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INTERACTION BETWEEN SELECTIVE CYCLOOXYGENASE INHIBITORS AND CAPSAICIN-SENSITIVE AFFERENT SENSORY NERVES IN PATHOGENESIS OF STRESS-INDUCED GASTRIC LESIONS. ROLE OF OXIDATIVE STRESS

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Gastric microcirculation plays an important role in the maintenance of the mucosal gastric integrity and the mechanism of injury as well as providing protection to the gastric mucosa. Disturbances in the blood perfusion, through the microcapillaries within the gastric mucosa may result in the formation of mucosal damage. Acute gastric mucosal lesions constitute an important clinical problem. Originally, one of the essential component of maintaining the gastric mucosal integrity was the biosynthesis of prostaglandins (PGs), an issue that has captured the attention of numerous investigations. PGs form due to the activity of cyclooxygenase (COX), an enzyme which is divided into 2 isoforms: constitutive (COX-1) and inducible (COX-2) ones. The inhibition of COX-1 by SC-560, or COX-2 by rofecoxib, reduces gastric blood flow (GBF) and impairs gastric mucosal integrity. Another detrimental effect on the gastric mucosal barrier results from the ablation of sensory afferent nerves by neurotoxic doses of capsaicin. Functional ablation of the sensory afferent nerves by capsaicin attenuates GBF and also renders the gastric mucosa more susceptible to gastric mucosal damage induced by ethanol, aspirin and stress. However, the role of reactive oxygen species (ROS) in the interaction between COX specific inhibitors and afferent sensory nerves has not been extensively studied. The aim of our present study was to determine the participation of ROS in pathogenesis of stress-induced gastric lesions in rats administered with SC-560 or rofecoxib, with or without ablation of the sensory afferent nerves. ROS were estimated by measuring the gastric mucosal tissue level of MDA and 4-HNE, the products of lipid peroxidation by ROS as well as the SOD activity and reduced glutathione (GSH) levels, both considered to be scavengers of ROS. It was demonstrated that exposure to 3.5 h of WRS resulted in gastric lesions, causing a significant increase of MDA and 4-HNE in the gastric mucosa, accompanied by a decrease of SOD activity and mucosal GSH level. Pretreatment with COX-1 and COX-2 inhibitors (SC-560 and rofecoxib, respectively) aggravated the number of gastric lesions, decreased GBF, attenuated GSH level without further significant changes in MDA and 4-HNE tissue levels and SOD activity. Furthermore, the capsaicin - inactivation of sensory nerves resulted in exaggeration of gastric mucosal damage induced by WRS and this was further augmented by rofecoxib. We conclude that 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 and the fall in GSH tissue level, may play an important role in the mechanism of interaction between the inhibition of COX activity and afferent sensory nerves releasing vasoactive neuropeptides. This is supported by the fact that the addition of specific COX-1 or COX-2 inhibitors to animals with capsaicin denervation led to exacerbation of gastric lesions, and further fall in the antioxidizing status of gastric mucosa exposed to stress.

Key words: *cyclooxygenase-1, cyclooxygenase-2, malondialdehyde, reduced glutathione, rofecoxib, SC-560, superoxide dismutase, water immersion and restraint stress*

INTRODUCTION

The physiological maintenance of the integrity of the gastric mucosal involves numerous factors and enzymes. Among the enzymes regulating the process of mucosal defence is cyclooxygenase (COX) which acts by producing endogenous prostaglandins (PGs). Arachidonic acid, a substrate for COX, is finally transformed to PG especially prostaglandin E_2 (PGE_2) (1, 2). PGs including PGE_2 were shown to prevent damage of deeper mucosal structures resulting in increase of mucus secretion and the intensification of bicarbonate anions production (HCO_3^-), which neutralises acidic gastric content and enhances gastric blood flow (3).

Two isoforms of COX were proposed: the constitutive isoform COX-1 which delivers PG for the physiological functions and the inducible isoform, COX-2 stimulated by inflammatory

conditions, lipopolysaccharide, growth factors and cytokines (4). Classic approach to their functions has established that COX-1 plays a gastro-protective role, because PGs generated by this pathway were shown to exert gastroprotective effects (5). On the other hand, high levels of PGs, produced by COX-2 activation, contribute to detrimental effects, such as inflammation, increase of vessels permeability, transmission of pain sensation and fever (6).

The classic non-steroidal anti-inflammatory drug (NSAID) aspirin acting as the non-selective COX-1 inhibitor, besides its therapeutic anti-inflammatory effects, resulting from COX-2 blockade, was reported to cause side effects due to COX-1 inhibition (7). However, the results of previous investigations revealed that a blockade of COX-2 worsened the ischemia-reperfusion injury to the stomach (8) and markedly delayed healing of chronic gastric ulcers (9-11), suggesting that PGs derived from COX-2 derivatives could exert a beneficial influence in the upper GI-tract *via* the strengthening of the gastric mucosal barrier. Therefore, it seems quite rational to examine the role of selective inhibitors of COX-1 (SC-560) and COX-2 (rofecoxib) in the mechanism of gastric injury induced by non-topical stimuli, such as stress, which represents an important clinical problem (2, 12, 13). Such bleeding stress erosions and mucosal damage associated with hemorrhage can be reproduced in experimental animals that were exposed to water immersion and restraint using the techniques originally described by Takagi *et al.* (14) and widely used to examine the consequences of stress on the pathology of the stomach (15, 16).

In addition to PGs, the capsaicin - sensitive afferent sensory nerves are involved in the regulation of blood circulation in the gastric mucosa. These nerves, that densely innervate gastric mucosa, contain vasodilator neuropeptides, such as calcitonin gene-related peptide (CGRP) and substance P (SP) (17, 18). Various doses of capsaicin affect these nerves in two different manners. Low doses of capsaicin resulted in the stimulation of sensory nerves, accompanied by the release of CGRP, whereas high doses of capsaicin lead to functional ablation of these nerves, which were unable to release vasodilatatory neuropeptides from their sensory nerve endings. Our previous studies revealed that the acute gastric damage induced by ethanol, ischemia-reperfusion or stress is aggravated in animals subjected to functional ablation of sensory nerves by high doses of capsaicin and this effect is accompanied by drastic fall in the gastric microcirculation (15, 16).

