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## GASTROPROTECTION BY PENTOXIFYLLINE AGAINST STRESS-INDUCED GASTRIC DAMAGE. ROLE OF LIPID PEROXIDATION, ANTIOXIDIZING ENZYMES AND PROINFLAMMATORY CYTOKINES

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Impairment of blood perfusion in gastric mucosa results in the formation of erosions and ulcers. Nitric oxide (NO), produced *via* activity of NO-synthase (NOS), appears to be a one of major factors, involved in the regulation of the gastric blood flow (GBF). Inhibition of this enzyme by N-nitro-L-arginine (L-NNA) results in local decrease of NO production, reduces GBF and impairs gastric mucosal integrity, the effects that can be reversed by the pretreatment with L-arginine, the NOS substrate. However, little information is available regarding the contribution of reactive oxygen species (ROS)-induced lipid peroxidation and NO to the mechanism of gastric mucosal integrity. Therefore, the aim of our present study was to determine the action of pentoxifylline (PTX), an inhibitor of tumor necrosis factor alpha (TNF $\alpha$ ) with or without NOS inhibition by L-NNA administration in rats with water immersion and restraint stress (WRS)-induced gastric lesions. Experiments were carried out on 100 male Wistar rats. The gastric blood flow (GBF) was measured using laser Doppler flowmeter. The area of gastric lesions was determined by planimetry and the levels of proinflammatory cytokines (IL-1 $\beta$  and TNF $\alpha$ ) were measured by ELISA. Colorimetric assays were employed to determine gastric mucosal levels of lipid peroxidation products, such as malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE) and antioxidant enzymes including superoxide dismutase (SOD) activity, as well as tissue concentration of reduced glutathione (GSH). Administration of PTX significantly attenuated the gastric lesions, induced by 3.5 h of WRS and this was accompanied by the rise in the GBF and a significant decrease in plasma proinflammatory cytokines (IL-1 $\beta$  and TNF $\alpha$ ) levels, as well as the reduction of lipid peroxidation. Exposure of rats to WRS suppressed the SOD and GSH activities and these effects were reversed by PTX. The protective and hyperemic effects of PTX, as well as an increase in mucosal SOD activity and GSH concentration were counteracted by pretreatment with L-NNA, but restored by the pretreatment with L-arginine, a NOS substrate. We conclude that PTX exerts beneficial, gastroprotective effect against WRS-induced gastric lesions due to enhancement in gastric microcirculation, possibly mediated by the enhanced NOS activity as well as local action of NO and by the attenuation of oxidative metabolism and generation proinflammatory cytokines.

Key words: *water immersion restraint stress, pentoxifylline, malondialdehyde, nitric oxide, interleukin-1 beta, tumor necrosis factor alpha*

## INTRODUCTION

Integrity of the gastric mucosa depends upon a variety of factors, such as particularly maintenance of microcirculation, mucus-alkaline secretion and activity of antioxidizing enzymes, such as superoxide dismutase (SOD) and glutathione peroxidase (GPx). These factors were also implicated in the gastroprotection against exogenous and endogenous irritants originating from lumen of stomach (1-3). Previous studies revealed that disturbances of blood perfusion of gastric mucosa may result in the formation of erosions and ulcers (4-6). Nitric oxide (NO) is widely accepted as a major factor, involved in control of the gastric blood flow (GBF) and in the maintenance of gastric mucosal integrity (7-10). NO is produced by cNOS and iNOS enzymatic pathways (11). Inhibition of NO-synthase (NOS) that causes decrease in local NO production, impairs gastric microcirculation and aggravates gastric lesions induced by noxious agents (12,13). N-nitro-L-arginine (L-NNA), the nonspecific NOS inhibitor, delays ulcer healing and the accompanying increase in GBF at ulcer margin and these effects can be reversed by application of L-arginine (14-16). However, little information is available regarding the contribution of reactive oxygen species (ROS) in mediation of lipid peroxidation and NO and proinflammatory cytokines expression in the mechanism of gastric mucosal integrity. Results of our previous studies indicate that the exposure of rats to 3.5 hours of water immersion restraint stress (WRS) leads to an increase in oxidative metabolism, comparable to that observed in ischemia-reperfusion model of gastric injury (17). WRS is known to provoke acute inflammation and injury of gastric mucosa. The interleukin-1 beta (IL-1 $\beta$ ) and tumor necrosis factor alpha (TNF $\alpha$ ) are the major proinflammatory cytokines, playing important role in the development of acute inflammation, mediated by neutrophil infiltration of gastric mucosa (17,18). Neutrophils produce superoxide radical anion (O<sub>2</sub><sup>•-</sup>), which belongs to group of ROS. Superoxide radical anion reacts with cellular membrane lipids, leading to the formation of lipid peroxides, that are metabolized to malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE). Gastric mucosa possesses an effective enzymatic system, which scavenges ROS and prevents their destructive action on this mucosa (*Fig. 1*). The first line antioxidative enzyme in gastric mucosa is superoxide dismutase (SOD), that catalyzes the dismutation of superoxide radical anion (O<sub>2</sub><sup>•-</sup>) into less noxious hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), that is further inactivated by degradation by glutathione peroxidase (GPx). The reduction of H<sub>2</sub>O<sub>2</sub> into water by GPx is accompanied by the conversion of glutathione from reduced form (GSH) into oxidized form (GSSG) (17,19) (*Fig. 1*).

