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ORIGINAL PAPER

The protective effect of apelin against water-immersion and restraint stress-induced gastric damage

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Abstract The aim of the present study was to investigate the gastroprotective effect of apelin on water-immersion and restraint stress (WIRS)-induced gastric lesions. Male Wistar rats were divided into four groups: control, WIRS, F13A + WIRS and F13A. APJ receptor antagonist F13A was administered to rats to determine the influence of apelin on stress-induced gastric injury. WIRS administered for 6 h resulted in the development of gastric mucosal lesions accompanied by a significant increase in plasma corticosterone. WIRS increased the concentration of 4-hydroxynonenol (4-HNE) + malondialdehyde (MDA) and the expression of apelin and hypoxia inducible factor-1α (HIF-1α) in gastric mucosa. In addition, WIRS reduced the mucosal blood flow and gastric prostaglandin E_2 (PGE_2) concentration. Plasma corticosterone, which was increased due to stress, was significantly decreased in the F13A + WIRS group. Gastric lesions and the 4-HNE + MDA concentration were also higher in the F13A + WIRS compared to the WIRS group. We conclude that apelin has a gastroprotective effect against stress-induced lesions possibly by reducing lipid peroxidation in gastric mucosa.

Keywords Gastric mucosal injury · Apelin · APJ antagonist · F13A · Water-immersion and restraint stress

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Introduction

Gastric stress ulceration occurs as a complication after prolonged anxiety, emotional stress, hemorrhagic surgical shock, burns, sepsis and trauma [1]. Stress-induced gastric mucosal lesions are frequently encountered in the clinical setting. The pathogenesis of stress-related acute gastric mucosal injury is multifactorial [2]. It has been demonstrated that acid back diffusion to the gastric mucosa [3], decrease in gastric mucosal blood flow [2, 4], inhibition of the production of mucosal prostaglandin [5], reduction of mucin [6], increase in oxidative stress and neutrophil activation [7, 8], suppression of the gastric mucosal cell growth and proliferation [9] are all involved in the development of stress-induced gastric mucosal injury.

Apelin has been identified as an endogenous ligand of the orphan G-protein coupled apelin receptor (APJ). Preproapelin consisting of 77 amino acids is cleaved to the C-terminal apelin-36 and to the more biologically active peptides, apelin-17 and apelin-13 [10, 11]. Apelin and APJ are highly synthesized in the gastrointestinal tract, brain, kidney, adipose tissue, lung, mammary gland and cardiovascular system [10–12]. Apelin peptides have been reported to regulate many activities, such as heart contractility and blood pressure, feeding behavior, fluid intake and secretion of insulin and cholecystokinin [11, 13]. In addition, apelin has been demonstrated to affect cell motility, proliferation and apoptosis [14].

Apelin and its receptor expression have been identified in the stomach and intestine [15]. Apelin is expressed in gastric mucous neck, parietal, chief and endocrine cells [16]. Furthermore, APJ immunostaining has been demonstrated on the surface epithelium of the rat stomach [17]. These data indicate that apelin is an important stomach peptide that potentially has a physiological role in the

gastrointestinal tract. Though apelin has been known to be involved in gastric acid secretion [16], its effects on the gastric mucosal barrier have not been previously described.

It has been demonstrated that APJ expression in the central nervous system is upregulated by stress and glucocorticoids [17, 18]. The increase in APJ expression in the hypothalamus under stressful conditions suggests that apelin has a pivotal role in the neuroendocrine response to stress. In addition, central administration of apelin increases the secretion of both plasma adrenocorticotropic hormone (ACTH) and corticosterone [17, 18]. Previous studies have reported that the effect of apelin on the neuroendocrine function of the hypothalamic–pituitary–adrenal (HPA) axis is mediated through corticotropin-releasing hormone (CRH)- and arginine vasopressin (AVP)-dependent mechanisms [17]. A role for APJ in stress is supported by studies showing increased levels of APJ mRNA expression in the parvocellular paraventricular nucleus (pPVN) in response to acute and chronic stress [19]. However, little is known about the role of apelin in the regulation of stress-induced gastric mucosal injury.

The aim of the present study was to investigate whether apelin has a gastroprotective effect on water-immersion and restraint stress (WIRS)-induced gastric lesions.

Materials and methods

Animals

A total of 64 adult male Wistar rats, weighing 250–300 g, were provided by Akdeniz University Faculty of Medicine Experimental Animals Care and Production Unit and used in this study. The rats were maintained in a temperature-controlled environment (23 ± 1 °C) and on a 12-h light/dark cycle, with free access to rat chow and tap water ad libitum. Animals were adapted for at least 7 days before the experiments. All experimental procedures were performed in accordance with mandated standards of human care and were approved by Animal Care and Use Committee of Akdeniz University (protocol no. B.30.2.AKD.0.05.07.00/20).

Experimental protocol

Following an 18-h fasting period, the animals were anesthetized with a single intraperitoneal (i.p.) injection of xylazine–ketamine (10 and 90 mg/kg, respectively, Alfasan International B.V., Woerden, Holland). The animals were divided into control ($n = 16$) and experimental ($n = 48$) groups. Rats in the experimental group were divided into 3 groups each consisting of 16 animals. In the WIRS group, the conscious rats were restrained individually in rectangular polypropylen cages (28 cm high × 8 cm wide × 8 cm depth) and immersed up to the depth of the xyphoid process in a 23 °C water bath to induce WIRS for 6 h as described in previous reports [1–4]. In the F13A group, the rats were injected with APJ receptor antagonist F13A (150 μg/kg i.v., Phoenix Pharmaceuticals, Burlingame, CA, USA, 057-29) into the tail vein. In the F13A + WIRS group, the rats were given 150 μg/kg of F13A by injection into the tail vein just before the application of WIRS. To further confirm the dose-dependent effect of F13A on WIRS-induced gastric mucosal injury formation, F13A (15, 75, or 150 μg/kg) was injected into the tail vein of rats before WIRS in a pilot study. We found an effect at F13A concentrations of 150 μg/kg and no significant effect at concentrations <150 μg/kg. After measuring gastric mucosal blood flow, a blood sample was withdrawn from the abdominal aorta and collected into chilled tubes containing ethylenediaminetetraacetic acid (EDTA, 1 mg/ml blood), centrifuged at 5,000*g* for 15 min at 4 °C. Then, the plasma was collected and stored at −80 °C until assayed. Plasma samples were used to measure corticosterone levels. Six of the stomach specimens in each group underwent immunohistochemistry, lesion index and apelin expression, while other samples were used to assay for prostaglandin E_2 (PGE_2), nitric oxide (NO) and malondialdehyde (MDA) plus 4-hydroxynonenal (4-HNE) concentration. Each of stomach specimens was confirmed to contain all of the anatomical sections of the stomach.