Little information is available regarding the contribution of reactive oxygen species (ROS) and selective COX-1 and COX-2 inhibition to the pathomechanism of gastric mucosal lesions induced by WRS. It has been recognized that WRS - induced damage is accompanied by neutrophil infiltration of the gastric mucosa (2, 15, 16). Neutrophils produce superoxide radical anion ($O_2^{\bullet -}$), which belongs to a group of reactive oxygen species (ROS). Superoxide radical anion reacts with cellular lipids, leading to the formation of lipid peroxides, that are metabolized to malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE). Several enzymatic antioxidizing systems, including superoxide dismutase (SOD), can scavenge ROS and thereby prevent its destructive action on target cells (18-20).

Three types of superoxide dismutase (SOD) can be differentiated into following categories: cytoplasmatic, mitochondrial and extracellular. SOD catalyzes the dismutation of superoxide radical anion ($O_2^{\bullet -}$) into less noxious hydrogen peroxide (H_2O_2), that is further degraded by catalase or glutathione peroxidase. Catalase is an enzyme which accelerates degradation of H_2O_2 into water and oxygen. The second pathway of H_2O_2 metabolism depend on the activity of glutathione peroxidase (GPx) cooperating with glutathione reductase. The reduction of H_2O_2 into water by GPx is accompanied by the conversion of glutathione from reduced forms (GSH) into its oxidized form (GSSG) (16, 19).

The aim of our present study was to examine the role of ROS as determined by the MDA concentration and SOD activity or GSH level within gastric mucosa that was exposed to WRS in the presence of selective or non-selective COX inhibitors. In separate group of rats with or without ablation of capsaicin - sensitive afferent nerves, we evaluated the possible interaction of ROS with COX-2 products and sensory neuropeptides, such as CGRP released from sensory afferent endings in the pathogenesis of WRS-induced lesions and the impairment of gastric microcirculation.

MATERIAL AND METHODS

Experiments were carried out on 90 male Wistar rats, which weighed about 200 g and were fasted for 24 h before the commencement of all studies. Studies were approved by the Ethic Committee for Animal Research of Jagiellonian University Medical College and run according the principles of Helsinki Declaration.

Production of gastric lesions

The animals were divided into 7 groups. Group 1 consisting of intact animals, served as a point of reference and did not undergo any procedures. In group 2, rats were exposed to 3.5 h of WRS within water with a temperature of 20°C, according to the method originally proposed by Takagi *et al.* (14). In group 3, SC-560 (Cayman, Chemical, Ann Arbor, USA, 5 mg/kg i.p.) was applied in graded doses from 1 mg/kg intraperitoneally (i.p.) to 10 mg/kg (i.p.), 30 min prior to WRS. In group 4, rofecoxib was applied in graded doses ranging from 2.5 mg/kg intragastrically (i.g.) up to 20 mg/kg (i.g.), given 30 min prior to WRS. In group 5, SC-560 was administered i.p. in a dose of 5 mg/kg, 30 min. prior to WRS. In group 6, rofecoxib (Vioxx, Merck, Glattbrugg, Switzerland) was administered i.g. in a dose of 10 mg/kg, 30 min prior to WRS.

Groups 7-9 consisting 6-9 rats each 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), approximately 2 weeks before the experiment (1, 15). 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 (16). Results in these capsaicin-denervated groups were compared with those obtained in groups 1-6, where the physiological function of sensory nerves was maintained and only vehicle (saline), instead of capsaicin, was administered to these animals.

Similarly as in groups with intact sensory nerves, animals of groups 7-9 with capsaicin denervation were exposed to 3.5 h of WRS in temperature 20°C, using the method originally proposed by Takagi *et al.* (14). In group 8, SC-560 (5 mg/kg i.p.) was administered to capsaicin-deactivated group of animals, 30 minutes prior to 3.5 h of WRS. In group 9, rofecoxib (10 mg/kg i.g.) was administered to capsaicin-deactivated group of animals, 30 min prior to 3.5 h of WRS.

Determination of gastric blood flow and number of gastric lesions

The evaluation of gastric lesions and gastric blood flow (GBF) was performed at the end of 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 (15,16). The animals were anaesthetized with pentobarbital 50 mg/kg (Biowet, Pulawy, 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 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 (19, 20).

Measurement of lipid peroxidation

To measure free radical activity in the gastric mucosa of rats with COX-1 and COX-2 inhibition as well as those with capsaicin denervation, the determination of malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE) levels was carried out and their levels were used as indicators of lipid peroxidation. The procedure of MDA and 4-HNE determination included excision of the gastric biopsy samples of about 600 mg from intact rats and those exposed to WRS with or without pretreatment with SC-560, rofecoxib and their combination. Then 20 µl 0.5 M BHT (butylated hydroxytoluene) was added, in order to prevent sample oxidation. This sample was subsequently homogenized in 20 mM Tris buffer for 15 sec in pH=7.4. Then the homogenate was centrifuged (3000 g at 4°C for 10 min) and clear supernatant was collected and stored at -80°C until the measurement.

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 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) as described in our previous studies (15, 16, 19).

Determination of superoxide dismutase (SOD) activity

To determine the activity of superoxide dismutase (SOD), a biopsy sample of gastric mucosa was obtained from each group of animals, 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) as described previously (2, 15, 16).

Measurement of glutathione level (GSH)

To determine the level of the reduced forms of glutathione (GSH), a sample of gastric mucosa was taken, as described above. Then 5% aqueous solution of metaphosphoric acid was added to each biopsy sample in order to evoke protein precipitation. Followed by colorimetric assay for assessment of reduced glutathione concentration (Bioxytech, GSH-400, Oxis, Portland, USA) was performed. The level of reduced glutathione was measured, with maximal absorbance at 400 nm, by spectrophotometry (Marcel s300, Warsaw, Poland). Results were expressed as micromole per gram of tissue (µmol/g) (20).

