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THE ROLE OF REACTIVE OXYGEN SPECIES AND CAPSAICIN-SENSITIVE SENSORY NERVES IN THE PATHOMECHANISMS OF GASTRIC ULCERS INDUCED BY STRESS

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Gastric microcirculation plays an important role in the maintenance of the gastric mucosal barrier and mucosal integrity. Sensory nerves are involved in the regulation of mucosal blood circulation and mucosal defense. Therefore, the ablation of these nerves by neurotoxic doses of capsaicin provides the possibility of determination of their role in gastric mucosal integrity. Stress ulceration represents a serious gastric lesions. Results of our previous experiments have indicated that water immersion and restraint stress (WRS) led to increased oxidative metabolism. Ablation of sensory nerves by high doses of capsaicin retards healing of gastric ulcers, but the role of reactive oxygen species (ROS) in the healing process has been little studied. Therefore, the aim of our present investigations was to determine the participation of ROS in sensory nerve activity during WRS. Experiments were caried out on 90 male Wistar rats and the area of gastric lesions was measured by planimetry.Colorimetric assays were used to determine gastric mucosal levels of malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE), as well as superoxide dismutase (SOD) activity. We demonstrated that inactivation of sensory nerves resulted in magnification of gastric mucosal damage induced by the WRS. In this process, oxidative stress, as reflected by an increase of MDA and 4-HNE tissue concentrations (an index of lipid peroxidation), as well as decrease of SOD activity, could play an important role. Aspirin, applied in a low dose, exerts a protective activity, possibly due to its metabolites, which possess the anti-oxidant and ROS scavanging properties. Pentoxyfilline-induced gastroprotection and hyperemia depends upon attenuation of the oxidative stress. This protection and hyperemia were, at least in part, attenuated by ASA.

Key words: *water immersion restraint stress, capsaicin, MDA, SOD, aspirin, pentoxyfilline*

INTRODUCTION

The integrity of the gastric mucosa depends on a variety of different factors. Gastric microcirculation plays an important role in the maintenance of the gastric mucosal barrier (1,2). Sensory nerves are involved in the regulation of blood circulation in gastric mucosa (3,4), that is densely innervated by capsaicin-sensitive afferent neurons, containing and releasing vasodilator peptides, such as calcitonin gene-related peptide (CGRP) (5). Capsaicin affects these nerves in two different ways. Low doses of capsaicin result in the stimulation of sensory nerves, accompanied by the release of CGRP, whereas high doses of capsaicin lead to ablation, or functional inactivation, of sensory nerves (6). Therefore, the ablation of sensory nerves by high doses of capsaicin provides the opportunity to determine their role in the gastric regulatory processes.

Stress ulceration represents a serious complication in patients under stress conditions. Experimental studies have demonstrated that the exposure of rat gastric mucosa to stress produces gastric mucosal lesions (7,8). Results of our previous experiments indicate that water immersion and restraint stress (WRS) is capable of raising oxidative metabolism, to the level comparable with that

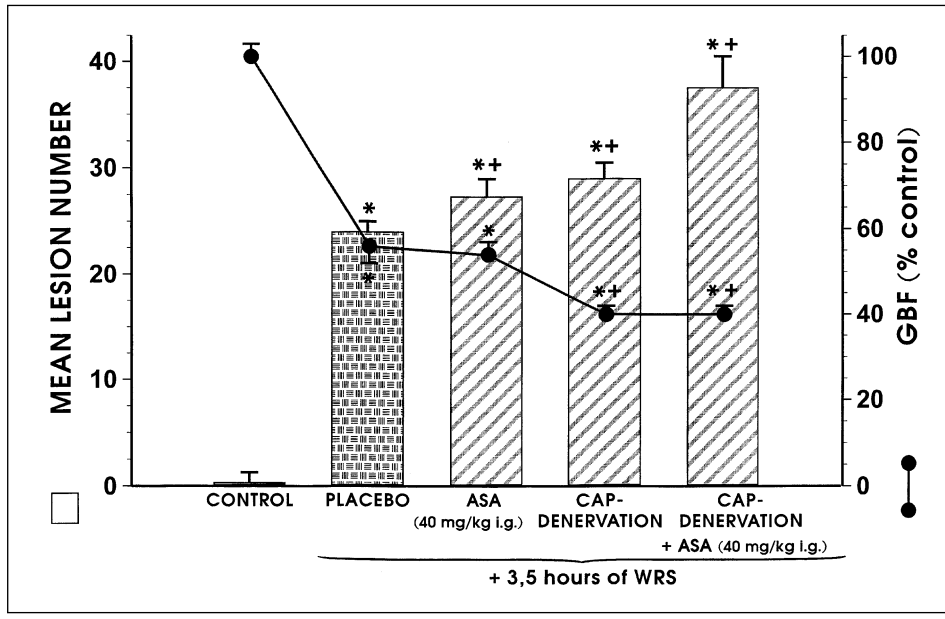

Fig.1. Mean number of gastric lesions and gastric blood flow (GBF) in rats exposed to 3.5 h of water immersion restraint stress (WRS) without or with capsaicin denervation (CAP-DENERVATION) and intragastric (i.g.) pretreatment with aspirin (ASA 40 mg/kg i.g.). Results are mean ± SEM of 8-10 rats. Asterisk (*) indicates significant change as compared with the respective values obtained in vehicle control group. Cross (+) indicates significant change as compared with the respective values obtained in rats exposed to 3.5 h of WRS alone.

observed in ischemia-reperfusion model. Two parameters are usually useful for the assessment of oxidative metabolism, namely the tissue levels of malondialdehyde (MDA) plus 4-hydroxynonenal (4-HNE) and the activity of superoxide dismutase (SOD). Tissue levels of MDA and 4-HNE are used as indicators of lipid peroxidation. SOD activity reflects the antioxidative properties of tissues (9).