Determination of plasma corticosterone

Plasma corticosterone levels were determined by using a commercially available Enzyme Immuno Assay (EIA) kit designed as a competitive immunoassay for the quantitative determination of corticosterone in body fluids (Enzo Life Sciences, Plymouth Meeting, PA 19462, USA, ADI-901-097). Steroid Displacement Reagent (2.5 part), which was supplied with the kit, was added to every 97.5 parts of plasma sample containing steroid binding proteins. Samples were diluted with assay buffer and the sample dilution ratio was verified as 1:1,000 after a few dilution trials by using a corticosterone standard curve; 100 μl of diluted samples and peptide standards were transferred into the immune plate, and the assay procedure was performed according to the manufacturer's instructions. The optical density of the wells was measured by a microplate reader (Biotek, ELx800, Highland Park, VT, USA) at 405 nm, and the concentration of corticosterone was calculated using a standard curve. The results were expressed as ng/ml.

Apelin protein expression

Samples were prepared by homogenizing the tissues in 300 µl lysis buffer [10 mM Tris (pH 7.4), 100 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM NaF, 20 mM $Na_4P_2O_7$, 2 mM Na_3VO_4, 1 % Triton-X, 10 % glycerol, 0.1 % SDS and 0.5 % deoxycholate] supplemented with 5-µl protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, USA, P2714), per 100 mg of gastric tissue. The samples were centrifuged at 15,000 rpm for 10 min at 4 °C. Then, the supernatant was mixed with reagents such as NuPAGE LDS sample buffer (4×) (Invitrogen, Carlsbad, CA, USA, NP0007) and reducing agent (10×) (Invitrogen, Carlsbad, CA, USA, NP0004). The protein concentration was determined with a commercial kit (Thermo Scientific, Rockford, IL 61101, USA, NJ176939) according to Bradford et al. [20]. Prior to electrophoresis, samples were boiled for 10 min at 70 °C; 35 µg of total protein was loaded onto 10 % NuPAGE-Bis–Tris Gel (Invitrogen, Carlsbad, CA, USA, NP0316BOX) along with a molecular weight marker (Fermentas, LT-02241 Vilnius, Lithuania, SM1811) and run at 200 mV for 45 min (XCell SureLock™ Mini-Cell Electrophoresis System, Carlsbad, CA, USA, EI0001) and transferred onto a PVDF membrane for 7 min (iBlot® Dry Blotting System, Invitrogen, Carlsbad, CA, IB1001). The membrane subsequently underwent processing for Western analysis (BenchPro Card Processing Station, Invitrogen, Carlsbad, CA, 4100) that included the following steps: 30 min blocking (5 % non-fat dry milk in 10 mM PBS, pH 7.4), 10 min rinse (double distilled water), 60 min apelin primary antibody (Santa Cruz Biotechnology, CA, sc-33805, dilution 1:200 and 5 % non-fat dry milk in 10 mM PBS, pH 7.4), 20 min washing (10 mM PBS, pH 7.4), 30 min HRP-conjugated secondary antibody (Santa Cruz Biotechnology, CA, sc-2004, dilution 1:4,000 and 5 % non-fat dry milk in 10 mM PBS, pH 7.4), 20 min washing and 6 min rinse. After stripping, the same procedure was performed using GAPDH antibody (Santa Cruz Biotechnology, CA, sc-47724, dilution 1:250) in BenchPro. Lastly, the blots were visualized using a chemiluminecent detection system kit according to the manufacturer's instructions (Chemicon, Temecula, CA, USA, 2600). The membranes were exposed to hyperfilm (Amersham Biosciences, Buckinghamshire, UK, RPN3103K), which was subsequently analyzed using image J, 1.37v software [21].

Immunohistochemistry

To determine the immunohistochemical expression of hypoxia-inducible factor (HIF)-1α, 5-µm-thick sections of formalin-fixed paraffin-embedded tissues were dried overnight at 56 °C. The samples were deparaffinized in xylene at room temperature for 10 min twice, dehydrated with graded ethanols and then washed in distilled water. Antigen retrieval was performed by soaking the slides in a citrate buffer (pH 6.0, 0.01 M) and then boiling in a microwave oven at 100 °C for 7 min. Endogenous peroxidase activity was blocked by incubating the sections with 0.3 % H_2O_2 for 15 min. Then, for blocking non-specific immunoglobulin binding, the slides were incubated with blocking serum for 7 min. The sections were incubated with anti-HIF-1α mouse monoclonal primary antibody (Santa Cruz Biotechnology, CA, sc-53546) at 1:400 overnight at 4 °C. Subsequently, sections were incubated with biotinylated goat anti-mouse IgG (Vector, Burlingame, CA, USA, BA-9200) at 1:400 for 45 min. Then, the sections were overlaid with peroxidase-labeled streptavidin (HRP LSAB-2 system, K0609, DakoCytomation, Glostrup, Denmark) for 25 min, which was followed by rinsing in PBS for 15 min. Diaminobenzidine (DAB, Vector, SK-4105) as a substrate of horseradish peroxidase (HRP) was added, and sections were observed until they turned brown simultaneously. The evaluation of the immunohistochemical labeling of HIF-1α in samples was performed using H-SCORE [22]. Briefly, sections were evaluated using an Axioplan microscope (Zeiss, Oberkochen, Germany) with a special ocular scale. Three randomly selected slides, each representing five different fields at 200 magnification, were evaluated for immunochemical labeling of HIF-1α. The labeling was scored in a semiquantitative fashion that included the intensity of the specific labeling in sections. The results were recorded as the percentage of labelled cells of all types in each of four intensity categories, denoted as 0 (no labeling), 1+ (weak labeling but detectable above control), 2+ (distinct labeling) and 3+ (intense labeling). For each tissue, an H-SCORE value was derived by summing the percentage of cells that were labeled at each intensity multiplied by the weighted intensity of the labeling: H-SCORE $= \sum$ Pi $(i + 1)$, where i is the intensity score and Pi is the corresponding percentage of cells. Two observers blinded to experimental groups performed the H-SCORE evaluations, and the average score was used.