Statistical analysis

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

RESULTS

Gastric lesions and gastric blood flow (GBF)

Table 1 shows the effect of administration of SC-560, applied i.p., in graded doses ranging from 1 mg/kg up to 10 mg/kg, on the mean lesion number and accompanying changes in the GBF, induced by WRS. The pretreatment with SC-560 caused dose-dependent increase of mean number of WRS lesions and significantly reduced the GBF. Maximal damaging effect was exerted by SC-560 injected in a dose of 5 mg/kg (i.p.). Higher dose, of 10 mg/kg of this agent, failed to cause a significant enhancement in damaging effect, comparing to that observed with SC-560 given in a dose of 5 mg/kg (i.p.).

Table 2 shows the effect of administration of rofecoxib, applied i.g., in graded doses ranging from 2.5 mg/kg up to 20 mg/kg, on the mean lesion number and accompanying changes in the GBF, induced by WRS. The pretreatment with rofecoxib caused dose-dependent increase of the mean number of the WRS lesions and significantly reduced the GBF. Maximal damaging effect followed by the significant fall in GBF was exerted by rofecoxib applied in a dose of 10 mg/kg (i.g.). Higher dose of 20 mg/kg of this COX-2 inhibitor failed to induce a further aggravation of the damaging effect and accompanied by a fall in the GBF, comparing to those observed with rofecoxib given in a dose of 10 mg/kg (i.g.).

Figs. 1 and *2* show mean ulcer number in the WRS model as well as accompanying alterations in the GBF. Intact mucosa (control) did not show any macroscopic lesions and the GBF in

Table 1. Mean number of gastric lesions and gastric blood flow (GBF) pretreated 30 min prior the exposure to 3.5 h of water immersion restraint stress (WRS) with placebo (saline) or SC-560 given intraperitoneally (i.p.) in graded doses ranging from 1 mg/kg up to 10 mg/kg. Asterisk indicates a significant change as compared to the value obtained in placebo-treated animals.

Type of test	Mean lesion number	Gastric blood flow (% Control)
Placebo	21 ± 2	62 ± 1
SC-560 (1 mg/kg)	24 ± 2	60 ± 3
SC-560 (2.5 mg/kg)	26 ± 4	58 ± 4.5
SC-560 (5 mg/kg)	27 ± 3 *	55 ± 2.3 *
SC-560 (10 mg/kg)	28 ± 3*	53 ± 1.5 *

Table 2. Mean number of gastric lesions and gastric blood flow (GBF) in rats pretreated 30 min prior the exposure to 3.5 h of water immersion restraint stress (WRS) with placebo (saline) or rofecoxib given intragastrically (i.g.) ranging from 1 mg/ kg up to 10 mg/kg. Asterisk indicates a significant change as compared to the value obtained in group: placebo+WRS.

Type of test	Mean lesion number	Gastric blood flow (% Control)
Placebo	21 ± 3	62 ± 1.3
Rofecoxib (2.5 mg/kg)	23 ± 4	61 ± 1.5
Rofecoxib (5 mg/kg)	24 ± 5	62 ± 4.2
Rofecoxib (10 mg/kg)	25 ± 2*	56 ± 1.8*
Rofecoxib (20 mg/kg)	27 ± 3*	53 ± 3.1*

this intact mucosa averaged 45±5 ml/min/100 g of tissue, being considered as the control value (100%). Following 3.5 h of WRS numerous gastric mucosal lesions were produced (mean lesion number was about 21±3) and GBF was reduced to 62±1% of control value. Ablation of sensory nerves by neurotoxic doses of capsaicin resulted in a significant increase in the number of gastric lesions and this effect was accompanied by a decrease of GBF, when compared with that obtained in rats with 3.5 h of WRS alone. Pretreatment with SC-560 in dose 5 mg/kg i.p. (*Fig. 1*), or rofecoxib in dose 10 mg/kg i.g. (*Fig. 2*), significantly increased the number of gastric lesions and also significantly decreased the GBF, when compared to the values of lesion number and GBF recorded after 3.5 h of WRS in placebo treated animals. The significant increase in the number of WRS lesions and a significant decrease in GBF were observed in a group of animals with capsaicin deactivation, which were administered with SC-560 (*Fig. 1*) or rofecoxib (*Fig. 2*). Administration of SC-560 and rofecoxib resulted in more pronounced changes in the number of WRS lesions and GBF, when compared with those in the group of capsaicin denervated rats without pretreatment with SC-560) or rofecoxib (*Figs. 1* and *2*).

Lipid peroxidation products

The concentration of MDA and 4-HNE within the intact mucosa (control) was very low and this value was, almost at the level of analytical limit of detection (5.0±0.1 nmol/g). After 3.5 h of WRS the level of MDA and 4-HNE increased by about three times, reaching the value of 15.85±1.27 nmol/g. Capsaicin denervation caused larger increment of MDA and 4-HNE concentration up to the level of 17.4±0.2 nmol/g of tissue (*Figs. 3* and *4*). After application of SC-560 in dose 5 mg/kg i.g. (*Fig. 3*) or rofecoxib in dose 10 mg/kg i.g. (*Fig. 4*), to the rats exposed to WRS, the concentration of MDA and 4-HNE remained in the same high level. In case of the animals denervated by capsaicin and exposed to WRS in the presence of SC-560 or rofecoxib,

