2 479937) to Dr. Tomasz Brzozowski as the principal investigator.

Conflict of interests: None declared.

REFERENCES

1. Dent J, El-Serag HB, Wallander MA, Johansson S. Epidemiology of gastroesophageal reflux disease: a systemic review. *Gut* 2005; 54: 710-717.
2. El-Searag HB. Time trends of gastroesophageal reflux disease: a systemic review. *Clin Gastroenterol Hepatol* 2007; 5: 17-26.
3. Vakil N. Disease definition, clinical manifestations, epidemiology and natural history of GERD. *Best Pract Res Clin Gastroenterol* 2010; 24: 759-764.
4. Boeckxstaens GE. Review article: the pathophysiology of gastro-oesophageal reflux disease. *Aliment Pharmacol Ther* 2007; 26: 149-160.
5. Farre R. Pathophysiology of gastro-esophageal reflux disease: a role for mucosa integrity? *Neurogastroenterol Motil* 2013; 25: 783-799.
6. Orlando RC. The integrity of the esophageal mucosa. Balance between offensive and defensive mechanisms. *Best Pract Res Clin Gastroenterol* 2010; 24: 873-878.
7. Nagahama K, Yamoto M, Nishio H, Takeuchi K. Essential role of pepsin in pathogenesis of acid reflux esophagitis in rats. *Dig Dis Sci* 2006; 51: 303-309.
8. Takeuchi K, Nagahama K. Animal model of acid-reflux esophagitis: pathogenic roles of acid/pepsin, prostaglandins, and amino acids. *BioMed Res Int* 2014; 2014: 532594.
9. Konturek SJ, Zayachkivska O, Havryluk XO, *et al.* Protective influence of melatonin against acute esophageal lesions involves prostaglandins, nitric oxide and sensory nerves. *J Physiol Pharmacol* 2007; 58: 371-387.
10. Naito Y, Uchiyama K, Kuroda M, *et al.* Role of pancreatic trypsin in chronic esophagitis induced by gastroduodenal reflux in rats. *J Gastroenterol* 2006; 41: 198-208.
11. Kikendall JW, Friedman AC, Oyewole MA, *et al.* Pill-induced esophageal injury: case reports and review of medical literature. *Dig Dis Sci* 1983; 28: 174-181.
12. Kahrilas PJ, Gupta RR. The effect of cigarette smoking on salivation and esophageal acid clearance in clinical esophagitis. *J Lab Clin Med* 1989; 114: 431-438.
13. Safaie-Shirazi S, Zike WL, Brubacher M, Den Bensten L. Effect of aspirin, alcohol, and pepsin on mucosal permeability of esophageal mucosa. *Surg Forum* 1974; 25: 335-337.
14. Heller SR, Fellows IW, Ogilvie AL, Atkinson M. Non-steroidal anti-inflammatory drugs and benign oesophageal stricture. *Br Med J* 1982; 285: 167-168.
15. Lanas A, Hirschowitz BI. Significant role of aspirin use in patients with esophagitis. *J Clin Gastroenterol* 1991; 13: 622-627.
16. Pawlik M, Pajdo R, Kwiecien S, *et al.* Nitric oxide (NO)-releasing aspirin exhibits a potent esophagoprotection in experimental model of acute reflux esophagitis. Role of nitric oxide and proinflammatory cytokines. *J Physiol Pharmacol* 2011; 62: 75-86.
17. Tobey NA, Hossein SS, Argote CM, Dobrucali AM, Awayda MS, Orlando RC. Dilated intercellular spaces and shunt permeability in nonerosive acid-damaged esophageal epithelium. *Am J Gastroenterol* 2004; 9: 13-22.
18. Abdulnour-Nakhoul S, Nakhoul NL, Wheeler SA, Wang P, Swenson ER, Orlando RC. HCO_3^- secretion in the esophageal submucosal glands. *Am J Physiol* 2006; 288: G736-G744.
19. Fujiwara Y, Higuchi K, Takashima, *et al.* Roles of epidermal growth factor and Na^+/H^+ exchanger-1 in esophageal epitheial defense against acid-induced injury. *Am J Physiol* 2006; 290: G665-G673.
20. Kaunitz JD, Tanaka S, Akiba YD. Gastroduodenal microcirculatory response to luminal acid. In: Organ Microcirculation. A Gateway to Diagnostic and Therapeutic Interventions. Series: Keio University International Symposia for Life Sciences and Medicine. Ishii H, Suematsu M, Tanishita K, Suzuki H. (eds). Vol. 13. Springer, 2005, pp. 79-88.
21. Tarnawski AS, Chai J, Jones MK. Esophageal and gastrointestinal microcirculation: essential for mucosal protection, a targed for injury, and critical component of injury and ulcer healing. In: Organ Microcirculation. A Gateway to Diagnostic and Therapeutic Interventions. Series: Keito University International Symposia for Life Sciences and Medicine, Ishii H, Suemastsu M, Tanishita K, Suzuki K. (eds). Vol 13. Springer, 2005, pp. 49-61.

