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ROLE OF SENSORY NERVES IN GASTROPROTECTIVE EFFECT OF ANANDAMIDE IN RATS

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Previous studies have shown that stimulation of cannabinoid 1 (CB1) receptor protects the gastric mucosa against stress-induced lesion. Aim of the present study was to examine the influence of anandamide on lipid peroxidation and antioxidant defense system in gastric mucosa and the role of sensory nerves in gastroprotective effects of cannabinoids. Studies were performed on rats with intact or ablated sensory nerves (by neurotoxic doses of capsaicin). Gastric lesions were induced by water immersion and restrain stress (WRS). Anandamide was administered at the dose of 0.3, 1.5 or 3.0 μmol/kg, 30 min before exposure to WRS. CB1 receptor antagonist, AM251 (4.0 μmol/kg) was administered 40 min before WRS. WRS induced gastric lesions associated with the decrease in gastric blood flow, mucosal DNA synthesis and mucosal activity of superoxide dismutase (SOD). Serum level of interleukin-1β (IL-1β) and mucosal level of malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE) were increased. Administration of anandamide reduced the ulcers area, generation of MDA+4-HNE and serum level of IL-1β, and this effect was associated with the reduction in the WRS-induced decrease in gastric mucosal blood flow, mucosal DNA synthesis and SOD activity. Ablation of sensory nerves increased the area of ulcers, serum level of IL-1β and mucosal content of MDA+4-HNE, whereas mucosal DNA synthesis, SOD activity and blood flow were additionally decreased. In rats with ablation of sensory nerves, administration of anandamide at the high doses (1.5 and 3.0 μmol/kg) partly reduced deleterious effect of WRS on gastric mucosa, but this effect was weaker than in animals with intact sensory nerves. Low dose of anandamide (0.3 μmol/kg) was ineffective in the protection of gastric mucosa against the WRS-induced lesions in rats with ablation of sensory nerves. In rats with intact sensory nerves and exposed to WRS, administration of AM251 exhibited deleterious effect. In rats with ablation of sensory nerves and exposed to WRS, AM251 failed to affect mucosal injury in the stomach. We conclude that anandamide reduces the mucosal oxidative stress and exhibits gastroprotective effect against WRS-induced ulcers. These effects are partly mediated by sensory nerves.

Key words: *cannabinoid, anandamide, stress-induced gastric lesions, lipid peroxidation, sensory nerves, capsaicin, reactive oxygen metabolites*

INTRODUCTION

Cannabinoids are group of oxygen containing aromatic hydrocarbon compounds, which were primary discovered in marijuana and hashish, herbal drugs derived from the female plant of *Cannabis sativa* (1). Cannabinoids have been for centuries used in native medicine for treatment of wide array of health concerns. Previous studies have shown that CB1 receptor activation reduces the ischemic myocardial necrosis area (2), inhibits gastric (3, 4) and intestinal (5) secretion, and gastrointestinal transit (6, 7), protects gastric mucosa against stress-induced ulcers (8) and affects the development of acute pancreatitis (9-12). Protection of gastric mucosa by cannabinoids has been also shown in ulcers evoked by pylorus ligation, aspirin or ethanol (13-15). Recent studies in the gut have found interaction between cannabinoids and cholecystokinin in the control of feeding (16-17). Moreover, clinical studies have shown that inhibition of endocannabinoid system in the gut seems to bee involved in pathogenesis of inflammatory bowel disease (18).

Exogenous and endogenous cannabinoids produce their biological effects by specific cannabinoid 1 (CB1) and cannabinoid 2 (CB2) receptors and both receptors are members of the superfamily of G protein coupled receptors (19). CB1 receptor was isolated in 1988 (20) and cloned in 1990 (21); whereas CB2 receptor was cloned in 1993 (22). CB1 receptors are distributed widely throughout the central and peripheral nervous system, including enteric nervous system (1). CB2 receptors are located peripherally and are closely linked with in the immune tissue, predominantly the spleen and macrophages (19). On the other hand, there are papers suggesting the presence of CB2 receptors also in neuronal tissue (23). Anandamide, a main endogenous cannabinoid, is structurally related to capsaicin and may directly act on TRPV1 receptors (transient receptor potential vanilloid 1 – TRPV1) (24, 25).

Primary capsaicin-sensitive sensory nerves serve for conduction of pain information to the central nervous system, but also are able to release neuromediators from the activated peripheral endings; this process is a basis for the local „axon

reflex" (26). Sensory fibers have a special sensitivity to capsaicin (27). Capsaicin, a main pungent ingredient of chili pepper, binds to specific vanilloid (capsaicin) receptors, TRPV1 on primary sensory neurons (28, 29). TRPV1 is a nonselective cation channel and belongs to the transient receptor potential family (28, 30). Low doses of capsaicin stimulate primary sensory nerves by opening the nonselective cation channels involved in TRPV1 receptors, resulting in local release of neurotransmitters such as calcitonin gene-related peptide (CGRP) and substance P (26, 31); whereas high neurotoxic doses of capsaicin lead to ablation of sensory nerves with decrease in plasma and tissue level of CGRP (32). Gastric mucosa is densely innervated by capsaicin-sensitive nerves. Sensory nerves and CGRP are involved in different aspects of the stomach pathology. The stimulation of sensory fibers, as well as, administration of exogenous CGRP was found to exert a protective effect in different experimental models of gastric ulcers (33, 34); whereas the ablation of sensory nerves aggravates gastric mucosal lesions (35) and prolongs gastric ulcer healing (36).

The aim of our present study was to examine the influence of anandamide on lipid peroxidation and antioxidant defense system in gastric mucosa, as well as to determine the role of sensory nerves in gastroprotective effects of cannabinoids.

MATERIALS AND METHODS

Studies were performed on male Wistar rats weighing 220-250 g and were conducted following the experimental protocol approved by the Local Commission of Ethics for the Care and Use of Laboratory Animals. Animals were housed in cages with wire mesh bottoms, in normal room temperature (22±1°C) and with a 12-h light-dark cycle. Rats were fasted for 24 hours prior to experiment with unlimited access to water.

Sensory nerve ablation was induced by capsaicin (Fluka, Buchs, Switzerland) injected s.c. at the total dose of 100 mg/kg over 3 consecutive days as described previously (37). Recovery period of 10 days was allowed before the final experiments. To assess the effectiveness of sensory nerves ablation, one day before the study experiment, a drop of 0.33 mM solution of capsaicin was installed into the eye of each rat and the presence of wiping movements was examined. All animals pretreated with capsaicin showed negative wiping movement test, thus confirming functional deactivation of capsaicin sensitive nerves.

Gastric ulcers were induced by rat immobilization in individual cage and immersion in water at 23°C for 3.5 hours to the level of xiphoid process (water immersion and restraint stress - WRS) as described previously by Takagi *et al*. (38).