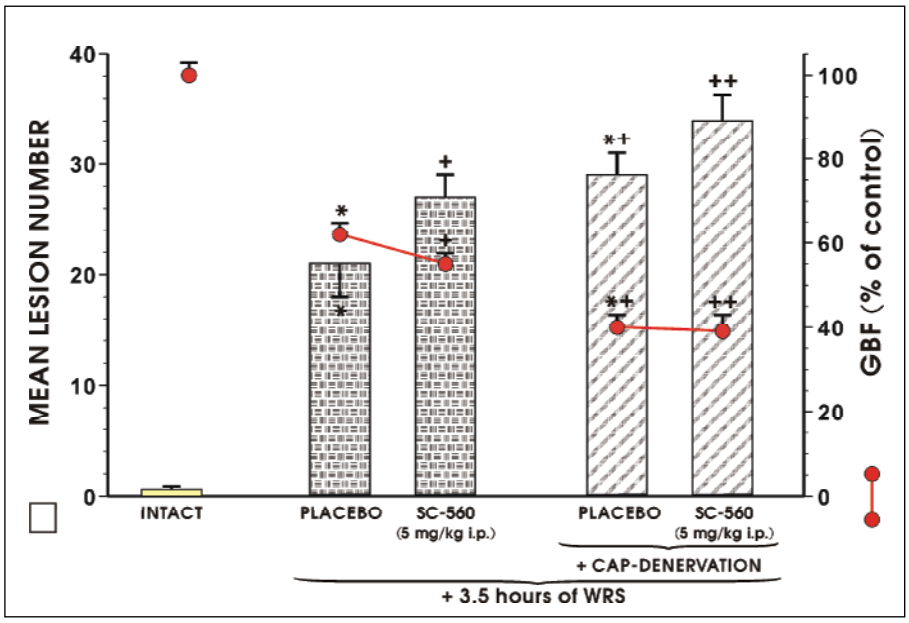

Fig. 1. Mean number of gastric lesions and gastric blood flow (GBF) in rats, without or with capsaicin denervation (CAP-DENERVATION), exposed to 3.5 h of water immersion restraint stress (WRS) with intraperitoneal (i.p.) pretreatment with SC-560 (5 mg/kg.) or placebo (saline). Results are mean ±S.E.M. of 8-10 rats. Asterisk indicates a significant change as compared with the respective values obtained in intact gastric mucosa. Cross indicates a significant change as compared with the respective values obtained in placebo (control) group. Asterisk and cross indicate a significant change as compared to respective values in placebo group without capsaicin denervation. Double crosses indicate a significant change as compared to placebo treated animals with capsaicin denervation.

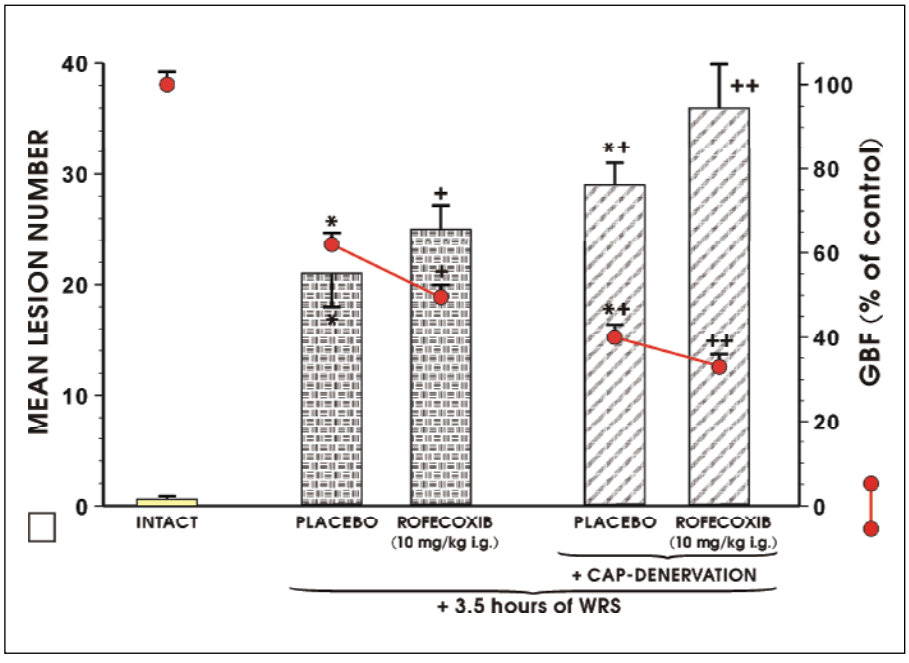

Fig. 2. Mean number of WRS-induced gastric lesions and gastric blood flow (GBF), without or with capsaicin denervation (CAP-DENERVATION) and intragastric (i.g.) pretreatment with rofecoxib (10 mg/kg) or placebo (saline). Results are mean ±S.E.M. of 8-10 rats. Asterisk indicates significant change as compared with the respective values obtained in intact gastric mucosa. Cross indicates significant change as compared with the respective values obtained in placebo control group. Asterisk and cross indicate a significant change as compared to respective values in rofecoxib group. Asterisk and cross indicate a significant change as compared to respective values in placebo and rofecoxib groups. Double crosses indicate a significant change as compared to the respective values in placebo-treated capsaicin denervated rats.

further significant rise of MDA+4-HNE has not been observed (*Figs. 3* and *4*).

Determination of superoxide dismutase (SOD) activity

As shown in *Fig. 5*, SOD activity averaged 400±25 U/g of tissue in intact gastric mucosa. Following exposure of rats to WRS, a significant decrease of SOD activity in the value of 245.2±22.0 U/g was observed. Functional ablation of sensory nerves with capsaicin led a to significant decrease of SOD activity, when compared to the respective values in healthy rats exposed to 3.5 h of WRS. After application of SC-560 in dose 5 mg/kg i.g. (*Fig. 5*) or rofecoxib in dose 10 mg/kg i.g. (*Fig. 6*), to the rats exposed to WRS, no significant change in activity of SOD was observed. In the case of animals denervated by capsaicin and exposed to WRS, the presence of SC-560 (*Fig. 5*) failed to affect a significant change, but addition of rofecoxib (*Fig. 6*), which produced a significant fall of SOD activity.