Functional ablation of sensory nerves by high doses of capsaicin was shown to aggravate healing of gastric ulcers. However, the role of reactive oxygen metabolites in the process of WRS-induced gastric damage has been little studied. Therefore, the aim of our present investigations was to assess the participation of reactive oxygen species in sensory nerve action during WRS.

MATERIAL AND METHODS

Experiments were carried out on 90 male Wistar rats, weighing about 200 g and fasted for 24 h before all studies. Studies were approved by the Ethic Committee for Animal Research of Jagiellonian University College of Medicine.

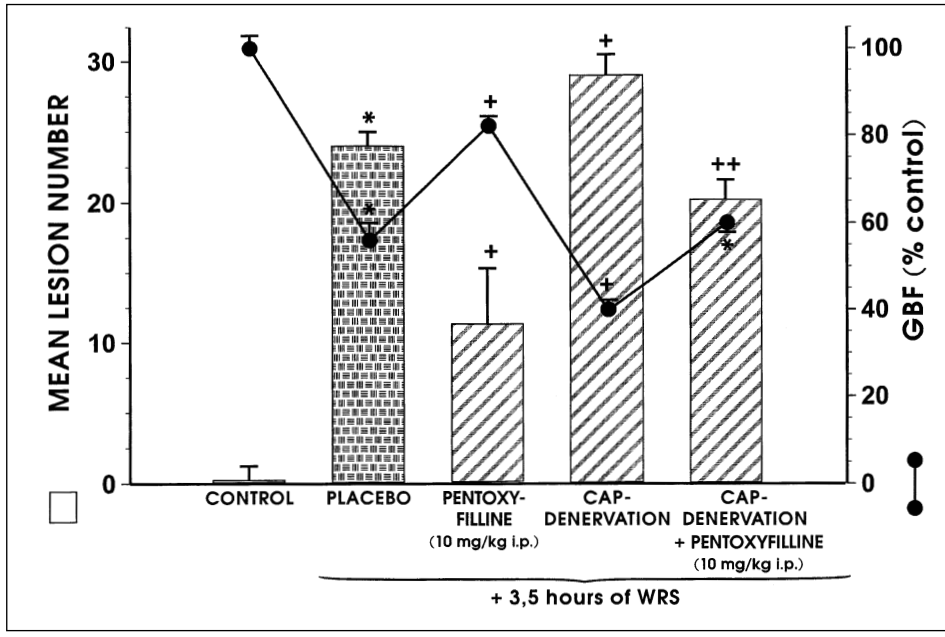

Fig.2. Mean number of WRS-induced gastric lesions and gastric blood flow (GBF) without or with capsaicin denervation (cap-denervation) or pentoxyfilline (10 mg/kg i.p.). Results are mean ± SEM of 8-10 rats. Asterisk (*) indicates significant change as compared with the respective values obtained in intact gastric mucosa. Cross (+) indicates significant change as compared with the respective values obtained in rats exposed to 3.5 h of WRS alone. Double cross (++) indicates a significant change as compared to the value obtained in rats with capsaicin denervation.