Our study was carried out on the following experimental groups: [1] control rats treated intraperitoneally (i.p.) with vehicle without exposure to WRS; [2] vehicle-treated rats exposed to WRS; [3-5] rats pretreated with anandamide (ALEXIS®Biochemicals, San Diego, CA, USA) at the dose of 0.3, 1.5 or 3.0 μmol/kg (104, 521 or 1024 μg/kg) i.p. and exposed to WRS; [6] rats with capsaicin-induced ablation of sensory nerves, pretreated with vehicle i.p. and exposed to WRS; [7-9] rats with capsaicin-induced ablation of sensory nerves, pretreated with anandamide at the dose of 0.3, 1.5 or 3.0 μmol/kg i.p. and exposed to WRS. [10] rats treated with CB1 antagonist, AM251 (ALEXIS®Biochemicals, San Diego, CA, USA) at the dose 4.0 μmol/kg (2.22 mg/kg) and exposed to WRS; [11] rats treated with combination of AM251 (4.0 μmol/kg, i.p.) plus anandamide (0.3 μmol/kg, i.p.) and exposed to WRS; [12] rats with capsaicin-induced ablation of sensory nerves, pretreated with AM251 (4.0 μmol/kg, i.p.) and exposed to WRS; [13] rats with capsaicin-induced ablation of sensory nerves, pretreated with combination of AM251 (4.0 μmol/kg, i.p.) and anandamide (0.3 μmol/kg, i.p.), and exposed to WRS. Experiments were repeated to obtain ten observations in each experimental group.

Anandamide and AM251 were primary dissolved in 100% ethanol (stock solution). Immediately, before administration, stock solution was diluted in saline to a final concentration of ethanol, 9%.

Anandamide or vehicle (9% solution of ethanol in saline) were administered i.p. 30 min before exposure to WRS.

AM251 was administered 40 min before WRS. Immediately after 3.5 of WRS, animals were anesthetized with ketamine (50 mg/kg i.p., Bioketan, Vetoquinol Biowet, Gorzow Wielkopolski, Poland). Abdominal cavity was opened and gastric mucosal blood flow was measured by a laser Doppler flowmeter using PeriFlux 4001 Master monitor (Perimed AB, Jarfalla, Sweden), as described previously (39). Data were presented as percent of control value obtained in vehicle-treated control rats without exposure to WRS.

After the measurement of gastric mucosal blood flow, blood was taken from the inferior caval vein and serum was collected and frozen at -60°C. Serum concentration of IL-1β was measured using the commercial BioSource Cytoscreen rat IL-1β kit (BioSource International, Camarillo, California, USA) based on ELISA.

The area of lesions of the oxyntic mucosa were measured, using computerized planimeter (Morphomat, Carl Zeiss, Berlin, Germany) as described previously (40). The stress-induced lesion was defined as a round or linear mucosal black or red defect of at least 0.1 mm in diameter.

Biopsy samples of gastric mucosa were taken for determination of mucosal DNA synthesis, concentration of malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE) and mucosal activity of superoxide dismutase (SOD).

The rate of DNA synthesis, an index of tissue damage and ability to the regeneration, was determined by measurement of labeled thymidine incorporation into DNA. Gastric mucosa scraped from the oxyntic gland area was incubated at 37°C for 45 min in 2 ml of medium containing 8 μCi/ml of [^{3}H]thymidine ([6-^{3}H]thymidine, 20-30 Ci/mmol; Institute for Research, Production and Application of Radioisotopes, Prague, Czech Republic), as described in details previously (41, 42). The rate DNA synthesis was expressed as [^{3}H]thymidine disintegrations per minute per microgram DNA (dpm/μg DNA).

Mucosal concentration of MDA and 4-HNE, an indicator of lipid peroxidation, was measured, using commercial kit Bioxytech ®LPO-586™ (OxisResearch™, OXIS Health Products, Inc., Portland, OR, USA), as described previously (43, 44). Prior to homogenization of tissue samples, 10 μl 0.5 M butylated hydroxytoluene in acetonitrate was added to prevent sample oxidation during homogenization. Mucosa was homogenized in ice-cold Tris buffer (20 mM, pH 7.4), centrifuged (3000 g at 4°C for 10 min) and supernatant was used for the assay. The Bioxytech ®LPO-586™ is a colorometric assay based on the reaction of a chromogenic reagent, N-methyl-2-phenylindole, with MDA and HAE at 45°C. One molecule of either MDA or 4-HNE) may react with 2 molecules of N-methyl-2-phenylindole to yield a stable chromophore with maximal absorbance at 586 nm. Results were expressed in nmol per g of gastric mucosa.

To determine mucosal activity of SOD, tissue was homogenized in 20 mM HEPES buffer, pH 7.2, containing 1 mM EGTA, 210 mM mannitol, and sucrose. Homogenate was centrifuged at 1500 g for five min at 4°C. Activity of SOD in the supernatant was measured using superoxide dismutase assay kit (Cayman Chemical Company, Ann Arbor, MI, USA). Results have been expressed in units per g of gastric mucosa.

Statistical analysis was carried out by one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test

using GraphPadPrism (GraphPad Software, San Diego, CA, USA). Results are expressed as mean ±S.E.M. Differences were considered to be statistically significant when *P* was less than 0.05.

RESULTS

Treatment with vehicle in rats without exposure to WRS did not induce any gastric mucosal damage (*Fig. 1A*). Exposure to WRS resulted in appearance of multiple erosions of gastric mucosa. The mean lesion area in vehicle-treated rats reached 7.5±0.6 mm^2. All doses of anandamide given alone significantly reduced the ulcer area in rats with intact sensory nerves; whereas ablation of sensory nerves by capsaicin significantly increased gastric mucosal damage induced by WRS (*Fig. 1A*) Ablation of sensory nerves reduced the protective effect of anandamid, but the ulcers area was still significantly smaller in rats treated with anandamide at the dose of 1.5 or 3.0 μmol/kg, than that in vehicle-treated rats with ablation of sensory nerves. The lowest dose of anandamide, 0.3 μmol/kg was ineffective in the protection of gastric mucosa against WRS in rats with ablation of sensory nerves (*Fig. 1A*).

Administration of AM251, a selective antagonist of CB1 receptors, significantly increased gastric ulcer area by 32% in rats with intact sensory nerves and abolished protective effect of anandamide given at the dose of 0.3 μmol/kg (*Fig. 1B*). On the other hand, administration of AM251 failed to affect gastric ulcer area in rats with ablation of sensory nerves.

Exposure to WRS reduced gastric mucosal blood flow by about 51% in vehicle-treated rats with intact sensory nerves (*Fig.*