Concentration of glutathione (GSH)

Figs. 7 and *8* shows the results of the concentration of reduced forms of glutathione (GSH) in intact gastric mucosa as well as in instances pretreated with placebo (saline) and exposed to WRS or

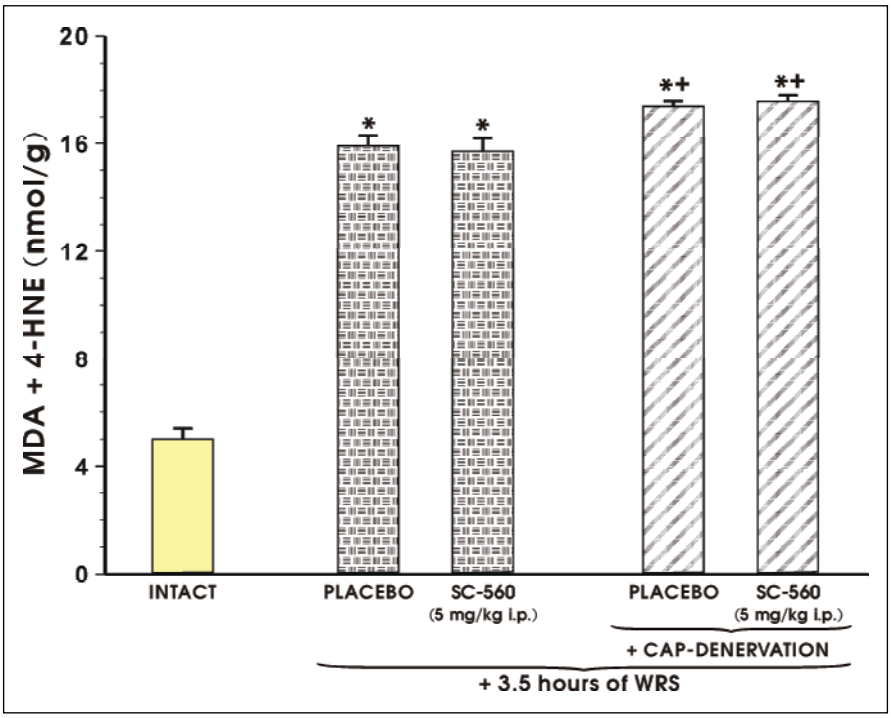

Fig. 3. Concentration of malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE) in the gastric mucosa of rats, without or with capsaicin denervation (CAP-DENERVATION) exposed to 3.5 h of WRS with intraperitoneal (i.p.) pretreatment with SC-560 (5 mg/kg.) or placebo (saline). Results are mean ±S.E.M. of 8-10 rats. Asterisk indicates a significant change as compared with the respective values obtained in intact gastric mucosa. Asterisk and cross indicate a significant change as compared with the respective values obtained in placebo and SC-560 (control) group without capsaicin denervation.

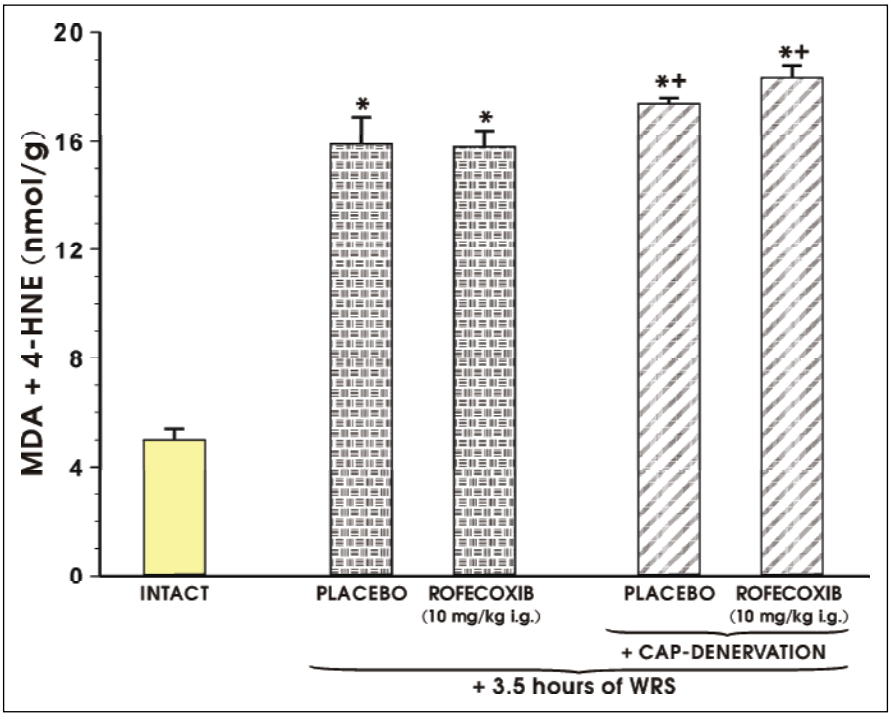

Fig. 4. Concentration of malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE) in the gastric mucosa of rats, without or with capsaicin denervation (CAP-DENERVATION) exposed to 3.5 h of WRS with intragastric (i.g.) with pretreatment with rofecoxib (10 mg/kg) or placebo (saline). Results are mean ±S.E.M. of 8-10 rats. Asterisk indicates a significant change as compared with the respective values obtained in intact gastric mucosa. Asterisk and cross indicate a significant change as compared with the respective values obtained in placebo (control) and rofecoxib group without capsaicin denervation.

in the gastric mucosa of rats treated with SC-560 or rofecoxib, with or without pretreatment of neurotoxic doses of capsaicin. Mucosal concentration of GSH in intact gastric mucosa reached the highest value and averaged 0.9±0.06 μmol/g. A significant decrease of GSH to the value of 0.63±0.02 μmol/g was measured in rats exposed to 3.5 h of WRS alone (placebo group), when compared with that in intact mucosa. Administration of SC-560 in dose 5 mg/kg i.g. (*Fig. 7*) or rofecoxib in dose 10 mg/kg i.g. (*Fig. 8*), resulted in a significant decrease of GSH level, as compared with those recorded in rats exposed to 3.5 h of WRS. Functional ablation of sensory nerves with capsaicin led to significant decrease of GSH concentration as compared with respective values in healthy rats exposed to 3.5 h of WRS. Administration of SC-560, or rofecoxib, to animals with capsaicin denervation, which underwent WRS, resulted in further significant fall of GSH tissue level, as compared to those with intact sensory nerves.