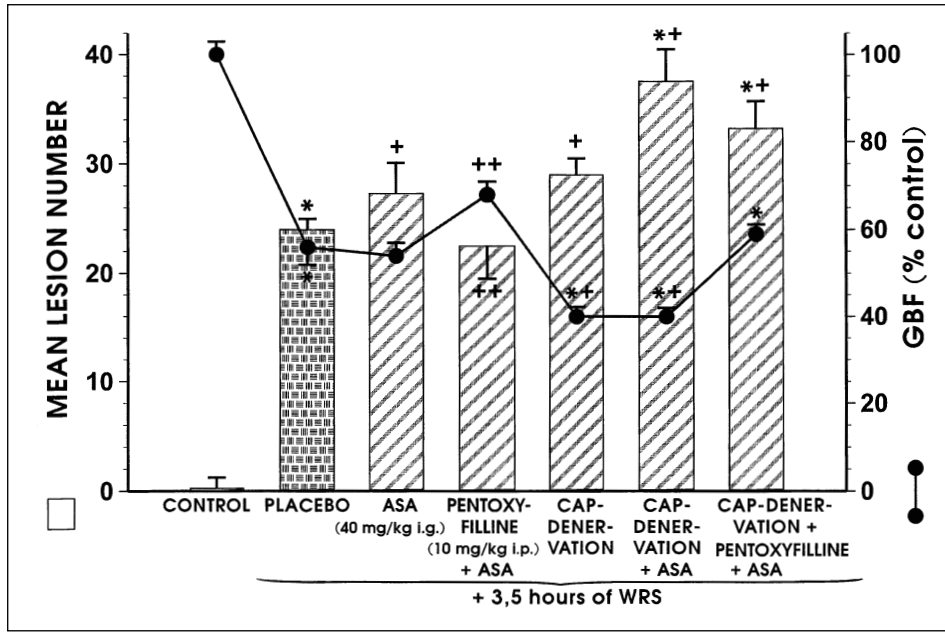

Fig.3. Mean number of gastric lesions and gastric blood flow (GBF) in rats exposed to 3.5 h of WRS without or with capsaicin denervation (cap-denervation) or pentoxyfilline (10 mg/kg i.p.) applied alone or given in the combination with aspirin (ASA 40 mg/kg i.g.). Results are mean ± SEM of 8-10 rats. Asterisk (*) indicates significant change as compared with the respective values obtained in intact gastric mucosa. Cross (+) indicates significant change as compared with the respective values obtained in rats exposed to 3.5 h of WRS alone. Double cross (++) indicates a significant change as compared to respective values obtained in rats with capsaicin denervation.

Production of gastric lesions

The animals were divided into 6 groups. Groups 1-2 underwent deactivation of sensory nerves by high doses of capsaicin (capsaicin denervation). For this purpose the animals were pretreated with capsaicin (Sigma Co., St Louis, USA) injected subcutaneously (s.c.) for 3 consecutive days at a dose of 25, 50 and 50 mg/kg (total of 125 mg/kg) about 2 weeks before the experiment. All injections of capsaicin were performed under ether anesthesia to counteract the pain reaction and respiratory impairment associated with injection of this agent. To check the effectiveness of the capsaicin denervation, a drop of 0.1 mg/ml solution of capsaicin was instilled into the eye of each rat and the protective wiping movement was counted as described previously (10).

In groups 3-6 the physiological function of sensory nerves was mantained and capsaicin was not administered to these animals.

In group 1 the ablation of sensory nerves was accompanied by 3.5 h of WRS in temperature 23°C, using the method originally proposed by Takagi et al. (11). In group 2 aspirin was administered intragastrically (ASA 40 mg/kg i.g.) to capsaicin-deactivated group of animals, 30 minutes prior to 3.5 h of WRS or pentoxyfilline was applied intragastrically (i.g.) in dose 10 mg/kg

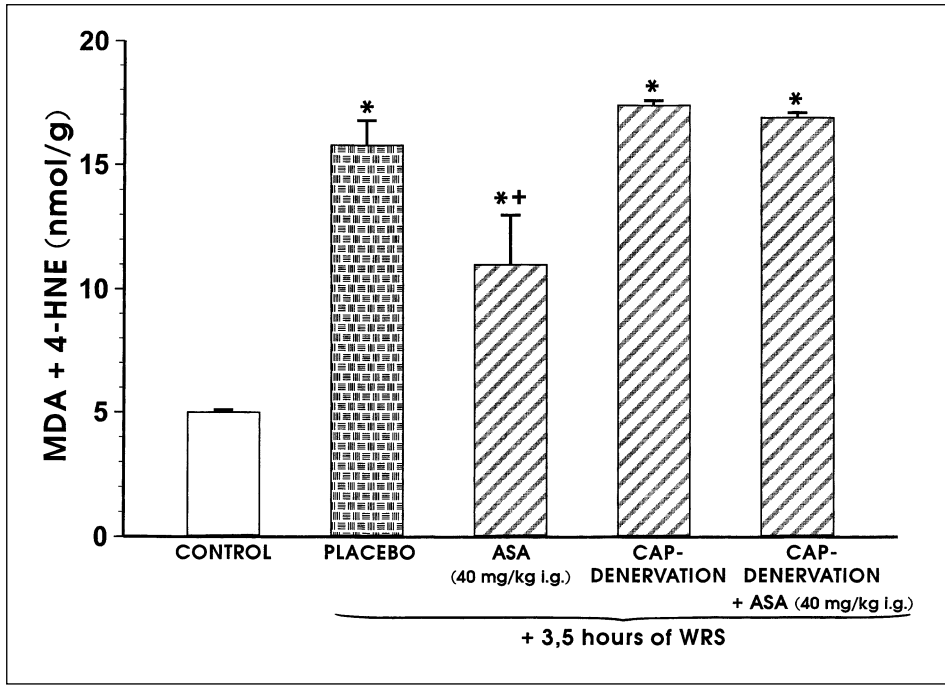

Fig.4. Concentration of malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE) in the gastric mucosa of rats exposed to 3.5 h of WRS without or with capsaicin denervation (cap-denervation) or aspirin (ASA 40 mg/kg i.g.). Results are mean ± SEM of 8-10 rats. Asterisk (*) indicates significant change as compared with the respective values obtained in vehicle control group. Cross (+) indicates significant change as compared with the respective values obtained in rats exposed to 3.5 h of WRS alone.

to rats with deactivated sensory nerves. In group 3 intraperitoneal pretreatment (i.p.) with pentoxyfilline (10 mg/kg), intragastrical administration of aspirin (40 mg/kg i.g.) was applied in animals before with intact sensory nerves before 3.5 h of WRS. In group 4 healthy intact rats underwent 3.5 h of WRS with or without pretreatment with aspirin (ASA 40 mg/kg i.g.) or pentoxyfilline (10 mg/kg i.p.) applied alone or in combination were administered prior to 3.5 h of WRS. In group 5 pentoxyfilline (10 mg/kg i.p.) applied in the combination with aspirin (40 mg/kg i.g.) were given to rats exposed to 3.5 h of WRS. In group 6 the animals served as a control group and did not undergo any procedures.