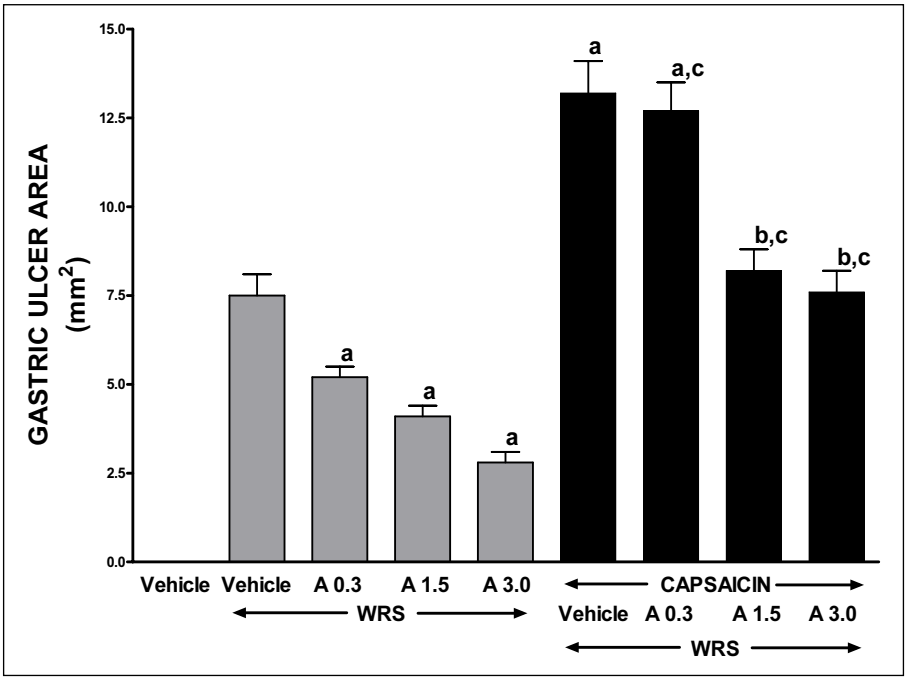

Fig. 1A. Influence of treatment with anandamide given at the dose of 0.3, 1.5 or 3.0 μmol/kg (A0.3, A1.5 or A3.0) and ablation of sensory nerves by capsaicin (capsaicin) on the area of water immersion and restrain stress (WRS)-induced gastric lesions. Mean ±S.E.M. *N*=10 rats in each experimental group. [a]*P*<0.05 compared to vehicle + WRS; [b]*P*<0.05 compared to capsaicin+WRS; [c]*P*<0.05 compared to WRS+anandamide given at the same dose in rats with intact sensory nerves.

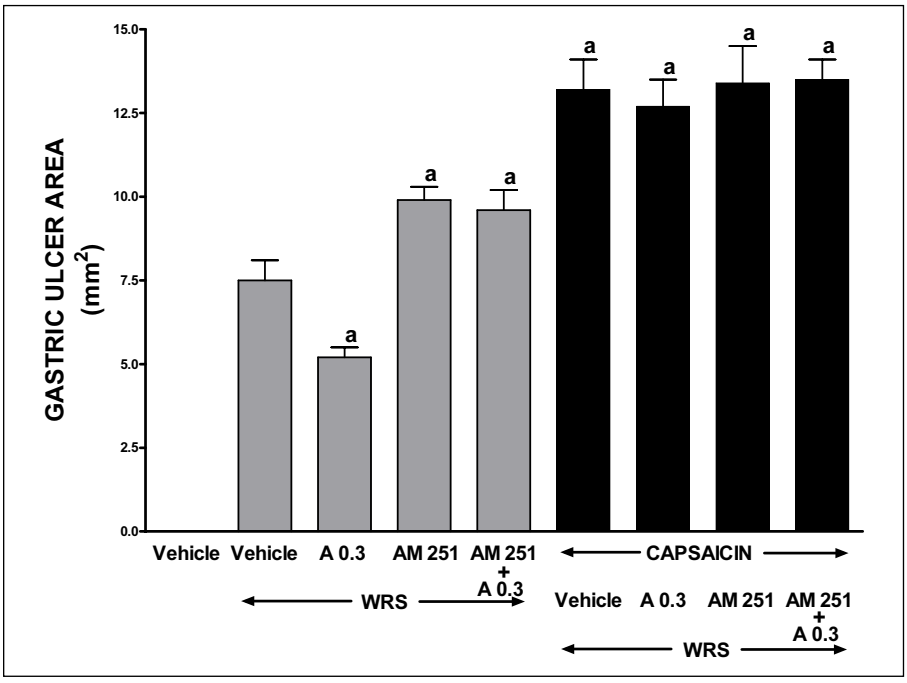

Fig. 1B. Influence of treatment with anandamide given at the dose of 0.3 μmol/kg (A0.3), AM251 given at the dose of 4.0 μmol/kg and ablation of sensory nerves by capsaicin (capsaicin) on the area of water immersion and restrain stress (WRS)-induced gastric lesions. Mean ±S.E.M. *N*=10 rats in each experimental group. [a]*P*<0.05 compared to vehicle+WRS.

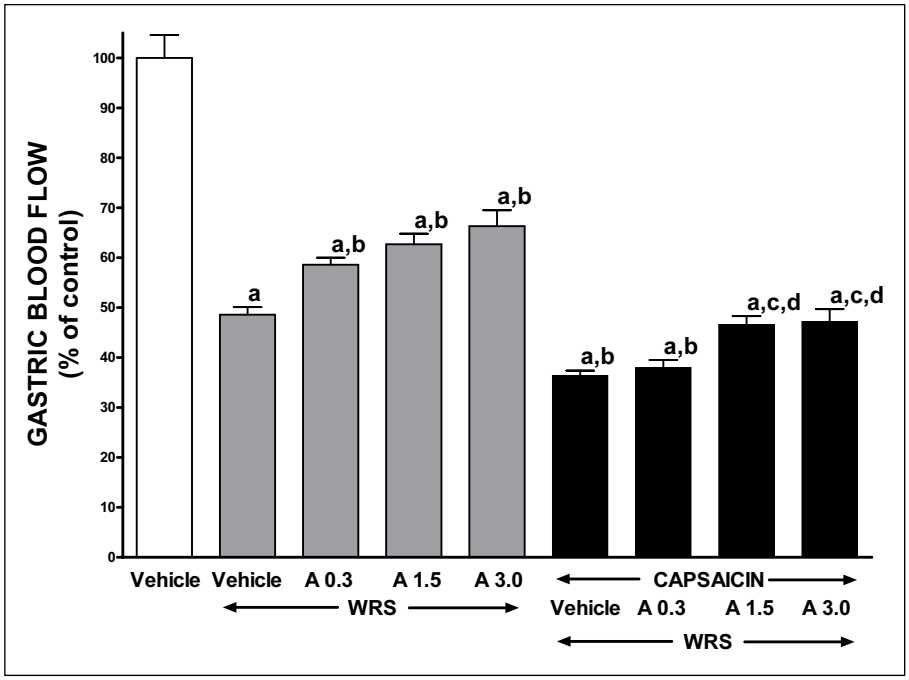

Fig. 2A. Influence of treatment with anandamide given at the dose of 0.3, 1.5 or 3.0 μmol/kg (A0.3, A1.5 or A3.0), ablation of sensory nerves by capsaicin (capsaicin) and water immersion and restrain stress (WRS) on gastric mucosal blood flow. Mean ±S.E.M. *N*=10 rats in each experimental group. $^{a}P<0.05$ compared to vehicle-treated control without exposure to WRS; $^{b}P<0.05$ compared to vehicle+WRS; $^{c}P<0.05$ compared to capsaicin+WRS; $^{d}P<0.05$ compared to WRS+anandamide given at the same dose in rats with intact sensory nerves.