DISCUSSION

This study was designed to determine the role of ROS in the gastric mucosa subjected to selective blockers of COX-1 and COX-2 activity, SC-560 (21) and rofecoxib (22), respectively. Our second goal was to examine the effect of capsaicin denervation which is known to deplete neuropeptides, such as CGRP released from sensory afferent nerves on the acute gastric lesions and lipid peroxidation and SOD activity in rats exposed to WRS. A few years ago, rofecoxib, which is highly selective inhibitor of COX-2, known as Vioxx (12, 21, 22), had been recommended as an agent to bring relief to patients with pain during acute rheumatoid arthritis, as well as inflammation and degenerative changes. Unfortunately, the severe side effects observed after administration of Vioxx, such as disturbances in cardiovascular system functions resulted in withdrawal of this

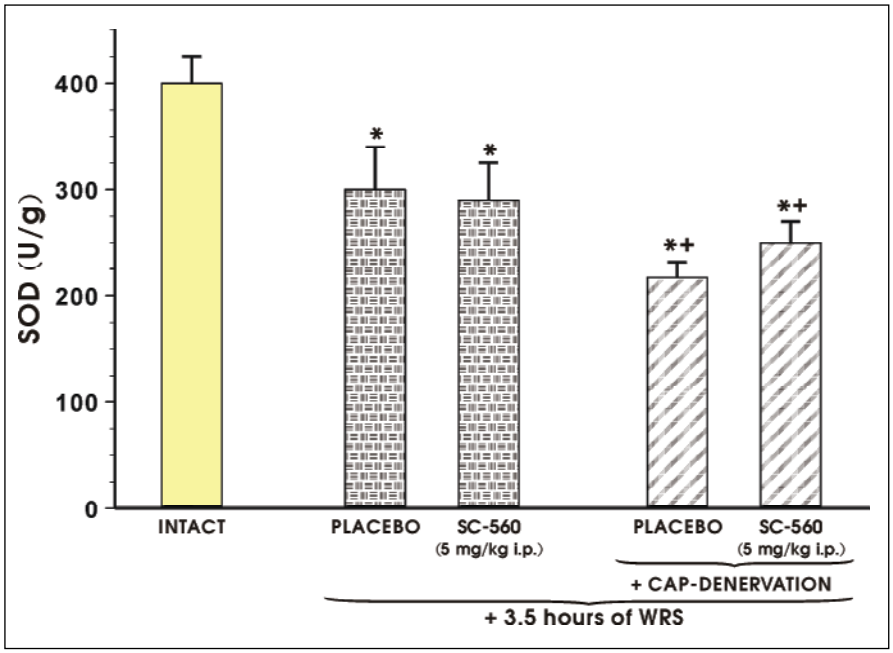

Fig. 5. Superoxide dismutase (SOD) activity in the gastric mucosa of rats, without or with capsaicin denervation (CAP-DENERVATION) exposed to 3.5 h of WRS with intraperitoneal (i.p.) pretreatment with SC-560 (5 mg/kg) or placebo (saline). Results are mean ±S.E.M. of 8-10 rats. Asterisk indicates a significant change as compared with the respective values obtained in intact gastric mucosa. Asterisk and cross indicate a significant change as compared with the respective values obtained in placebo (control) and SC-560 groups without capsaicin denervation.

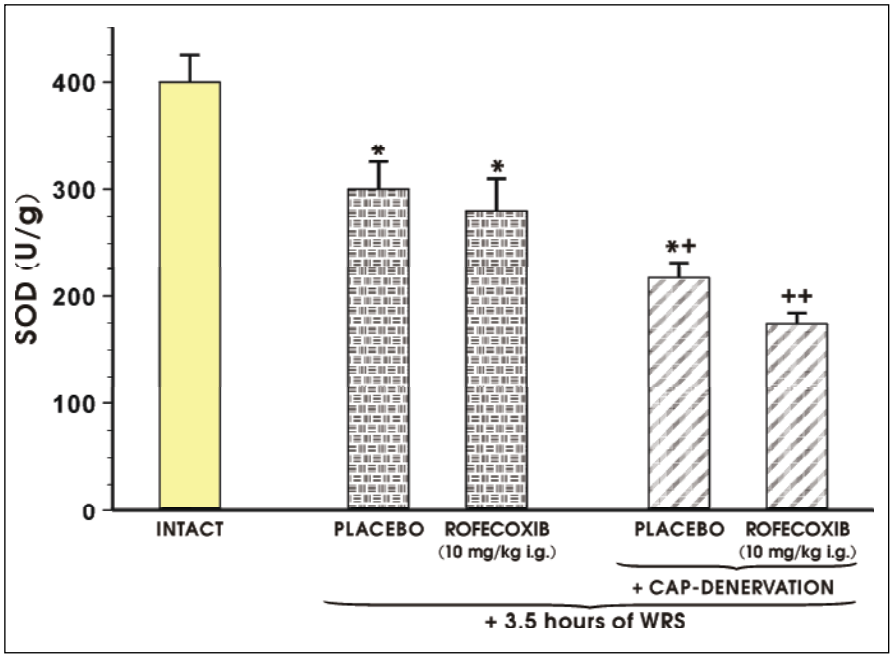

Fig. 6. Superoxide dismutase (SOD) activity in the gastric mucosa of rats, without or with capsaicin denervation (CAP-DENERVATION) exposed to 3.5 h of WRS with intragastric (i.g.) pretreatment with rofecoxib (10 mg/kg i.g.). Results are mean ±S.E.M. of 8-10 rats. Asterisk indicates significant change as compared with the respective values obtained in intact gastric mucosa. Asterisk and cross indicate a significant change as compared with the respective values obtained in placebo (control) and rofecoxib groups without capsaicin denervation. Double crosses indicate a significant change as compared to the respective values in placebo-treated capsaicin denervated rats.

drug from the market (24). Despite withdrawal of Vioxx from treatment, this substance is still useful under experimental conditions for its selectivity in COX-2 blockade (21, 23). We confirmed that the blockade of either COX-1 and COX-2 activity by administration of SC-560 and rofecoxib, respectively, worsened the WRS-induced gastric lesions and this effect was accompanied by a fall in the GBF.