Measurement of gastric lesions and histological evaluation of gastric lesions

To determine the lesion index, the stomach was removed, incised along the greater curvature and pinned onto a platform. Photos were taken and transferred to a computer. By using 'Spot Advanced Analysis Program,' total hemorrhagic erosions were determined as the lesion index (in mm^2).

Gastric lesions were evaluated with classical routine hematoxylin and eosin (H&E) staining. After fixation of the stomach specimens with 4 % formalin for 25 h, they were embedded in paraffin wax. Five-micrometer-thick sections were obtained and stained with H&E.

Measurement of gastric mucosal blood flow

Gastric mucosal blood flow (GMBF) was assessed using laser Doppler flowmetry (LDF 100C, Model TSD 145, Biopac, Goleta, CA, USA), which is based on the principle of light reflectance from the moving red blood cells [23]. Briefly, rats were anesthetized with xylazine–ketamine, and the abdomen was opened. The stomach was incised and the gastric contents were gently evacuated. The stomach was then exposed to determine the GMBF. An optical probe immobilized by using a micromanipulator was gently placed 0.5 mm above and perpendicular to the mucosal surface in the oxyntic gland area to monitor GMBF, which was displayed in mV (value of the Doppler signal voltage) on the digital panel of the flowmeter. When GMBF was stable, four points were selected for measurement (one point for 1 min), and the average value was calculated as BPU (blood Per unit).

Nitrate/nitrite assay

Nitrate/nitrite (NOx) concentration in the stomach was determined using a colorimetric assay kit (Cayman, Ann Arbor, MI, USA, 780001). Briefly, stomach sections were weighed and homogenized in 1 ml PBS (pH 7.4) per 100 mg of tissue. Exudates were centrifuged 2,500 rpm for 10 min. The supernatant was passed through a 30-kDa filter by centrifugation at 29,000*g* for 60 min. According to the manufacturer's instructions, the filtrate was incubated with nitrate reductase and cofactor mixtures for 3 h at room temperature to convert nitrate in the sample to nitrite. The optical density of the wells was measured by microplate reader (Biotek, ELx800, Highland Park, VT, USA) at 540 nm, and the concentration of total nitrite, reflecting the NOx concentration in the sample, was calculated by using a standard nitrate curve after the reaction with Griess reagent. The results are expressed as μM per mg of protein.

Determination of PGE_2 concentrations

PGE_2 assay was performed according to the protocol supplied with the PGE_2 Enzyme Immunoassay Kit (Cayman, Ann Arbor, MI, USA, 514010). At the end of the experimental period, the stomachs were opened to the greater curvature, the mucosa was scraped with glass slides, immediately frozen in liquid nitrogen and stored at −80 °C. During analysis, the tissue scraping samples were weighed and homogenized in 1 ml homogenization buffer [0.1 M phosphate buffer (pH 7.4), containing 1 mM EDTA and 10 μM indomethacin] per 100 mg of tissue. Then, the tissue homogenates were spun at 8,000*g* for 10 min. According to the protocol, resultant supernatants were diluted with assay buffer to a proportion of 1:500; protein concentrations were determined, and PGE_2 levels were assayed in 96-well plates. The PGE_2 level in the gastric mucosa is expressed as pg per mg of tissue.

Determination of 4-HNE–MDA concentrations

To evaluate lipid peroxidation in the gastric mucosa, MDA plus 4-HNE concentration was determined according to the protocol supplied with the colorimetric assay (Oxis Research Bioxytech LPO 586, Burlingame, CA, USA, 21012). Briefly, tissue was homogenized in 10 ml ice-cold phosphate-buffered saline (PBS, 20 mM, pH 7.4) per 1 g of tissue. Prior to homogenization, 10 μl of 0.5 M butylated hydroxytoluene in acetonitrile was added to 1 ml of tissue homogenate to prevent sample oxidation during homogenization. Homogenate was centrifuged at 3,000*g* at 4 °C for 10 min. Clean supernatant was used for the assay. Samples were transferred to a colorimetric assay kit according to the manufacturer's instructions. 4-HNE plus MDA concentration was calculated by using a standard curve, and the results are expressed as nM per mg of tissue.

Statistical analysis

Data are presented as the mean ± SD. Statistical analyses were performed with SPSS version 13.0 software by using the Kruskal-Wallis and Mann-Whitney *U* test. The level of significance was accepted as $p < 0.05$.

Results

Plasma corticosterone level

The plasma corticosterone concentration in rats exposed to WIRS for 6 h was significantly higher than those in control rats without stress (Fig. 1). To investigate whether apelin played a role in regulating the release of corticosterone, the effect of i.v. administration of apelin receptor antagonist F13A was determined. F13A administered at a dose of 150 μg/kg just before the onset of WIRS reduced the increase in plasma corticosterone concentration in stressed rats. The same dose of F13A similarly given to rats without WIRS did not affect the plasma corticosterone concentration (Fig. 1).