Determination of gastric blood flow and number of lesions

The evaluation of gastric lesions and gastric blood flow (GBF) was performed 3.5 hours after the start of WRS. To measure GBF the laser Doppler flowmeter (Laserflo, model BPM 403A, Blood Perfusion Monitor, Vasamedics, St. Paul, Minnesota, USA) was employed. The animals were anaesthetized with pentobarbital 50 mg/kg (Biowet, Puławy, Poland), then the abdomen was opened and the stomach was exposed to determine the GBF. The GBF was measured on the anterior and posterior walls of the stomach not involving gastric lesions. The mean values of three measurements

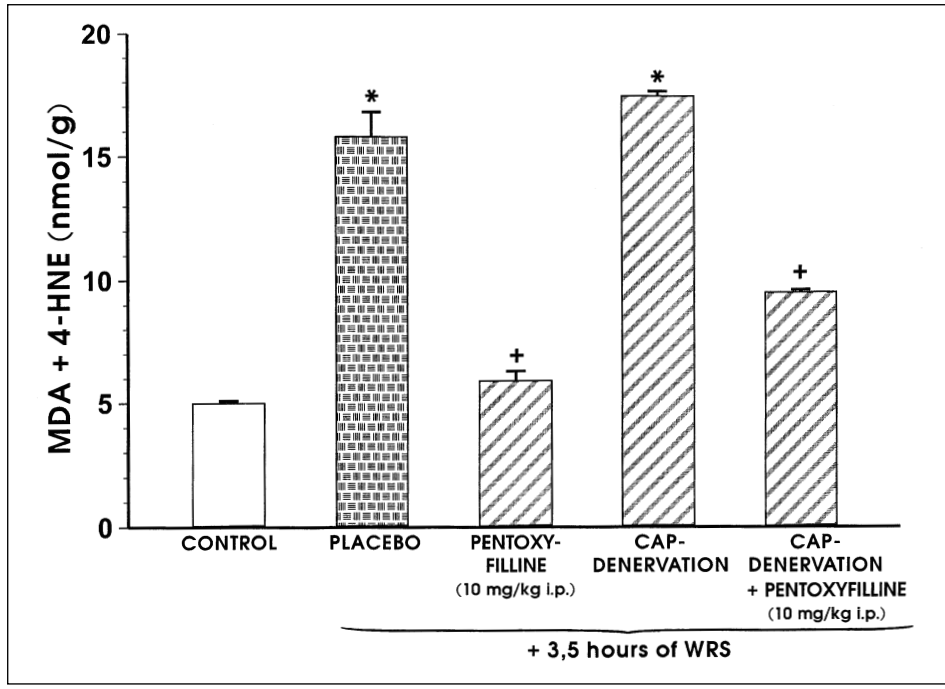

Fig. 5. Concentration of malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE) in the gastric mucosa of rats exposed to 3.5 h of WRS without or with capsaicin denervation (CAP-DENERVATION) or pentoxyfilline (10 mg/kg i.p.). Results are mean ± SEM of 8-10 rats. Asterisk (*) indicates significant change as compared with the respective values obtained in intact control group. Cross (+) indicates significant change as compared with the respective values obtained in rats exposed to 3.5 h of WRS alone without pretreatment with pentoxyfilline.

were calculated and expressed as percent change from value recorded in intact mucosa. The number of gastric lesions were determined by computerized planimetry (Morphomat, Carl Zeiss, Berlin, Germany), as described previously (2,9).

Measurement of lipid peroxydation

For lipid peroxydation in the investigated groups, the determination of the levels of malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE) was carried out and their levels were used as indicators of lipid peroxidation. The procedure of MDA and 4-HNE determination included excision of about 600 mg of gastric mucosa from intact rats and those exposed to WRS with or without pretreatment with ASA, pentoxyfilline and their combination. Then 20 ml 0.5 M BHT (butylated hydroxytoluene) was added in order to prevent sample oxidation. This sample was subsequently homogenized in 20 mM Tris for 15 s. in pH=7.4. Then the homogenate was centrifuged (3000 g at 4°C for 10 min). Obtained clear supernatant was stored at -80°C prior to testing.

The colorimetric assay for lipid peroxidation (Bioxytech LPO-586, Oxis, Portland, USA) was used to determine of MDA and 4-HNE tissue concentrations. This assay is based on the reaction of a chromogenic reagent N-methyl-2-phenylindole with MDA and 4-HNE at 45°C. This reaction