22. Hollwarth ME, Smith ME, Kvietys PR, Granger DN. Esophageal blood flow in the cat. Normal distribution and effects of acid perfusion. *Gastroenterology* 1986; 90: 622-28.
23. Bass BL, Schweitzer EJ, Harmon JW, Kraimer J. H^+ back diffusion interferes with intrinsic reactive regulation of esophageal mucosal blood flow. *Surgery* 1984; 96: 404-413.
24. Szentpali K, Kaszaki J, Eros G, *et al*. Changes in microcirculation of esophageal mucosa during acute experimental reflux. Pathogenic role of the biliary component. *Magy Seb* 2003; 56: 61-67.
25. Magierowski M, Jasnos K, Pawlik M, *et al.* Role of angiotensin-(1-7) in gastroprotection against stress-induced ulcerogenesis. The involvement of mas receptor, nitric oxide, prostaglandins, and sensory neuropeptides. *J Pharmacol Exp Ther* 2013; 374: 717-726.
26. Mine S, Iida T, Tabata T, Okada Y, Tanaka Y. Increased esophageal mucosal/submucosal blood flow in patients with gastroesophageal reflux disease: normalization by treatment with proton pump inhibitor. *J Gastroenterol Hepatol* 2008; 23: 303-309.
27. McKie LD, Dunkin BJ, Pennanen MF, Dunlap KW, Harmon JW, Bass BL. Esophageal blood flow: a central role for calcitonin gene related peptide. *Surgery* 1994; 116: 409-437.
28. Holzer P. Role of visceral afferent neurons in mucosal inflammation. *Curr Opin Pharmacol* 2007; 7: 563-569.
29. Bass BL, Trad KS, Harmon JW, Hakki FZ. Capsaicin sensitive nerves mediate esophageal mucosal protection. *Surgery* 1991; 110: 419-425.
30. Pawlik MW, Kwiecien S, Pajdo R, *et al.* Esophagoprotective activity of angiotensin-(1-7) in rat model of acute reflux esophagitis. Role of sensory neuropeptides and proinflammatory cytokines. *Gastroenterology* 2012: 142 (Suppl 1): 592.
31. Paul M, Poyan Mehr A, Kreutz R. Physiology of local renin-angiotensin systems. *Physiol Rev* 2006; 86: 747-803.
32. Fyhrquist F, Saijonmaa O. Renin-angiotensin system revisited. *J Intern Med* 2008; 264: 224-236.
33. Mehta PK, Griendling KK. Angiotensin II cell signaling: physiological and pathological effects in the cardiovascular system. *Am J Physiol* 2007; 292: C82-C97.
34. Casselbrant A, Edebo A, Hallersund P, *et al.* Angiotensin II receptors are expressed and functional in human esophageal mucosa. *Am J Physiol Gastrointest Liver Physiol* 2009; 297: G1019-G1027.
35. Fandriks L. The renin-angiotensin system and the gastrointestinal mucosa. *Acta Physiol* (*Oxf*) 2011; 201: 157-167.
36. Bjorkman E, Edebo A, Casselbrant A, *et al.* The renin-angiotensin system in the esophageal mucosa of healthy subjects and patients with reflux disease. *Scan J Gastroenterol* 2013; 48: 147-159.
37. Garg M, Angus PW, Burreli LM, Herath C, Gibson PR, Lubel JS. Review article: the pathophysiological roles of the renin-angiotensin system in the gastrointestinal tract. *Aliment Pharmacol Ther* 2012; 35: 414-428.
38. Santos RA, Ferreira AJ, Verano-Braga T, Bader M. Angiotensin-converting enzyme 2, angiotensin-(1-7) and Mas: new players of the renin angiotensin system. *J Endocrinol* 2013; 216: R1-R17.
39. Santos RA. Angiotensin-(1-7). *Hypertension* 2014; 63: 1138-1147.