Expression of apelin in gastric mucosa

Western blot analysis revealed that 6-h WIRS promoted gastric apelin expression (Fig. 2). There was no difference in apelin protein expression between the WIRS and F13A plus WIRS groups. F13A had no effect on the expression of gastric apelin.

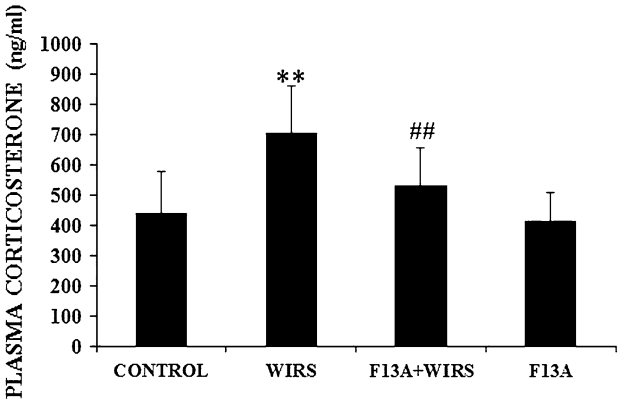

Fig. 1 Effect of F13A (APJ receptor antagonist) on plasma corticosterone levels in rats subjected to water-immersion and restraint stress (WIRS). F13A (150 μg/kg) was administered into the tail vein before the application of WIRS. After 6 h of WIRS, the plasma corticosterone level was determined as described in "Materials and methods." The results are the mean ± SD of 10 per each group. $^{**}p < 0.01$ vs. control group, $^{\#\#}p < 0.01$ vs. WIRS group

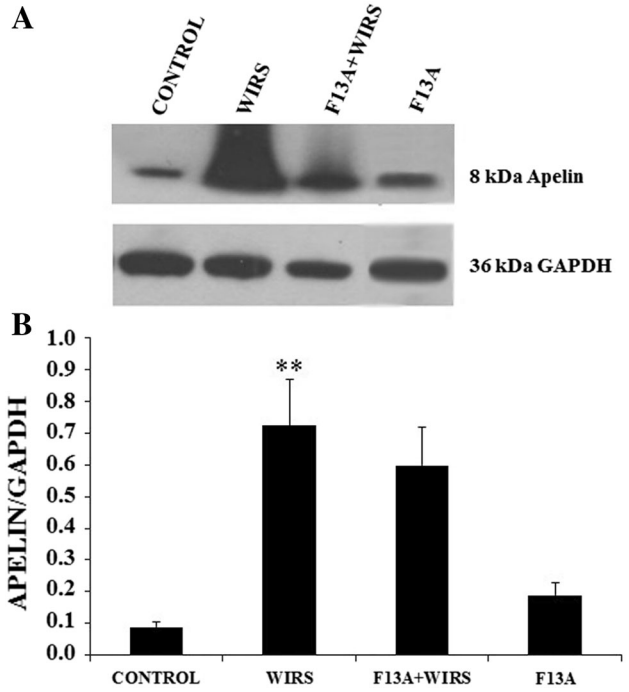

Fig. 2 Effect of F13A (APJ receptor antagonist) on apelin expression in rat gastric mucosa subjected to water-immersion and restraint stress (WIRS). F13A (150 μg/kg) was administered into the tail vein before the application of WIRS. After 6 h of WIRS, apelin expression in gastric mucosa was determined as described in "Materials and methods." The changes in apelin expression in gastric mucosa. The results are the mean ± SD of 6 per each group. $^{**}p < 0.01$ vs. control group

Expression of HIF-1α in gastric mucosa

Photomicrographs of HIF-1α staining in the gastric tissues of all groups are shown in Fig. 3. In the WIRS group, more HIF-1α-positive gastric cells were observed compared to control rats. Gastric HIF-1α expression was increased in density and intensity in the WIRS group. F13A administration (150 μg/kg) before the onset of WIRS did not change HIF-1α expression in gastric tissue. The same dose of F13A given to rats without WIRS did not affect gastric HIF-1α expression compared to control rats.

Gastric mucosal injury

WIRS for 6 h induced marked hemorrhagic lesions in the gastric mucosa (Fig. 4). F13A administered just before the onset of WIRS significantly increased gastric lesions.

Histologically, few neutrophils were observed in the gastric mucosa in the control group. The application of WIRS for 6 h induced the infiltration of neutrophils into the surface epithelium as well as necrosis in the gastric mucosa. Injection of F13A to rats exposed to WIRS increased the number of neutrophils and lesions in the gastric mucosa compared to the WIRS group (Fig. 5).

Gastric mucosal blood flow

Figure 6 shows GMBF in the WIRS model. GMBF in intact mucosa was 315 ± 50 BPU, and this value was considered as basal flow (100 %). Following 6 h of WIRS, the GMBF was reduced to 53 ± 6 % of the control value. Pre-treatment with F13A did not change GMBF in rats exposed to 6-h WIRS.

Gastric NOx content

As shown in Fig. 7, 6-h WIRS did not change the NOx content within the stomach. Further, pre-treatment with F13A of rats before the onset of WIRS for 6 h did not alter the NOx concentration in the gastric mucosa.

Gastric PGE_2 content

The mean gastric PGE_2 content in rats exposed to WIRS was significantly lower compared to the control group. However, there was no difference in gastric PGE_2 in either the F13A + WIRS or F13A group (Fig. 8).

Lipid peroxidation in gastric mucosa

The concentration of MDA plus 4-HNE in the control group was very low, near to analytical limit of detection. After 6 h of WIRS, the level of MDA plus 4-HNE nearly tripled. Administration of F13A plus WIRS resulted in a significant increase in MDA plus 4-HNE concentrations when compared with the animals that had only been exposed to 6 h of WIRS (Fig. 9).