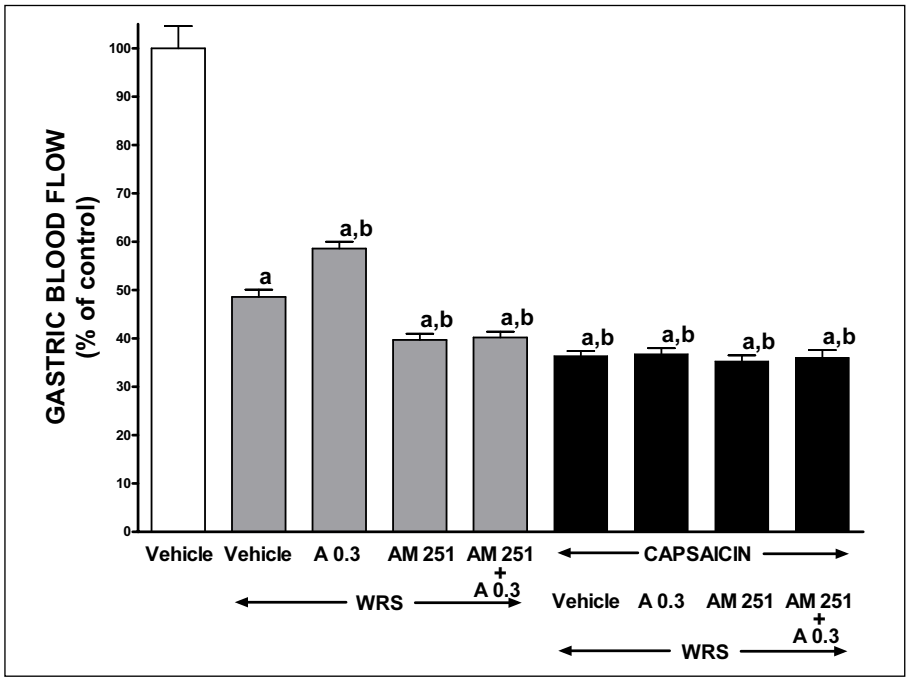

Fig. 2B. Influence of treatment with anandamide given at the dose of 0.3 μmol/kg (A0.3), AM251 given at the dose of 4.0 μmol/kg, ablation of sensory nerves by capsaicin (capsaicin) and water immersion and restrain stress (WRS) on gastric mucosal blood flow. Mean ±S.E.M. N=10 rats in each experimental group. $^{a}P<0.05$ compared to vehicle-treated control without exposure to WRS; $^{b}P<0.05$ compared to vehicle+WRS.

2A). Pretreatment with anandamide alone partly, but significantly reversed this effect. Ablation of sensory nerves prior to WRS, additionally and significantly reduced gastric mucosal blood flow. In rats with ablation of sensory nerves and exposed to WRS, treatment with low doses of anandamide (0.3 μmol/kg) was without effect on gastric mucosal blood flow; whereas higher doses of anandamide partly and significantly reversed the WRS-evoked decrease in gastric blood flow, but this effect was markedly lower than in rats with intact sensory nerves (*Fig. 2A*).

Pretreatment with AM251 caused additional and significant reduction in gastric mucosal blood flow in rats with intact sensory nerves and exposed to WRS, as well as abolished the effect of anandamide administration on mucosal blood flow in these rats. Contrary to that, AM251 was without effect on gastric mucosal blood flow in rats with ablation of sensory nerves (*Fig. 2B*).

In vehicle-treated control rats without exposure to WRS, gastric mucosal DNA synthesis reached a value of 44.7±2.1 dpm/μg DNA (*Fig. 3A*). Exposure to WRS reduced gastric mucosal DNA synthesis by 47% in vehicle-treated rats. Pretreatment with anandamide at all doses used, partly but significantly and dose dependently reversed the WRS-induced decrease in gastric mucosal DNA synthesis. In vehicle-treated rats exposed to WRS, ablation of sensory nerves significantly reduce gastric mucosal DNA synthesis. In these rats, pretreatment with anandamide given at the dose of 0.3 μmol/kg was without effect on mucosal DNA synthesis. Higher doses of anandamide partly reversed the WRS-evoked reduction in gastric DNA in rats with ablation of sensory nerves, but this effect was significantly weaker than in rats with intact sensory nerves (*Fig. 3A*).

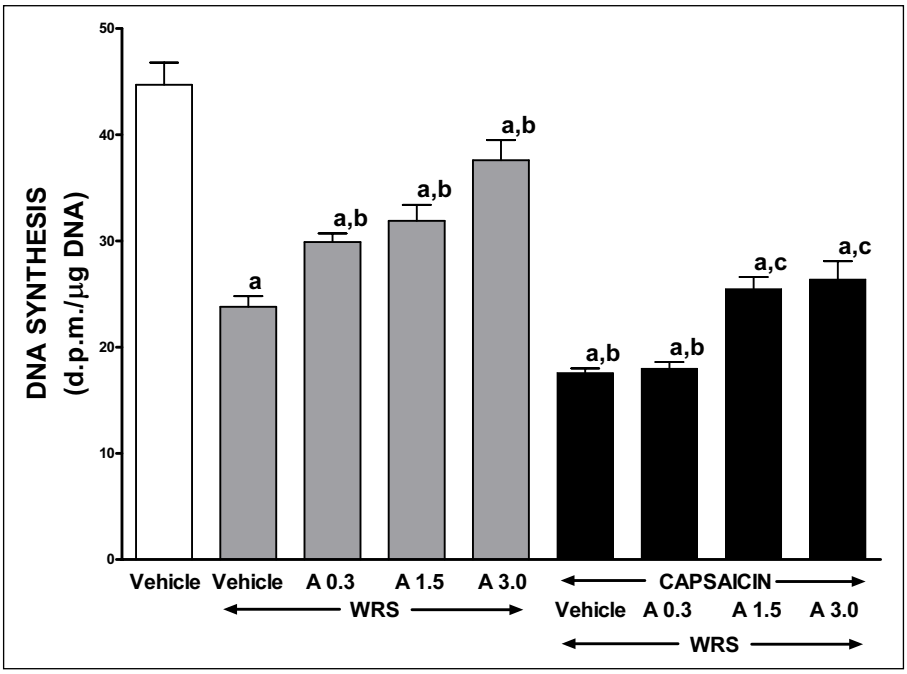

Fig. 3A. Influence of treatment with anandamide given at the dose of 0.3, 1.5 or 3.0 μmol/kg (A0.3, A1.5 or A3.0), ablation of sensory nerves by capsaicin (capsaicin) and water immersion and restrain stress (WRS) on gastric mucosal DNA synthesis. Mean ±S.E.M. N=10 rats in each experimental group. $^{a}P<0.05$ compared to vehicle-treated control without exposure to WRS; $^{b}P<0.05$ compared to vehicle+WRS; $^{c}P<0.05$ compared to capsaicin+WRS.