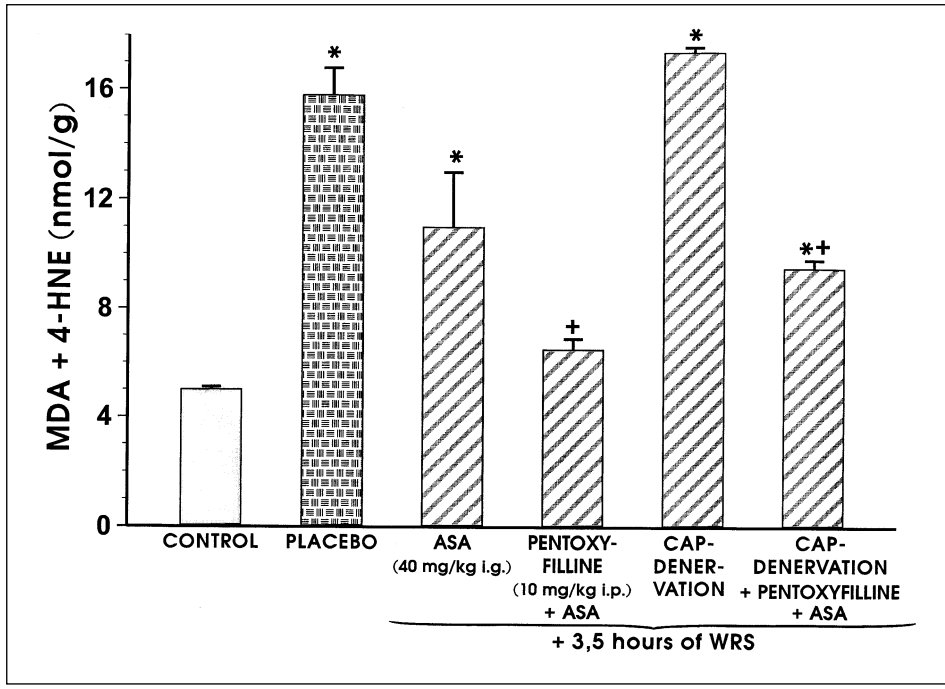

Fig.6. Concentration of malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE) in the gastric mucosa of rats exposed to 3.5 h of WRS without or with capsaicin denervation (cap-denervation) or pentoxyfilline (10 mg/kg i.p.) combined with aspirin (ASA 40 mg/kg i.g.). Results are mean ± SEM of 8-10 rats. Asterisk (*) indicates significant change as compared with the respective values obtained in vehicle control group. Cross (+) indicates significant change as compared with the respective values obtained in rats exposed to 3.5 h of WRS alone.

yields a stable chromophore with maximal absorbance at 586 nm. This absorbance was measured by spectrophotometer Marcel s300 (Warsaw, Poland). Results were expressed as nanomoles per gram of tissue (nmol/g) (9).

Determination of SOD activity

To determine the activity of superoxide dismutase (SOD), a sample of gastric mucosa was obtained, as described above. The colorimetric assay for assessment of SOD activity (Bioxytech SOD-525, Oxis, Portland, USA) was used. This method is based on the SOD-mediated increase in the rate of autooxidation of tetrahydrobenzofluorene in aqueous alkaline solution to yield a chromophore with maximum absorbance at 525 nm. This absorbance was measured by spectrophotometer Marcel s300 (Warsaw, Poland) and the results were expressed as units per gram of gastric tissue (U/g) (9).

Statistical analysis

Results are expressed as means ±SEM. Statistical analysis was done using nonparametric Mann-Whitney test. Differences with $p<0.05$ were considered significant.

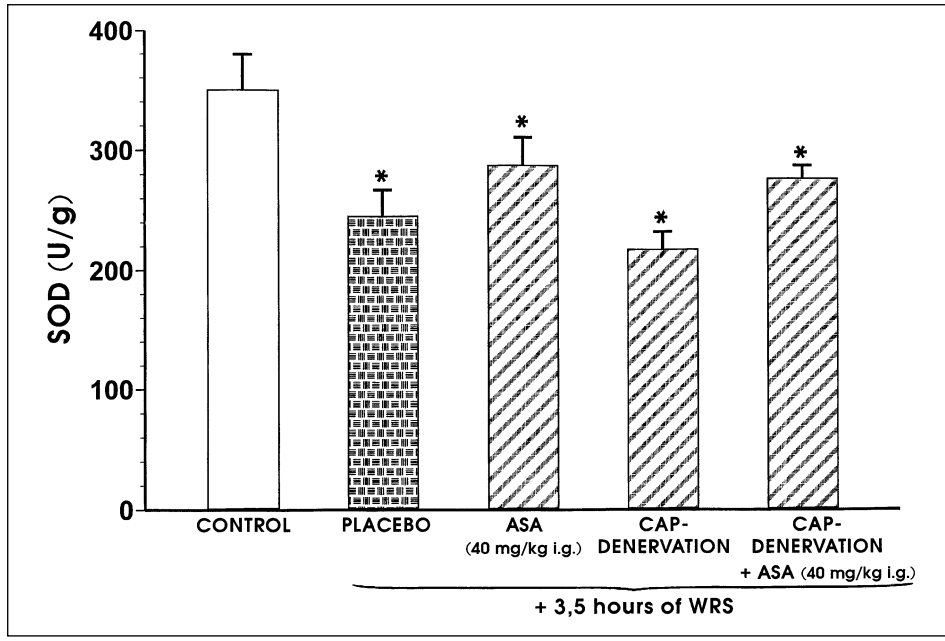

Fig.7. Superoxide dismutase (SOD) activity in the gastric mucosa of rats exposed to 3.5 h of WRS without or with capsaicin denervation (cap-denervation) or ASA 40 mg/kg i.g.. Results are mean ± SEM of 8-10 rats. Asterisk (*) indicates significant change as compared with the respective values obtained in vehicle control group.