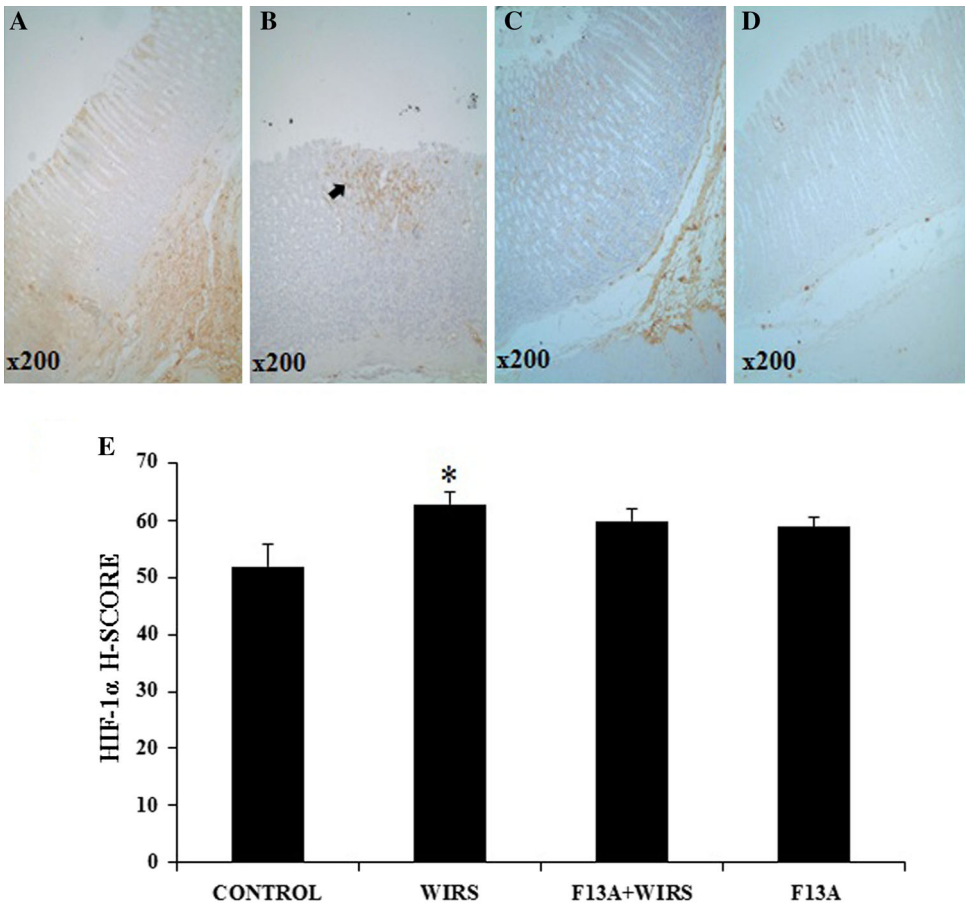

Fig. 3 Effect of F13A (APJ receptor antagonist) on the HIF1-α expression in rat gastric mucosa subjected to water-immersion and restraint stress (WIRS). F13A (150 μg/kg) was administered into the tail vein before the application of WIRS. After 6 h of WIRS, the HIF1-α expression in gastric mucosa was determined as described in "Materials and methods." **a** Shows stomach specimen of control, **b** shows stomach specimen of WIRS group, **c** shows stomach specimen of F13A + WIRS, **d** shows stomach specimen of F13A and **e** shows HIF1-α *H* scores of groups. The results are the mean ± SD of 6 per each group. $^{*}p < 0.05$ vs. control group

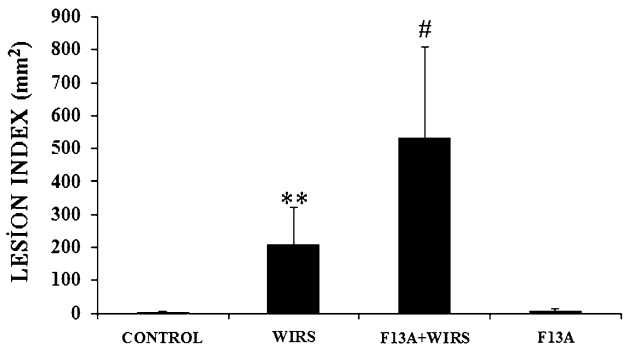

Fig. 4 Effect of F13A (APJ receptor antagonist) on the area of gastric lesions in rats subjected to water-immersion and restraint stress (WIRS). F13A (150 μg/kg) was administered into the tail vein before the application of WIRS. After 6 h of WIRS, the lesion index in gastric mucosa was determined as described in "Materials and methods." The results are the mean ± SD of 10 per each group. $^{**}p < 0.01$ vs. control group, $^{\#}p < 0.05$ vs. WIRS group

Discussion

In the present study, we demonstrated that the administration of the specific APJ receptor antagonist F13A to rats exposed to WIRS significantly augmented the lesion area by increasing lipid peroxidation in gastric tissue. These results provide strong evidence for a role of apelin in gastroprotection against gastric lesions induced by WIRS.

WIRS, a form of emotional stress, has been widely used as an experimental model for the development acute gastric mucosal lesions [24, 25]. This model mimics clinical acute gastric lesions that are observed as a result of major trauma, sepsis or surgery and is commonly used for studying the mechanism of stress-induced gastric damage [26–28]. Previous studies have shown that increasing gastric lipid peroxidation and oxidative stress play an important role in the pathogenesis of WIRS-induced gastric hemorrhage and ulceration [27, 29].