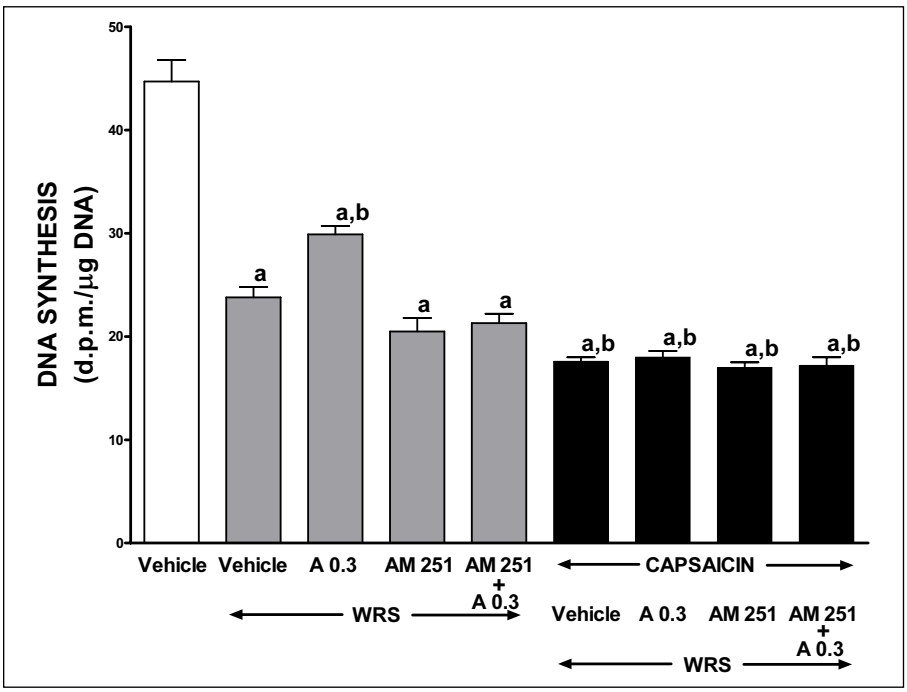

Fig. 3B. Influence of treatment with anandamide given at the dose of 0.3 μmol/kg (A0.3), AM251 given at the dose of 4.0 μmol/kg, ablation of sensory nerves by capsaicin (capsaicin) and water immersion and restrain stress (WRS) on gastric mucosal DNA synthesis. Mean ±S.E.M. *N*=10 rats in each experimental group. $^{a}P<0.05$ compared to vehicle-treated control without exposure to WRS; $^{b}P<0.05$ compared to vehicle+WRS.

In sensory nerves intact rats exposed to WRS, pretreatment with AM251 reduced gastric mucosal DNA synthesis and abolished effect of anandamide administration on this parameter (*Fig. 3B*). In rats with ablation of sensory nerves, neither AM251 nor anandamide given at the dose of 0.3 μmol/kg significantly affected gastric mucosal DNA synthesis (*Fig. 3B*).

In vehicle-treated control rats without exposure to WRS, serum concentration of pro-inflammatory IL-1β reached a value of 42.5±2.1 pg/ml (*Fig. 4A*). Exposure to WRS led to twofold increase in serum concentration of IL-1β. Pretreatment with anandamide significantly and dose-dependently attenuated the WRS-evoked increase in serum concentration of IL-1β in rats with intact sensory nerves. This effect reached statistical significance after each used doses of anandamide (*Fig. 4A*). Ablation of sensory nerves by capsaicin additionally and significantly enhanced the WRS-induced increase in serum level of IL-1β. Pretreatment with anandamide was without significant effect on serum level of IL-1β in rats with ablation of sensory nerves and exposed to WRS (*Fig. 4A*).

In rats with intact sensory nerves and exposed to WRS, pretreatment with AM251 additionally and significantly increased serum concentration of pro-inflammatory IL-1β and abolished inhibitory effect of anandamide on this parameter (*Fig. 4B*). In rats with ablation of sensory nerves and exposure to WRS, anandamide and AM251 given alone or in their combination failed to affect significantly serum concentration of IL-1β (*Fig. 4B*).

In vehicle-treated control rats without exposure to WRS, total mucosal concentration of MDA and 4-HNE reached a value of 4.1±0.3 nmol/g of wet weight of gastric mucosa (*Fig.*

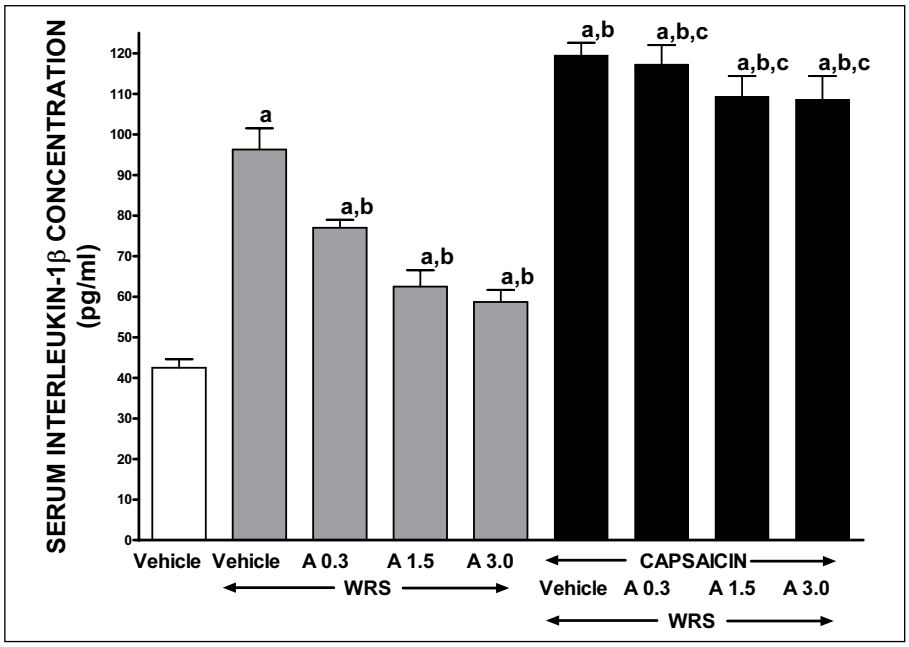

Fig. 4A. Influence of treatment with anandamide given at the dose of 0.3, 1.5 or 3.0 μmol/kg (A0.3, A1.5 or A3.0), ablation of sensory nerves by capsaicin (capsaicin) and water immersion and restrain stress (WRS) on serum concentration of interleukin-1β. Mean ±S.E.M. N=10 rats in each experimental group. ^{a}P<0.05 compared to vehicle-treated control without exposure to WRS; ^{b}P<0.05 compared to vehicle+WRS; ^{c}P<0.05 compared to WRS+anandamide given at the same dose in rats with intact sensory nerves.