The animal WRS model seems to reproduce both local and systemic consequences of stress exposure to the upper GI-tract resulting in the formation of bleeding gastric erosions and a fall in the microcirculation. These effects could be due to an increase of the production of catecholamines by suprarenal glands, which lead to gastric vasoconstriction, local ischemia and resulted in gastric lesions (14, 15, 20). This restraint animal model mimics clinical acute gastric ulcerations in humans caused by major trauma, surgery or sepsis, which is being widely accepted for studying the mechanism of stress-induced gastric lesions (19, 25). Acute inflammation in gastric mucosa contributes to the pathomechanism of WRS ulcerogenesis, which seems to be responsible for the enhanced permeability of blood vessels to neutrophils (2, 19, 20). It is known that neutrophils are equipped in the NADPH oxidase in their membrane, and they are considered to be a principal source of a superoxide radical anion ($O_2^{\bullet -}$). In our present study, the suppression of the antioxidative mechanisms, in particular the reduction of the mucosal SOD activity and mucosal GSH content induced by COX-1 and COX-2 blockade, could contribute to the augmentation of ROS-induced lipid peroxidation (expressed as an increase of MDA+4-HNE concentration) and exacerbation of WRS-induced gastric lesions (15, 19, 20, 26). This is in keeping with the results of our previous studies that ROS are involved in the formation of WRS-induced gastric mucosal damage due to an enhancement of

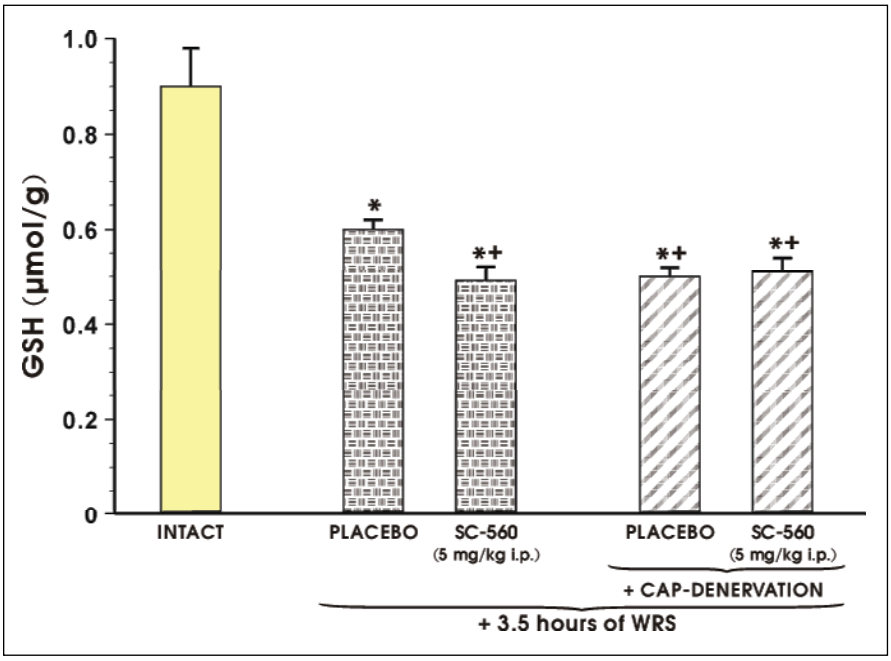

Fig. 7. Concentration of reduced glutathione (GSH) in the gastric mucosa of rats, without or with capsaicin denervation (CAP-DENERVATION) exposed to 3.5 h of WRS with intraperitoneal (i.p.) pretreatment with SC-560 (5 mg/kg) or placebo (saline). Results are mean ±S.E.M. of 8-10 rats. Asterisk indicates significant change as compared with the respective values obtained in intact gastric mucosa. Asterisk and cross indicate a significant change as compared with the respective values obtained in placebo (control) group without capsaicin denervation.

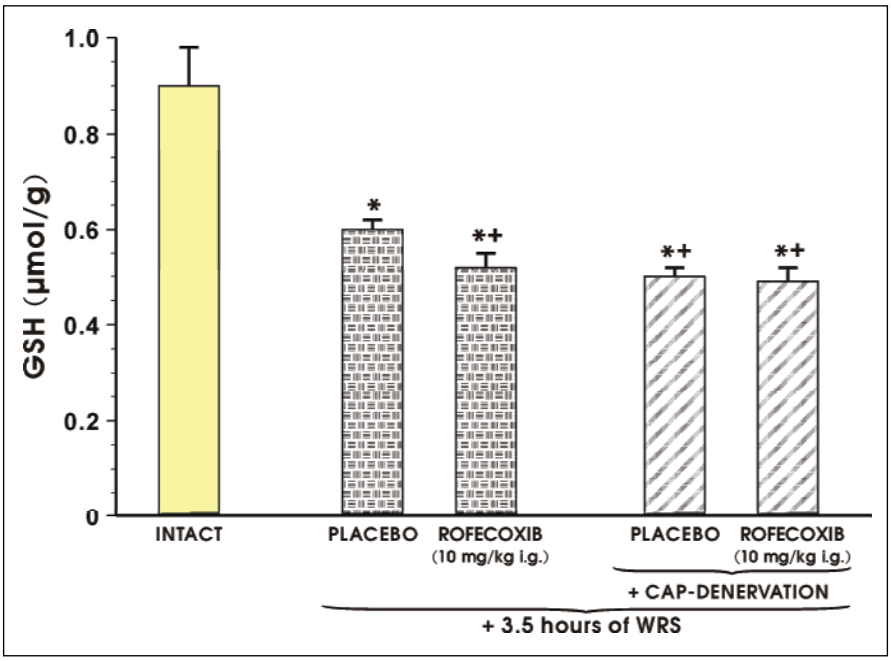

Fig. 8. Concentration of reduced glutathione (GSH) in the gastric mucosa of rats, without or with capsaicin denervation (CAP-DENERVATION) exposed to 3.5 h of WRS with intragastric (i.g.) pretreatment with rofecoxib (10 mg/kg i.g.) or placebo (saline). Results are mean ±S.E.M. of 8-10 rats. Asterisk indicates significant change as compared with the respective values obtained in intact gastric mucosa. Asterisk and cross indicate a significant change as compared with the respective values obtained in placebo (control) group without capsaicin denervation.

lipid peroxidation and attenuation of mucosal antioxidative mechanisms (19, 20). In addition, we found that the severe impairment of the gastric mucosa in rats subjected to capsaicin denervation could be also due to the increase in both the production of free oxygen radical and lipid peroxidation as well as an impairment to the antioxidizing enzyme activity by a decreasing GSH tissue level.