Activation of the HPA axis is one of the main characteristics of stress [30]. Glucocorticoids released during activation of the HPA axis are the major mediators of the stress response [29, 31]. In this study, we measured the levels of corticosterone as the main glucocorticoid in rats under WIRS conditions. Consistent with previous investigations [29, 31], rats exposed to 6-h WIRS showed evidence of a stress response through activation of the HPA axis, judging from the high level of plasma corticosterone. Blocking the apelin effect with an APJ receptor antagonist

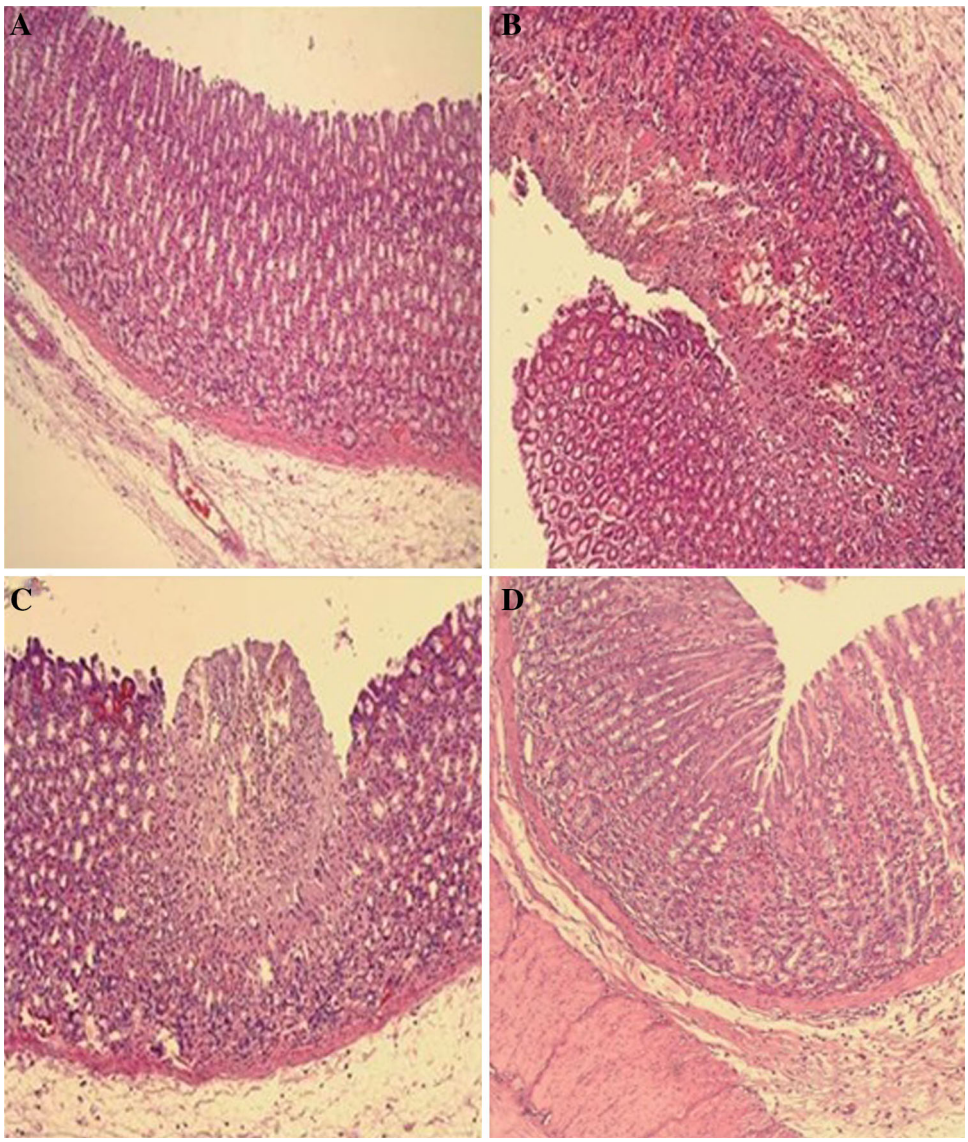

Fig. 5 Microscopic appearance of gastric lesions. The rats were subjected to water-immersion and restraint stress (WIRS). F13A (150 μg/kg) was administered into the tail vein before the application of WIRS. After 6 h of WIRS, gastric lesions were examined histologically as described in "Materials and methods." H&E staining (objective ×100). **a** Shows stomach specimen of control, **b** shows stomach specimen of WIRS group, **c** shows stomach specimen of F13A + WIRS and **d** shows stomach specimen of F13A

resulted in a reduction of the WIRS-induced corticosterone release. Our findings are consistent with other studies demonstrating that apelin promotes the release of stress hormones such as CRH [29, 31], ACTH and corticosterone [31]. Therefore, based on the present findings, it is reasonable to assume that stress-induced corticosterone release is upregulated by apelin.

Apelin is a novel gastric peptide isolated from bovine stomach extracts that is the endogenous ligand of the orphan G-protein coupled receptor APJ [32]. Apelin and APJ are widely distributed in the central nervous system (CNS) and peripheral tissues. In the CNS, apelin and APJ mRNAs have been found in the brain, cerebellum, pituitary and spinal cord [13]. In peripheral tissues, expression of the apelin/APJ system has been demonstrated in the stomach, intestine, lung, heart, kidney, adrenal gland, etc. [13, 32]. An earlier report indicated that the highest level of apelin in the gastrointestinal tract was expressed in the stomach [15]. While the regulation of apelin expression is still unknown, previous studies have shown that apelin gene expression may be controlled by oxygen levels and HIF-1α [14, 33]. HIF-1α is activated under conditions of hypoxia because of inhibition of oxygen-dependent HIF-1α prolyl-4-hydroxylase-mediated proline hydroxylation, which under normoxic conditions targets the HIF-1α subunit for ubiquitination and proteosomal degradation. Additionally, in gastric tissue, reactive oxygen species (ROS) act as signaling molecules that stimulate HIF-1α protein synthesis via activation of the PI3K/AKT and p42/p44 MAPK pathways [34]. HIF-1α regulates the expression of several hypoxia-inducible genes including erythropoietin, adrenomedullin, atrial natriuretic peptide and apelin [35–37]. It has been reported that the expression of apelin is rapid in response to hypoxia, i.e., <3 h in isolated atria and <2 h in