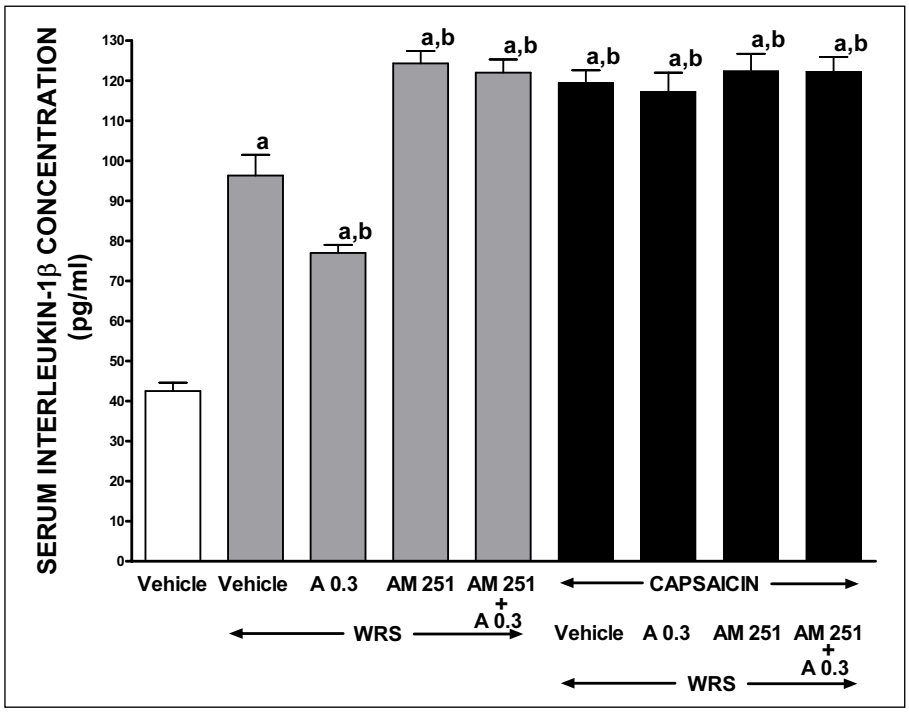

Fig. 4B. Influence of treatment with anandamide given at the dose of 0.3 μmol/kg (A0.3), AM251 given at the dose of 4.0 μmol/kg, ablation of sensory nerves by capsaicin (capsaicin) and water immersion and restrain stress (WRS) on serum concentration of interleukin-1β. Mean ±S.E.M. N=10 rats in each experimental group. ^{a}P<0.05 compared to vehicle-treated control without exposure to WRS; ^{b}P<0.05 compared to vehicle+WRS.

5A). Exposure to WRS caused fourfold increase in gastric mucosal concentration of MDA and 4-HNE. Treatment with anandamide significantly reduced the WRS-induced increase in gastric mucosal concentration of MDA and 4-HNE in rats with intact sensory nerves. This effect was dose-dependent and statistically significant after each used dose of anandamide. In rats exposed to WRS, ablation of sensory nerves tended to increase gastric mucosal concentration of MDA and 4-HNE, but this effect was statistically insignificant. On the other hand, ablation of sensory nerves abolished the anandamide-evoked decrease in gastric concentration of MDA and 4-HNE in rat exposed to WRS (*Fig. 5A*).

In rats with intact sensory nerves and exposed to WRS, pretreatment with AM251 increased mucosal concentration of MDA and 4-HNE, but this effect was insignificant (*Fig. 5B*). On the other hand, AM251 abolished the effect of anandamide administration on mucosal concentration of MDA and 4-HNE in these rats. In rats with ablation of sensory nerves and exposure to WRS, neither AM251 nor anandamide affected significantly mucosal concentration of MDA and 4-HNE (*Fig. 5B*).

In saline-treated control rats with intact sensory nerves and without exposure to WRS, gastric activity of SOD reached a value of 311±12 U/g (*Fig. 6A*). In rats with intact sensory nerves, exposure to WRS led to decrease in gastric activity of SOD by 38%. Pretreatment with anandamide significantly attenuated the WRS-evoked decrease in gastric activity of SOD in rats with intact sensory nerves. In rats exposed to WRS, ablation of sensory nerves by capsaicin tended to additional reduction in gastric activity of SOD, but this effect was statistically

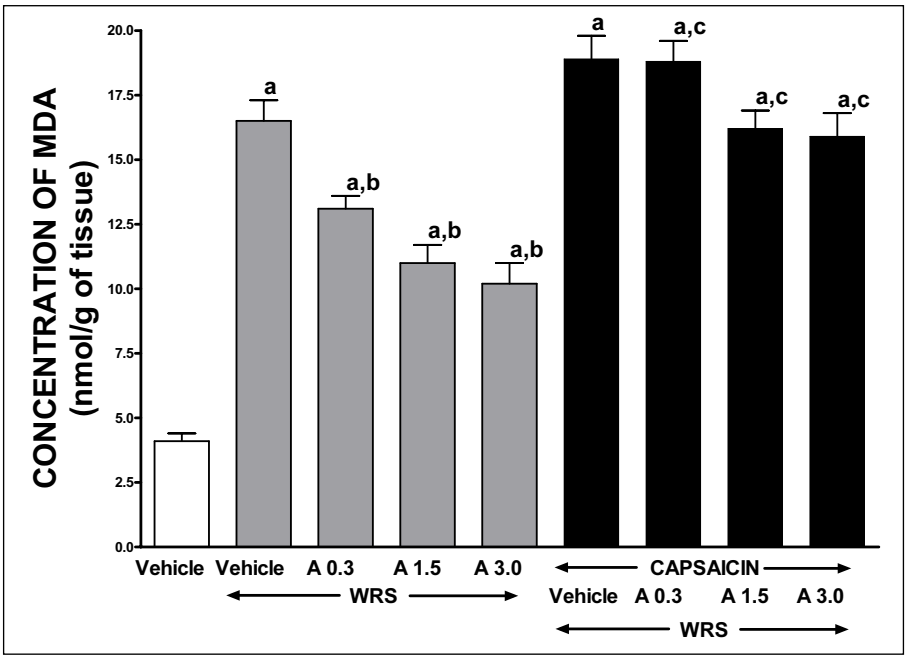

Fig. 5A. Influence of treatment with anandamide given at the dose of 0.3, 1.5 or 3.0 μmol/kg (A0.3, A1.5 or A3.0), ablation of sensory nerves by capsaicin (capsaicin) and water immersion and restrain stress (WRS) on gastric mucosal concentration of MDA and 4-HNE. Mean ±S.E.M. *N*=10 rats in each experimental group. $^{a}P<0.05$ compared to vehicle-treated control without exposure to WRS; $^{b}P<0.05$ compared to vehicle+WRS; $^{c}P<0.05$ compared to WRS+ anandamide given at the same dose in rats with intact sensory nerves.

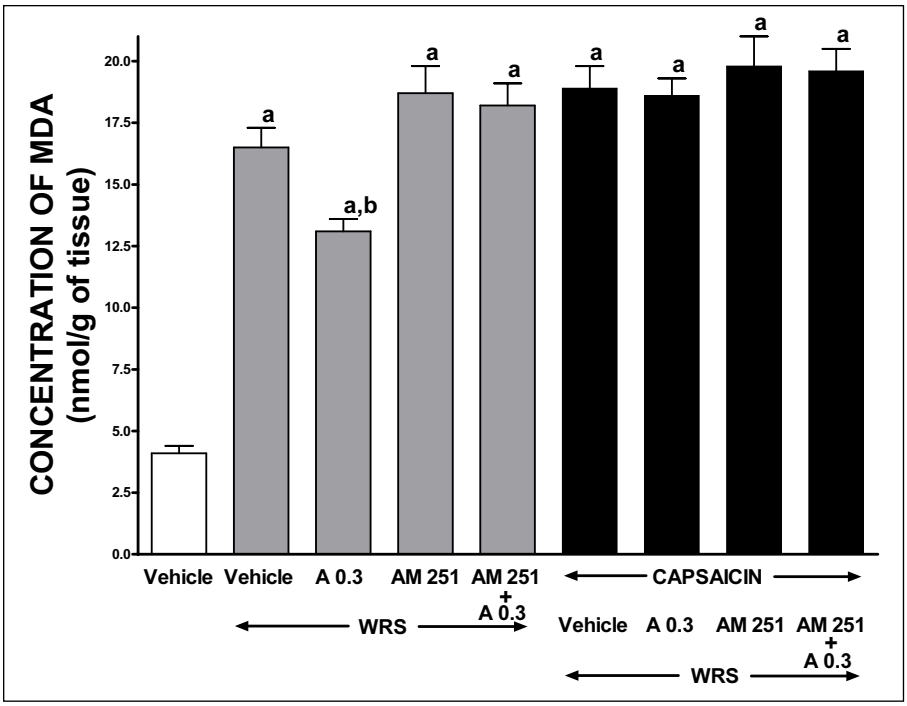

Fig. 5B. Influence of treatment with anandamide given at the dose of 0.3 μmol/kg (A0.3), AM251 given at the dose of 4.0 μmol/kg, ablation of sensory nerves by capsaicin (capsaicin) and water immersion and restrain stress (WRS) on gastric mucosal concentration of MDA and 4-HNE. Mean ±S.E.M. N=10 rats in each experimental group. $^{a}P<0.05$ compared to vehicle-treated control without exposure to WRS; $^{b}P<0.05$ compared to vehicle+WRS.