RESULTS

Gastric lesions and gastric blood flow

Figs 1 and *2* show mean ulcer number in the WRS model as well as accompaning alterations in the GBF. Intact mucosa (control) did not show any macroscopic lesions and the GBF in this intact mucosa averaged 45±5 ml/min/100 g of tissue, being accepted as the control value (100%). Following 3.5 h of WRS numerous gastric mucosal lesions were produced (mean lesion number was about 24±1) and GBF was reduced to 56±4 % of control value. Ablation of sensory nerves by neurotoxic doses of capsaicin resulted in a significant increase of number of gastric lesion and this effect was accompanied by a decrease of GBF as compared with that obtained in rats with 3.5 h of WRS alone. Pretreatment with ASA (40 mg/kg i.g.) tended to increase the number of gastric lesions and decreased the GBF as compared to the values of lesion number and GBF recorded after 3.5 h of WRS. Significant changes of these parameters were observed in a group of animals with capsaicin deactivation and ASA application before 3.5 h of WRS and these changes were significantly more pronounced as compared with those in group of capsaicin denervated rats without pretreatment with ASA (*Fig.*

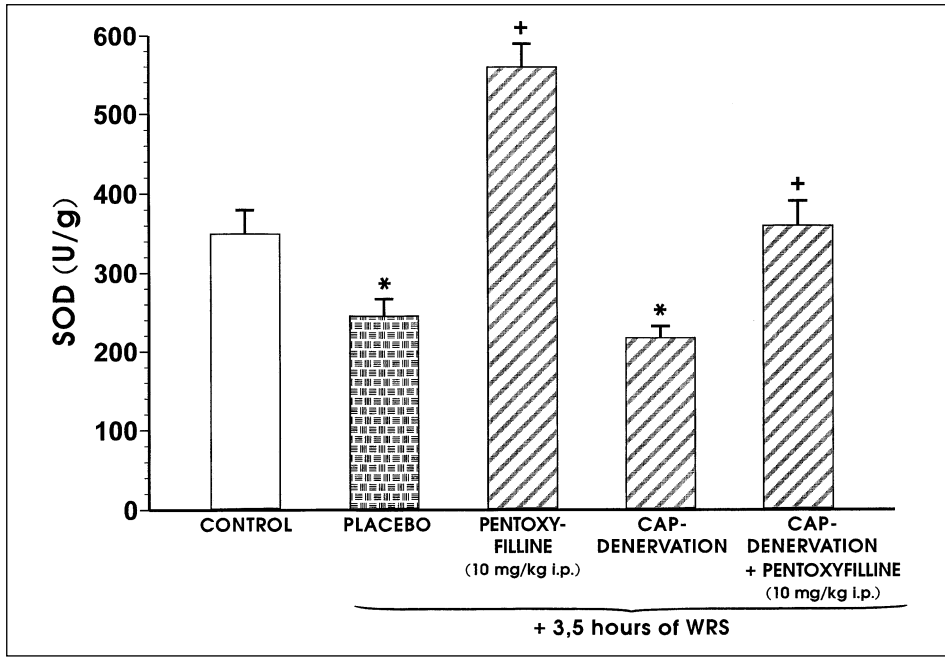

Fig.8. Superoxide dismutase (SOD) activity in the gastric mucosa of rats exposed to 3.5 h of WRS without or with capsaicin denervation (cap-denervation) or pentoxyfilline (10 mg/kg i.p.) applied alone or in the combination with capsaicin denervation. Results are mean ± SEM of 8-10 rats. Asterisk (*) indicates significant change as compared with the respective values obtained in non stressed animals. Cross (+) indicates significant change as compared with the respective values obtained in rats exposed to 3.5 h of WRS alone.

1). Administration of pentoxyfilline (10 mg/kg i.p.) produced a significant decrease of gastric lesion number and an increase of GBF, as compared with the 3.5 h of WRS group. The changes occurred in both, the healthy animals and those with functional ablation of sensory nerves. However, the augmentation of these changes in group of rats with intact sensory nerves was observed (*Fig. 2*). Combination of pentoxyfilline (10 mg/kg i.p.) with ASA (40 mg/kg i.g.) decreased significantly the gastric lesion number and raised significantly the GBF as compared with WRS group of animals with intact sensory fibers treated with ASA. The combined pretreatment with pentoxyfilline and ASA in capsaicin-denervated animals resulted in a significant increase of gastric lesion number as compared with those in rats exposed to WRS (*Fig. 3*).

Lipid peroxidation products

Concentration of MDA and 4-HNE in intact mucosa (control) was very low, almost at the level of analytical limit of detection (5,0±0,1 nmol/g). After 3.5 h of