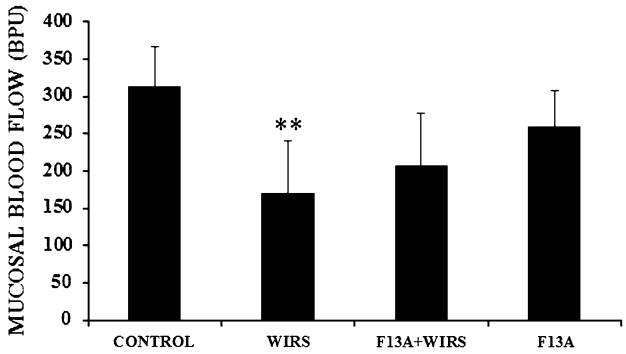

Fig. 6 Effect of F13A (APJ receptor antagonist) on gastric mucosal blood flow in rats subjected to water-immersion and restraint stress (WIRS). F13A (150 μg/kg) was administered into the tail vein before the application of WIRS. After 6 h of WIRS, gastric mucosal blood flow was determined as described in "Materials and methods." The results are the mean ± SD of 10 per each group. $^{**}p < 0.01$ vs. control group

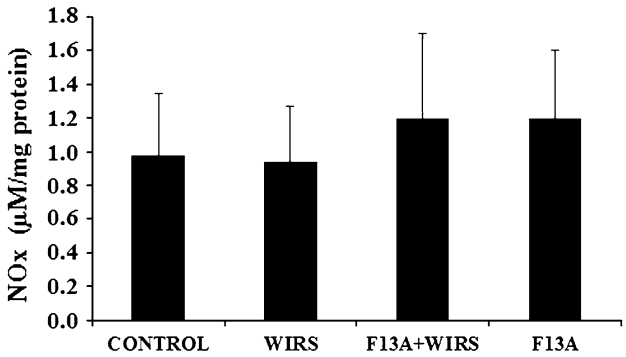

Fig. 7 Effect of F13A (APJ receptor antagonist) on gastric nitrite/nitrate (NOx) content in rats subjected to water-immersion and restraint stress (WIRS). F13A (150 μg/kg) was administered into the tail vein before the application of WIRS. After 6 h of WIRS, gastric NOx content was determined as described in "Materials and methods." The changes in gastric NOx content. The results are the mean ± SD of 10 per each group

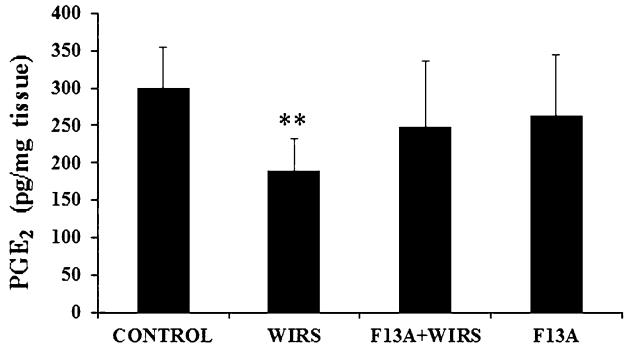

Fig. 8 Effect of F13A (APJ receptor antagonist) on gastric PGE_2 content in rats subjected to water-immersion and restraint stress (WIRS). F13A (150 μg/kg) was administered into the tail vein before the application of WIRS. After 6 h of WIRS, gastric PGE_2 content was determined as described in "Materials and methods." The results are the mean ± SD of 10 per each group. $^{**}p < 0.01$ vs. control group

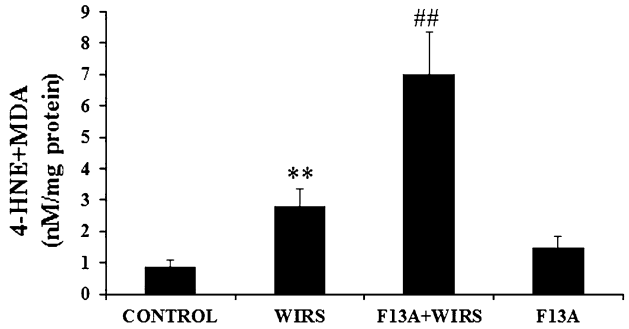

Fig. 9 Effect of F13A (APJ receptor antagonist) on gastric lipid peroxidation (4-HNE plus MDA content) in rats subjected to water-immersion and restraint stress (WIRS). F13A (150 μg/kg) was administered into the tail vein before the application of WIRS. After 6 h of WIRS, gastric 4-HNE plus MDA content was determined as described in "Materials and methods." The results are the mean ± SD of 10 per each group. $^{**}p < 0.01$ vs. control group, $^{\#\#}p < 0.01$ vs. WIRS group

cultured neonatal cardiomyocytes [38]. Our findings demonstrated for the first time that the expression of gastric apelin was regulated by WIRS. In our study, WIRS for 6 h induced the expression of apelin in the stomach. This result suggests that the increase in apelin expression in rat gastric mucosa exposed to WIRS may be due to hypoxia-induced HIF-1α induction. Based on our current knowledge, stress causes both sympathetic and parasympathetic stimulation of the stomach, thereby increasing gastric motility and muscular contraction. These changes ultimately lead to vascular compression and mucosal ischemia. Sympathetic stimulation also causes arteriolar vasoconstriction and thus reduces the blood flow to the gastric mucosa leading to local hypoxia and ischemia [26].

In the present study, we determined that exposure to WIRS for 6 h leads to the development of lesions in the glandular part of the gastric mucosa. These lesions were characterized by erythema, hemorrhages and erosion of the gastric mucosa. This result was in agreement with previous studies that demonstrated the importance of balance between aggressive and defensive factors in the gastric mucosa [39, 40]. We demonstrated that WIRS for 6 h led to the development of gastric lesions, a reduction in mucosal blood flow, a decrease in prostaglandin production and an increase in the products of lipid peroxidation in the gastric mucosa. Blocking the apelin effect with F13A at a dose of 150 μg/kg resulted in a further increase in WIRS-induced gastric injury. Injection of the same dose of F13A to rats without WIRS did not cause lesions to develop in the gastric mucosa.