insignificant. On the other hand, in rats exposed to WRS, ablation of sensory nerves made the influence of anandamide on gastric activity of SOD statistically insignificant. Activity activity of SOD in gastric mucosa of rats with ablation of sensory nerves and treated with anandamide was significantly lower than in rats with intact sensory nerves and treated with anandamide (*Fig. 6A*).

In rats with intact sensory nerves and exposed to WRS, administration of AM251 alone was without significant influence on gastric mucosa activity of SOD, but abolished the influence of anandamide on this parameter (*Fig. 6B*). Neither anandamide nor AM251 given alone nor in their combination significantly affected mucosal activity of SOD in the stomach of rats with ablation of sensory nerves and exposed to WRS (*Fig. 6B*).

DISCUSSION

Previous studies have shown that peripheral pretreatment with cannabinoids decreases formation of experimental gastric ulcers evoked by stress, pylorus ligation, aspirin or ethanol (10, 13-15, 45). Moreover, Shujaa *et al*. (15) have found that cannabinoids given centrally (intracerebroventricularly) result in gastroprotective effect. Our present study confirms and extends these observations. According to our previous study, protective effect of anandamide has involved an improvement of gastric mucosal blood flow and mucosal DNA synthesis, as well as a reduction in serum level of pro-inflammatory IL-1β.

Exposure to WRS has reduced gastric mucosal blood flow; whereas pretreatment with anandamide has partly reversed the WRS-evoked fall in mucosal blood flow. Gastric blood flow

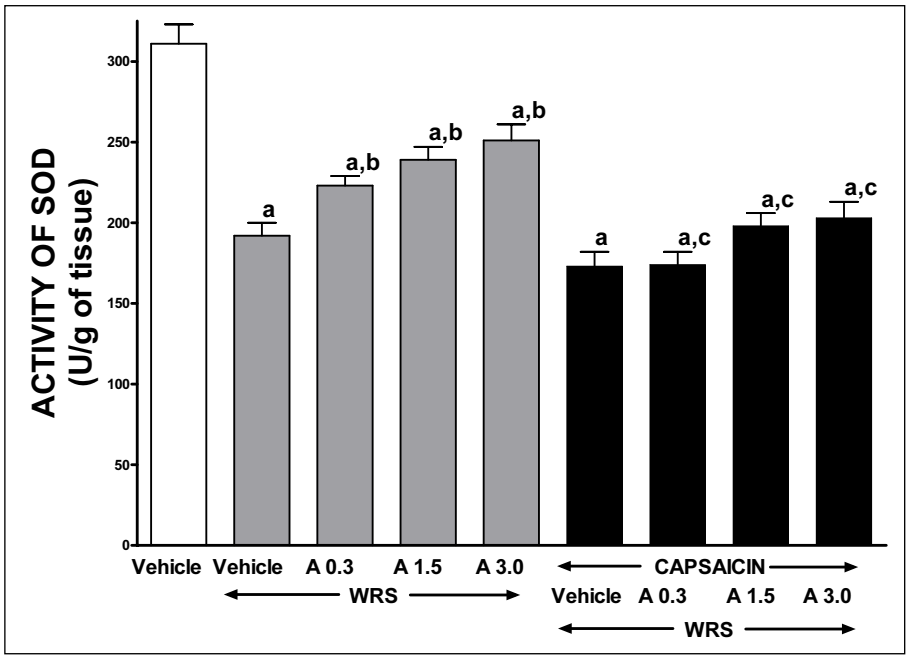

Fig. 6A. Influence of treatment with anandamide given at the dose of 0.3, 1.5 or 3.0 μmol/kg (A0.3, A1.5 or A3.0), ablation of sensory nerves by capsaicin (capsaicin) and water immersion and restrain stress (WRS) on gastric mucosal activity of superoxide dismutase (SOD). Mean±S.E.M. N=10 rats in each experimental group. $^{a}P<0.05$ compared to vehicle-treated control without exposure to WRS; $^{b}P<0.05$ compared to vehicle+WRS; $^{c}P<0.05$ compared to WRS+anandamide given at the same dose in rats with intact sensory nerves.

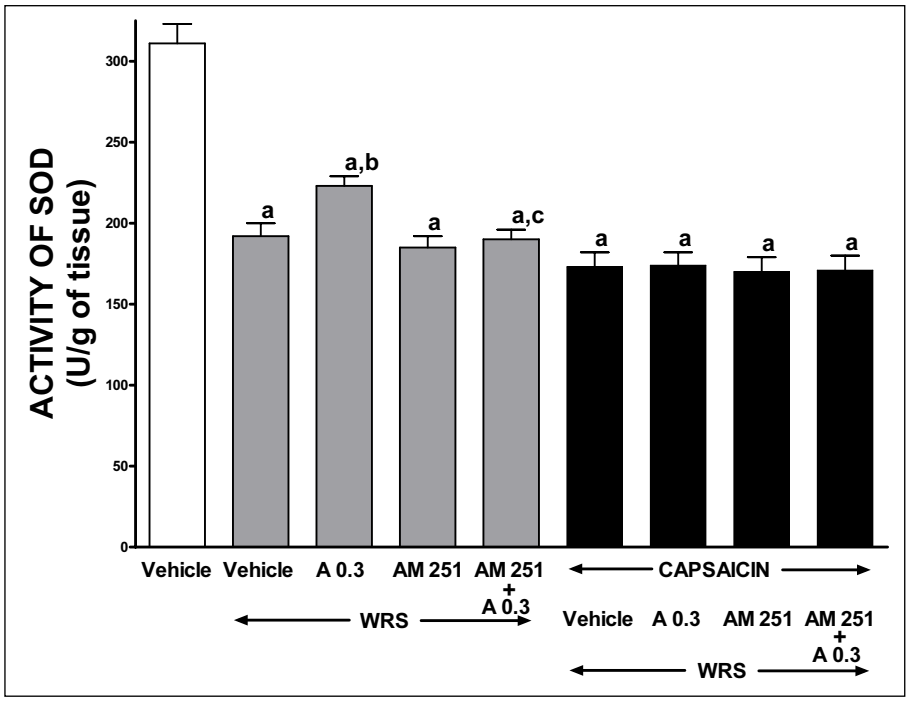

Fig. 6B. Influence of treatment with anandamide given at the dose of 0.3 μmol/kg (A0.3), AM251 given at the dose of 4.0 μmol/kg, ablation of sensory nerves by capsaicin (capsaicin) and water immersion and restrain stress (WRS) on gastric mucosal activity of superoxide dismutase (SOD). N=10 rats in each experimental group. $^{a}P<0.05$ compared to vehicle-treated control without exposure to WRS; $^{b}P<0.05$ compared to vehicle+WRS; $^{c}P<0.05$ compared to WRS+ anandamide.

plays an important role in the protection and healing of gastric mucosa (46). Blood flow supplies the mucosa with oxygen and bicarbonates, and removes hydrogen ions, carbon dioxide and toxic agents diffusing from the gastric lumen. Mucosal ischemia has been found to contribute to gastric ulceration in various clinical and experimental conditions; whereas hyperemia protects against mucosal damage (40, 46).