40. Hamaguchi M, Fujiwara Y, Takashima T, *et al.* Increased expression of cytokines and adhesion molecules in rat chronic esophagitis. *Digestion* 2003; 68: 189-197.
41. Orlando RC, Peterson WG, Harnett KM, *et al.* Esophageal disease: updated information on inflammation. *Ann NY Acad Sci* 2011; 1232: 369-375.
42. Altomare A, Guarino MP, Cocca S, Emerenziani S, Cicala M. Gastroesophageal reflux disease: update on inflammation and symptom perception. *World J Gastroenterol* 2013; 19: 6523-6528.
43. Omura N, Kashiwagi H, Chen G, Suzuki Y, Yano F, Aoki T. Establishment of surgically induced chronic acid reflux esophagitis in rats. *Scand J Gastroenterol* 1999; 34: 948-953.
44. Konturek PC, Brzozowska I, Targosz A, *et al.* Esophagoprotection mediated by exogenous and endogenous melatonin in an experimental model of reflux esophagitis. *J Pineal Res* 2013; 55: 46-57.
45. Chomczynski P, Sacchi N. Single-step method of RNA isolation by acid guanidinum thiocyanate-phenol-chloroform extraction. *Anal Biochem* 1987; 162: 156-159.
46. Brzozowski T, Kwiecien S, Szlachcic A, *et al.* Involvement of renin-angiotensin system and angiotensin-(1-7) metabolite of angiotensin II in gastric mucosal injury and gastroprotection. In: Cell/Tissue Injury and Cytoprotection/Organoprotection in the Gastrointestinal Tract: Mechanisms, Prevention and Treatment. Filaretowa LP, Takeuchi K. (eds). Basel, Karger 2012, pp. 181-190.
47. Pawlik MW, Sendur R, Biernat J, *et al.* Involvement of nitric oxide and prostaglandins in the angiotensin 1-7 induced acceleration of the healing of experimental gastric ulcers. *Gastroenterology* 2010; 138 (Suppl. 1): S-131.
48. Brzozowski T, Ptak Belowska A, Kwiecien S, *et al.* Novel concept in the mechanism of injury and protection of gastric mucosa: role of renin-angiotensin system and active metabolites of angiotensin. *Curr Med Chem* 2012; 19: 55-62.
49. Tarnawski AS, Ahluwalia A. Molecular mechanisms of epithelial regeneration and neovascularization during healing of gastric and esophageal ulcers. *Curr Med Chem* 2012; 19: 16-27.
50. Szlachcic A, Majka J, Strzalka M, *et al.* Experimental healing of preexisting gastric ulcers induced by hormones controlling food intake ghrelin, orexin-A and nesfatin-1 is impaired under diabetic conditions. A key to understanding the diabetic gastropathy? *J Physiol Pharmacol*. 2013; 6: 625-637.
51. Mokrowiecka A, Sokolowska M, Luczak E, *et al.* Adiponectin and leptin receptors expression in Barrett's esophagus and normal squamous epithelium in relation to central obesity status. *J Physiol Pharmacol* 2013; 64: 193-199.
52. Tanaka F, Tominaga K, Sasaki E, *et al.* Cytoglobin may be involved in the healing process of gastric mucosal injuries in the late phase without angiogenesis. *Dig Dis Sci* 2013; 58: 1198-1206.

R e c e i v e d: May 15, 2014
A c c e p t ed: November 22, 2014

Author's address: Dr. Michal Pawlik, Department of Physiology Jagiellonian University Medical College, 16 Grzegorzecka Street, 31-531 Cracow, Poland
e-mail: mppawlik@m4-medizin.de

Prof. Tomasz Brzozowski, Chair, Department of Physiology Jagiellonian University Medical College, 16 Grzegorzecka Street, 31-531 Cracow, Poland
e-mail: mpbrzozo@cyf-kr.edu.pl