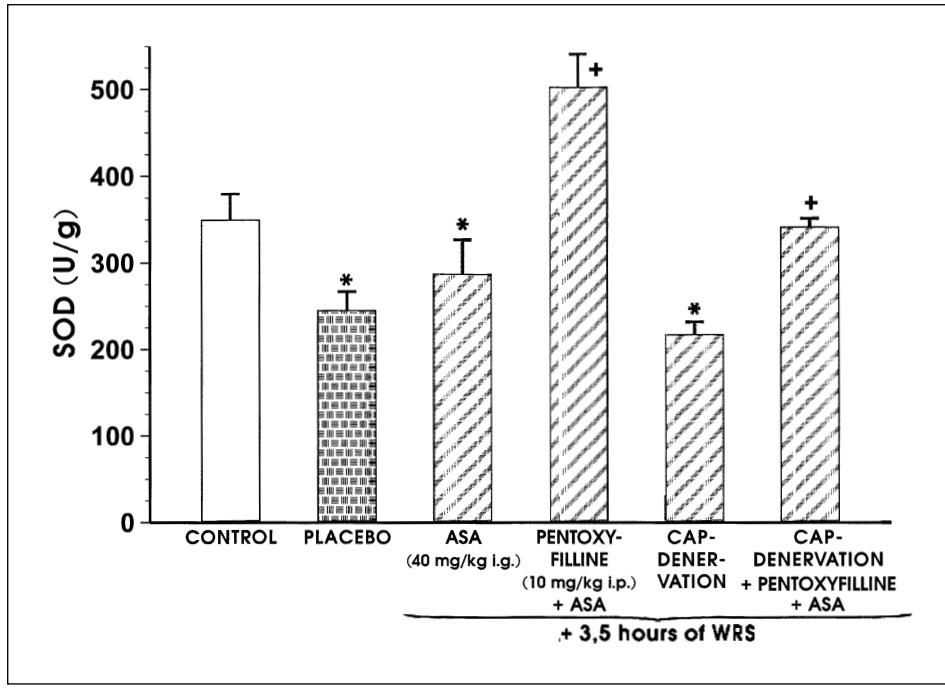

Fig.9. Superoxide dismutase (SOD) activity in the gastric mucosa of rats exposed to 3.5 h of WRS without or with capsaicin denervation (cap-denervation) or pentoxyfilline (10 mg/kg i.p.) applied without or with the combination with aspirin (ASA 40 mg/kg i.g.). Results are mean ± SEM of 8-10 rats. Asterisk (*) indicates significant change as compared with the respective values obtained in intact gastric mucosa. Cross (+) indicates significant change as compared with the respective values obtained in rats exposed to 3.5 h of WRS alone without pretreatment with pentoxyfilline.

WRS the level of MDA and 4-HNE increased by about three times, reaching the value of 15.8±1.0 nmol/g. Capsaicin deactivation caused larger increment of MDA and 4-HNE concentration up to the level of 17.4 ± 0.2 nmol/ g of tissue. Application of aspirin (ASA 40 mg/kg i.g.) resulted in a significant decrease of lipid peroxidation products. ASA in the same dose given to rats with ablation of sensory nerves established the concentration of MDA and 4-HNE at the level similar to that observed in the 3.5 h of WRS group (*Fig. 4*). Pretreatment with pentoxyfilline (10 mg/kg i.p.) led to a significant fall in lipid peroxidation in rats with or without capsaicin denervation and further exposed to WRS (*Fig. 5*). The similar reduction in MDA plus 4-HNE levels were observed in non-denervated and capsaicin-denervated rats with the combination of pentoxyfilline and ASA (*Fig. 6*).

SOD activity

As shown in *Fig. 7*, SOD activity averaged 352 ± 30 U/g of tissue in intact gastric mucosa. Following exposure of rats to WRS, a significant decrease of

SOD activity to the value 245.2±22.0 U/g was observed. Functional ablation of sensory nerves with capsaicin did not lead to any significant changes as compared with respective values in healthy rats exposed to 3.5 h of WRS. Administration of aspirin (ASA) to both groups (intact and capsaicin deactivated) failed to cause any significant elevation of SOD activity (*Fig. 7*). Parenteral application of pentoxyfilline to rats with maintained sensory nerves evoked high, significant increment of SOD activity that reached the value 559.6 ± 30.0 U/g. Pentoxyfilline in this dose injected to capsaicin-denervated animals did not significantly elevate SOD activity, as comparedto the value observed in the control group (*Fig. 8*). Combination of pentoxyfilline with ASA in rats with intact sensory nerves, resulted in a significant increase of SOD activity as compared to vehicle controls, but in capsaicin-deactivated animals, the application of these agents did not cause further increase in SOD activity, and the SOD activity remained similar to the value detected in the intact mucosa (*Fig. 9*).

DISCUSSION

Previous studies, related to the role of C fibers, focused mainly on their function in visceral circulation. It was documented that capsaicin-sensitive nerves played a role in the physiology of the gut, especially in the modulation of GI blood flow and metabolism (12). Numerous experiments were carried out on animal, with the deactivated sensory nerves. The aim of our present investigations was to determine role of sensory nerves in pathogenesis of WRS-induced gastric damage with the special focus on NSAID influence of gastric lesions and gastric blood flow. The assessment of C fibers in the development of lesions caused by ASA and WRS was carried out. Previous studies revealed that high doses of capsaicin, which led to sensory nerve ablation, resulted in delay of gastric ulcer healing (13). These changes were attributed, at least in part, to the release of endogenous prostaglandins Delay of healing, caused by capsaicin deactivation, was also observed in chronic model of gastric ulcers induced by acetic acid (10). Deleterious effect of sensory nerves ablation by capsaicin was recently demonstrated in the ischemia-reperfusion model of gastric mucosa damage (14). The WRS model seems to be especially interesting due to well-known systemic mode of the damaging action of stress. The aggravation of WRS damage by capsaicin denervation was attributed to attenuation of tissue release of nitric oxide (NO), resulting in the fall in the gastric microcirculation (15). Role of NO in the mechanism of afferent C fibers action was also shown in other models of gastric damage induction (16-18). However, the participation of reactive oxygen species (ROS) in the pathogenesis acute gastric lesions induced by concomitant action of two aggressive factors, such as ASA and WRS, by capsaicin deactivation has not been studied. In our previous investigations we have documented an important role of ROS in the formation of WRS-induced gastric damage (9). We have also

demonstrated the gastroprotective effect of NO-donors on the gastric lesions induced by WRS (19).