The rate of DNA synthesis is an index of tissue damage and ability to regeneration. Several reports have demonstrated that after exposure to stress, the gastric mucosa exhibits a decrease in cell proliferation and DNA synthesis (47, 48). The same effect has been observed in our present study. Additionally, we have found that pretreatment with anandamide partly reversed the WRS-induced fall in gastric mucosal DNA synthesis. This finding is the evidence that gastroprotective effect of anandamide involves defense of cellular vitality.

Interleukin-1β is a well-known mediator of acute inflammation and plays a crucial role in the release of other members of pro-inflammatory cytokine cascade (49). IL-1β stimulates the synthesis and release of inflammatory mediators such as TNF-α, PAF, prostaglandins and pro-inflammatory interleukins (49, 50). The essential role of IL-1β in inflammatory process is evidenced by the observation that administration of IL-1β receptor antagonist prevents the release of pro-inflammatory mediators and reduces the severity of inflammation and tissue damage (49). These data are in agreement with our present observation. Exposure to WRS,

leading to gastric mucosa damage has increased serum concentration of IL-1β. Pretreatment with anandamide has reduced the WRS-evoked increase in serum level of IL-1β. This observation indicates that reduction of inflammation is involved in gastroprotective effect of anandamide. On the other hand, it must be pointed out that there are some reports showing that pretreatment with IL-1β may reduce gastric damage evoked by different noxious agents (51, 52). This protective effect of IL-1β seems to be some kind of phenomenon called adaptive cytoprotection. Numerous studies have shown that exposure of gastric mucosa to mild irritants results in enhanced mucosal resistance against subsequent exposure to severe damaging factors (53). Similar protective effect can be also evoked by ischemic preconditioning. Brief periods of warm ischemia and subsequent short reperfusion, before long-term ischemic insults or exposure to other damaging factor, enhance resistance and reduce injury in various organs, such as the heart (54), liver (55) stomach (56) or pancreas (57, 58).

Important finding of present study is observation that pretreatment with anandamine affects mucosal reactive oxygen species (ROS) production and antioxidant defense. ROS are involved in the pathogenesis of gastric mucosal injury. There are papers showing that ROS are implicated in gastric mucosal damage evoked by ischemia/reperfusion in (59), aspirin (60) or stress (44). ROS may be formed from several sources, but two enzymes, xanthine oxidase, and neutrophilic NADPH oxidase play the most important role in generation of ROS and development of tissue damage (59). ROS react with cellular lipids, leading to the formation of lipid peroxidation products, MDA and 4-HNE (61). Nature has provided a myriad of antioxidant enzymes and scavengers to protect against the deleterious effects of ROS. The primary defense against oxidative insults to the tissue includes the enzymes, superoxide dismutase (SOD), catalase, and glutathione peroxidase (59).

Oxidative stress was originally defined as the disequilibrium between prooxidants and antioxidants in biological systems (61). In agreement with previous reports (44), our present study, has shown that induction of gastric ulcers by WRS is associated with mucosal oxidative stress. WRS has increased concentration of MDA and 4-HNE, and reduced activity of SOD in gastric mucosa. New finding of our study is observation that gastroprotective effect of anandamide involves reduction of lipid peroxidation and enhancement of activity of antioxidative enzymes.

Peptic ulcer has been attributed to an imbalance between ulcer-promoting factors, such as excessive secretion of acid and pepsin, reduction in mucosal blood flow, and mucosal protective factors, such as: mucus, alkaline and prostaglandin production, or rapid mucosal cell turnover. Since Schwartz's dictum in 1910 „no acid - no ulcer", overproduction of gastric acid was considered to be the most important factor in the pathogenesis of peptic ulcer and treatment of this disease was based mainly on the inhibition of gastric acid secretion (62, 63). Gastric acid plays an important role in the development of stress-induced ulcer (64). Cannabinoids inhibit gastric acid secretion and it is most likely that gastroprotective effect of anandamide involves its antisecretory effect in the stomach. However, this effect of anadamide has not been studied in the present study.

In harmony with previous study (35), we have observed that ablation of sensory nerves prior to exposure to WRS increases gastric damage and reduces gastric mucosal blood flow. We have also found that deleterious effect of sensory nerves ablation is associated with an increase in serum level of pro-inflammatory IL-1β and mucosal concentration of MDA and 4-HNE, as well as with a decrease in mucosal DNA synthesis and SOD activity.

The most important finding of our present study is the role of sensory nerves in gastroprotective effect of anandamide. Previous studies have shown that gastroprotective effect of cannabinoids is related to CB1 receptors (8, 10, 14). CB1 receptors are widely distributed in the central and peripheral nervous system (19). Cannabinoids share structural similarities and they are all polyunsaturated fatty acids/arachidonic acid derivatives (65). According to their lipophilic nature, cannabinoids are nearly insoluble in water but are soluble in lipids. For this reason, cannabinoids can cross over the blood-brain barrier and act on receptors located in central nervous system. These properties of cannabinoids suggest that gastroprotective effect of anandamide is dependent on activation of CB1 receptors located peripherally and centrally. Neurotoxic doses of capsaicin deactivate sensory nerves but are not able to affect CB1 receptor in central nervous system. Probably for this reason, ablation of sensory nerves only partly attenuates gastroprotective effect of peripherally administered high doses of anandamide. On the other hand, anandamide given at the low dose of 0.3 μmol/kg was ineffective in protection of gastric mucosa against WRS in rats with ablation of sensory nerves; whereas this low dose of anandamide was still effective in rats with intact of sensory nerves. This concept of peripheral and central action of anandamide after intraperitoneal administration is also supported by observation that anandamide and other CB1 receptors agonists exhibit gastroprotective effect after peripheral and central, intraventricular administration (15). Moreover, study performed by Wang *et al*. has shown that administration of methananandamide, a CB1 receptor agonist, stimulates a release of sensory nerves mediator, CGRP (66). This observation additionally indicates involvement of sensory nerves in the effects of cannabinoids.

Our present study has shown that administration of AM251, a selective antagonist of CB1 receptors, significantly increased gastric ulcer area, serum concentration of pro-inflammatory IL-1β and additionally reduced gastric blood flow in rats with intact sensory nerves and abolished protective effect of anandamide. On the other hand, administration of AM251 failed to affect gastric ulcer area in rats with ablation of sensory nerves. These data indicate that endogenous cannabinoids are involved in the protection of gastric mucosa against noxious agents and sensory nerves are implicated in this effect.

Finally, our present study has demonstrated that anandamide reduces the mucosal oxidative stress, inhibits inflammatory process and protects gastric mucosa in stress-induced gastric ulcers. These effects are partly mediated by capsaicin-sensitive sensory nerves.

Conflict of interests: None declared.

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