The expression of apelin and APJ receptor in the paraventricular nucleus in the hypothalamus has been demonstrated previously [18]. In response to acute stress, CRH

and vasopressin expression increases in the hypothalamic paraventricular nucleus (PVN), and both hormones are released into the hypophysial portal blood. CRH acts in synergy with vasopressin to release ACTH from the anterior pituitary, and ACTH stimulates production of corticosterone by the adrenal cortex [17–19].

The present study is the first to demonstrate that apelin has a protective effect against stress-induced gastric mucosal injury. According to our findings, apelin is most likely involved in the defense mechanisms of the gastric mucosa. This gastroprotective effect could be related to the mounting effect of apelin on glucocorticoid release during stress. It has been recently demonstrated that an acute increase in corticosterone protects the stomach against stress-induced injury [30]. The gastroprotective action of glucocorticoids is due to the maintenance of gastric mucosal blood flow, mucus secretion and repair processes as well as the attenuation of harmful factors such as increased gastric motility and microvascular permeability [41]. Furthermore, glucocorticoids exert a compensatory gastroprotective role under conditions when the gastroprotective mechanisms provided by PGs, NO and capsaicin-sensitive sensory neurons are impaired [30]. However, our findings demonstrated that the gastroprotective effects of apelin were independent of gastric mucosal blood flow, PGE_2 content and NO production. Further studies are needed to further investigate whether the gastroprotective effect of apelin is related to glucocorticoids.

Our data showing the expression of apelin and the APJ receptor in the stomach suggest that the gastroprotective effect of apelin under conditions of stress might be related to local defense mechanisms in the gastric mucosa. The main components of gastric mucosal defense mechanisms are submucosal microcirculation and the release of mediators such as PGE_2 and NO. Mucosal microcirculation is essential for distributing oxygen and nutrients and for removing metabolic wastes from the tissue [28]. Functional and morphological alterations in the microcirculation may produce gastric hemorrhages, erosions and ulcers. A disturbed mucosal microcirculation is an extremely important factor in the pathogenesis of stress ulcers [39]. Physiological effects of apelin have been studied in the peripheral venous and arterial circulation, indicating that is has a powerful vasodilator effect [42]. According to Maenhaut and Van de Voorde [40], i.v. injection of apelin led to a reduction in blood pressure in rats. This effect was abolished in the presence of a nitric oxide synthase inhibitor, suggesting that apelin lowers blood pressure by increasing NO production [40, 43]. In our study, the decrease in gastric mucosal blood flow in the WIRS group was not significantly affected by administration of APJ receptor antagonist, suggesting that the gastroprotective effect of apelin is independent of mucosal blood flow under WIRS for 6 h. In addition, gastric contents of NO were not affected by either WIRS or the blocking effect of apelin with F13A. However, Nishida et al. [44] demonstrated that WIRS over a 6-h period increased the concentrations of nitrite/nitrate, the breakdown products of NO via iNOS in the gastric mucosa. In our study, WIRS for 6 h did not cause an increase in NO content in gastric samples. One explanation for this finding may be that the stomach samples used in the present study contained all layers of the stomach wall.

Prostaglandins, especially PGE_2, have cytoprotective effects on gastric mucosa. The gastroprotective effects of PGE_2 include increased epithelial mucus and bicarbonate secretion, inhibition of free radical production and enzyme release from neutrophils, amelioration of mucosal blood flow and regulation of gastric muscle activities [42, 45]. Previous studies have shown that the decrease in PGE_2 levels after 3.5 h of water-immersion and restraint stress was associated with a significant formation of lesions [46]. In agreement with these studies, 6 h of WIRS caused a decrease in gastric PGE_2 in our study. However, F13A applied before stress did not change the gastric PGE_2 level suggesting that the gastroprotective effect of apelin was independent of the production of mucosal PGE_2.

It is known that superoxide radical anion reacts with cellular membrane lipids, leading to the formation of lipid peroxides, and increases the production of MDA and 4-HNE [28, 47, 48]. Levels of MDA plus 4-HNE are useful for determining biological effects of reactive oxygen species [49]. It has been demonstrated that MDA plus 4-HNE contents of gastric mucosa are considered indicators of lipid peroxidation [50]. We reported in previous studies that lipid peroxidation was involved in the formation of stress-induced gastric mucosal damage [49, 50]. In this study, we determined the mucosal content of MDA plus 4-HNE, reflecting the relationship between gastric apelin and lipid peroxidation after WIRS in rats with or without F13A treatment. WIRS-induced gastric damage was associated with augmented ROS-induced lipid peroxidation manifested by an increase in the MDA plus 4-HNE concentration. In agreement with our results, it has been shown that apelin reduced oxidative stress and also significantly stimulated the mRNA expression and activity of antioxidant enzymes such as catalase and superoxide dismutase in cardiomyocytes [51]. Further, it has been recently shown that apelin, through its interaction with the APJ receptor, suppresses the production and release of ROS in adipocytes [52]. According to the findings of Than et al. [52], apelin promotes the expression of antioxidant enzymes via the MAPK kinase/ERK and AMPK pathways and inhibits the expression of prooxidant enzymes via the AMPK pathway. Our present study provides evidence for the first time that blocking of

the apelin effect with APJ receptor antagonist enhances the number of WIRS-induced gastric lesions and that the increase in gastric lesions is accompanied by a marked rise in the gastric MDA plus 4-HNE concentration.

Conclusion

We conclude that apelin may play an important role in the gastroprotective mechanism against stress-induced gastric damage because of the suppression of lipid peroxidation in gastric mucosa. The important findings of the current study are (1) 6-h WIRS increases gastric apelin expression; (2) this upregulation of apelin expression by WIRS may be dependent on HIF-1α expression; (3) stress-induced corticosterone release is upregulated by apelin; (4) apelin has a gastroprotective effect against stress-induced gastric damage, which may be related to the suppression of lipid peroxidation.

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Conflict of interest The authors declare that they have no conflict of interest.

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