In our present investigations we attempted to assess biological effects of ROS activity, as expressed by tissue levels of MDA + 4-HNE, an index of lipid peroxidation, and SOD activity in rats exposed to WRS with or without capsaicin denervation. We also looked for changes in the ROS metabolism after administration of aspirin, which is well-known as a non-selective inhibitor of cyclooxygenase (COX). We also attempted to study effect of pentoxyfilline - a compound that was shown to improve tissue blood flow and to counteract the proinflammatory effects of tumor necrosis factor alpha (TNFα).

As expected, an increase of oxygen metabolism in the WRS model occurred and this effect was accompanied by induction of typical bleeding erosions in the gastric mucosa. Capsaicin denervation resulted in further elevation of mucosal MDA and 4-HNE levels, indicating oxydative-dependent tissue damage. The apparent decrease in SOD activity was enhanced in capsaicin-denervated animals again supporting the notion that lack of sensory innervation additionaly contributes to the attenuation of antioxidative properties of gastric tissues. Our results provided evidence for the participation of ROS in the detrimental effect of capsaicin applied in high doses on gastric mucosa exposed to WRS.

Erin *et al.*(20) failed to show an unequivocal association between lipid peroxidation and induction of gastric ulcers in thermal stress in rat. However, these authors (20) performed thermal stress under different experimental conditions, including different duration of stress and temperature applied to produce gastric lesions. Moreover, capsaicin was administered topically to the region of the coeliac plexus (20). On the contrary, we used animals with systemic application of capsaicin in order to ablate functionally the sensory nerves, as proposed previously (13-15).

Our results apparently disagree with those obtained by Kogure (21), who showed a potent antioxidative property of capsaicin on liver mitochondria. The reasons of this discrepancy is not clear, but Kogure *et al.* carried out his experiments in cell culture *in vitro*, while our experiments were performed on animal model in vivo. Moreover, in their study (21) capsaicin was used in low, non-cytotoxic doses, that are known to play a role of radical scavanger in a region of mitochondrial membranes. Whereas in our work, capsaicin was administered in high doses in order to cause deactivation of C fibers in the WRS model.

It was of particular interest to determine the synergism between a NSAID such as ASA and the stress in animals with capsaicin deactivation. This issue was not studied carefully since previous studies focused on the effect of low doses of capsaicin against ASA or WRS applied separately. Low doses of capsaicin associated with ASA administration had beneficial effects on gastric mucosa evoked gastroprotective effect against ASA-induced damage (22-24). In case of high doses of capsaicin we expected aggravation of gastric lesions, induced by WRS with or without treatment with ASA, accompanied by increase of lipid

peroxidation and depletion of antioxidative enzyme (SOD) following ASA. Indeed, we observed the aggravation of WRS lesions in capsaicin-deactivated animals, but only decrease in SOD activity after ASA application was noticed. This phenomenon could be explained by partial scavanger activity of ASA, applied in low doses, due to action of its metabolites, such as salicylic acid, that could exert a partial scavanging effect against free radicals. On the contrary to high cytotoxic doses of capsaicin, ASA could exert an antiinflammatory and radical scavanging activities in the WRS model (25). ASA exhibited here only partial scavanging effect, as documented by the still elevated level of MDA and 4-HNE,over that recorded in non-stressed animals. Moreover, Konturek *et al.*(26) documented that rats adapted to prolonged ASA treatment retain the mucosal resistance to repeated ASA insult, even after capsaicin deactivation due to the reduction in neutrophil activity, observed after repeated administration of this NSAID.

Administration of pentoxyfilline exerted beneficial effects manifested by the decrease of number of gastric lesions, followed by an increase of gastric blood flow, and finally accompanied by a large decrement in MDA plus 4-HNE tissue concentration and a potent increase in SOD activity. ASA application resulted in partial reversal gastroprotective effect of pentoxyfilline and slightly aggravated parameters of antioxidative defense mechanisms in tissues, an overall effect achieved was, however, better than that obtained in WRS animals pretreated with vehicle. This finding is in keeping with our previous observations (25).

We conclude that inactivation of C fibers, that induced functional impairment of afferent endings releasing vasoactive neurotransmitters, such as CGRP, augmented the gastric mucosal damage induced by the WRS. The oxidative stress, as documented by an increase of MDA and 4-HNE tissue concentrations as well as the fall in SOD activity, appears to play an important role in this process of the enhanced susceptibility of gastric mucosa to WRS in rats with capsaicin-induced deactivation of sensory nerves. ASA, applied in low dose, exerts protective activity, possibly due to its metabolites, which possess the anti-oxidant and ROS scavanging properties. Pentoxyfilline-induced gastroprotection and hyperemia depends upon attenuation of the oxidative stress. This protection and hyperemia were, at least in part, attenuated by ASA, suggesting that endogenous prostaglandins contribute to the beneficial effects of this agent on the gastric mucosa.

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