One of our primary goal was to determine the antioxidative potential of gastric mucosa, where the depletion of GSH pool in tissues takes place, perhaps after administration of gastric mucosa to COX inhibitors in animals exposed to WRS. The GSH contributes to the mechanism of gastric integrity by GSH-4HNE conjugates production, as an effective mechanism of toxic products of lipid peroxidation elimination by reduced form of glutathione (26, 27). Moreover, Miyamoto *et al.* (28) documented, that excess of lipid peroxide was hydrolysed by phospholipase A_2 and then reduced by glutathione peroxidise. It was important, that glutathione peroxidise required large amounts of GSH for its function and the action of this enzyme led to depletion of cellular GSH store. These results were in part confirmed by Altinkaynak *et al.* (29), who reported that rofecoxib is capable of decreasing the GSH pool in rats with indomethacin-induced gastric lesions. However, in our study this evidently deleterious effect of rofecoxib on gastric lesions induced by WRS was accompanied by a lack of any macroscopic changes in intact, healthy mucosa not exposed to WRS, as well as it did not produce alterations in the parameters of oxidative stress, such as GSH in intact gastric mucosa. This indicates that depletion of the GSH storage pool observed in animals exposed to WRS and administered with COX-2 inhibitor is not a prerequisite for development of gastric lesions. Peskar *et al.* (30). observed that in the absence of ulcerogenic factors, rofecoxib had no influence on the intact gastric mucosa. This could be explained by the fact that there is a lack of COX-2 expression in healthy gastric mucosa, while in other organs such as heart and kidneys, COX-2 is a constitutive target for rofecoxib action.

Previous studies, related to the involvement of capsaicin-sensitive afferent fibers, focused mainly on their function in visceral circulation. It was documented that capsaicin-sensitive nerves play an important a role in the physiology of the gut, especially in the modulation of gastrointestinal blood flow (GBF) and metabolism (31). This action could be inhibited by capsaicin applied in large doses to these animals. The aim of our present investigations was to determine role of sensory nerves in the pathogenesis of WRS-induced gastric damage and GBF under conditions where COX-2 was inhibited by selective COX-2 inhibitor or COX-1 inhibitor. For this purpose, animals were treated with high doses of capsaicin. Previous studies documented that sensory nerve ablation caused exacerbation of acute gastric lesions and delayed gastric ulcer healing (16). On the other hand, small or moderate dose of capsaicin increased GBF and exerted a gastro-protective effect and these changes were attributed, at least in part, to the release of endogenous prostaglandins (1, 25).

As shown in this study, an increase of oxygen metabolism evoked by WRS was manifested by an increase of lipid peroxidation products, a decrement of SOD activity and depletion of GSH content. Capsaicin denervation resulted in further elevation of mucosal MDA and 4-HNE levels, indicating oxidative-dependent tissue damage. Interestingly, capsaicin-denervated animals exhibited the decrease in SOD activity, again, supporting the notion that lack of sensory innervation could contribute to the attenuation of antioxidative properties of gastric tissues in animals exposed to WRS. Additional application of rofecoxib resulted in further decrease of SOD activity in capsaicin-deactivated animals. These results provided evidence for the participation of ROS in the pathomechanism of gastric lesions associated with a fall in the GBF evoked by capsaicin denervation in animals exposed to WRS.

Our results differ with those published by Erin *et al.* (32), who failed to show an univocal association between lipid peroxidation and the formation of gastric lesions induced by thermal stress in rats. However, these authors (32) performed thermal stress under different experimental conditions, including different duration of stress and temperature conditions applied to produce gastric lesions. Moreover, in their study capsaicin was administered topically to the region of the coeliac plexus in the dose range smaller then in our study (32). On the contrary, we used animals with systemic application of capsaicin in order to ablate functionally the sensory nerves, as proposed previously (16, 21).

The effect of capsaicin on gastric cells might differ depending on whether the studies were carried out using *in vivo* or *in vitro* techniques. Kogure *et al.* (33), have demonstrated a potent antioxidative property of capsaicin on liver mitochondria. However, their study (33) was carried out in cell cultures *in vitro*, while our experiments were performed *in vivo* conditions. Moreover, in their study (33) capsaicin was used in low, non-cytotoxic dose, that is known to play a role of radical scavenger in a region of mitochondrial membranes. In contrast we have employed, capsaicin in high dose, capable of causing the deactivation of capsaicin-sensitive fibers in animals exposed to WRS. Harada *et al.* (34) have demonstrated that capsaicin-sensitive sensory neurons released CGRP, there by increasing the gastric tissue levels of PGE_2 and PGI_2 by activating COX-1 through stimulation of the constitutive forms of NOS in rats subjected to stress.

In summary we conclude, that WRS caused a significant increase of MDA and 4-HNE mucosal level, accompanied by a decrease of SOD activity and GSH level. Pretreatment with specific COX-1 and COX-2 inhibitors (SC-560, rofecoxib) exacerbated of gastric mucosal injury accompanied by a decrement of GBF, fall in the GSH, with a further significant change of MDA and 4-HNE tissue levels as well as SOD activity. Moreover, we demonstrated that inactivation of sensory nerves by capsaicin, also aggravated gastric mucosal damage induced by WRS and increased 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 and decrement of the GSH tissue level. Addition of COX specific inhibitors in animals with ablation of these fibers led to a further aggravation of these changes, especially in the cases of rofecoxib administration suggesting that COX-2 products and neuropeptides released from sensory nerves interact for the protection of the gastric mucosa, especially when challenged by noxious stimuli such as stress.

Conflict of interests: None declared.

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