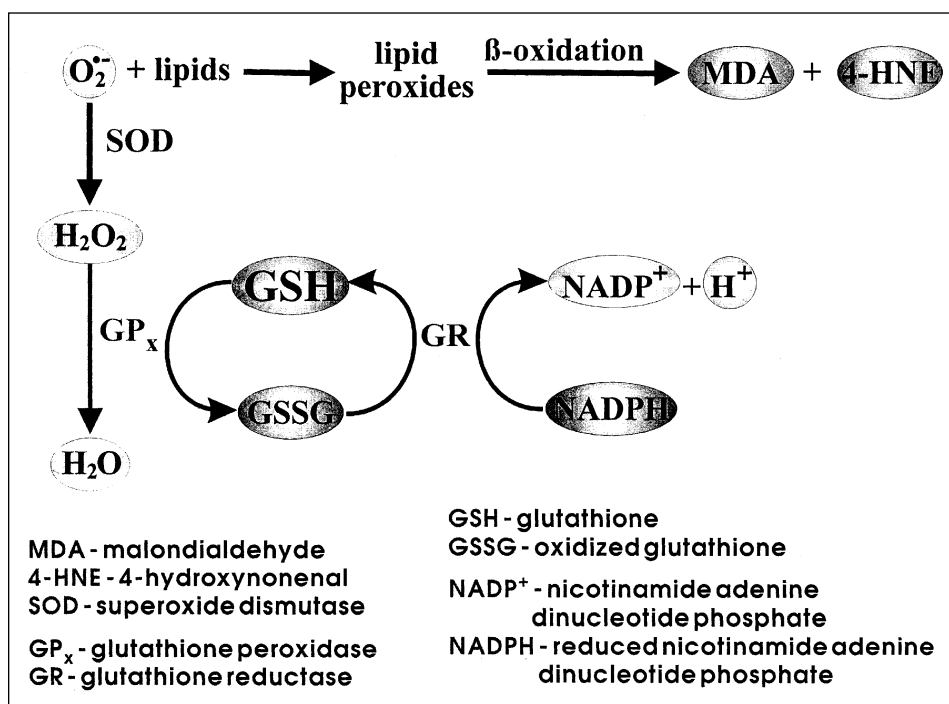


Fig. 1 Transformation pathways of a superoxide radical anion ( $O_2^{\bullet -}$ ) in the body. The role of glutathione metabolism in neutralization of reactive oxygen species is also emphasized.

We have demonstrated that the inhibition of NO synthesis by suppressing of NOS results in delay of the healing of gastric ulcers induced by WRS. However, the role of ROS mediated lipid peroxidation in WRS ulcerogenesis has been little studied. Therefore, the aim of our present investigations was to determine the role of oxidative stress in gastroprotection by using pentoxifylline (PTX), an inhibitor of  $TNF\alpha$  activity, against WRS-induced gastric lesions and accompanied changes in the GBF.

#### MATERIAL AND METHODS

Experiments were carried out on 100 male Wistar rats, weighing 200-240 g and fasted for 24 h before all studies. Studies were approved by the Ethic Committee for Animal Research of Jagiellonian University.

#### *Production of gastric lesions*

The animals were divided into 7 groups. In group 1, rats underwent 3.5 h of WRS in water temperature of  $23^\circ\text{C}$ , using the method originally proposed by Takagi et al. (20). In group 2, PTX was applied in graded doses from 2.5 mg/kg (i.p.) to 20 mg/kg (i.p.) prior to WRS. In group 3, PTX

was administered intraperitoneally (i.p.) in a dose of 10 mg/kg 30 min. prior to WRS. In group 4, N<sup>ϵ</sup>-nitro-L-arginine (L-NNA) was applied intraperitoneally (20 mg/kg i.p.) alone or in the combination with PTX (10 mg/kg i.p.) prior to WRS. Group 5 was pretreated with L-arginine, applied intragastrically (i.g.), in a dose of 50 mg/kg followed 30 min. later by L-NNA (20 mg/kg i.p.) without or with following administration of PTX (10 mg/kg i.p.), in animals subsequently exposed to 3.5 h of WRS. Group 6 of intact 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 at the end of 3.5 h 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 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 (17,19).

#### *Determination of plasma IL-1 $\beta$ and TNF $\alpha$ levels*

A venous blood sample was withdrawn from the vena cava into EDTA-containing vials in order to determine the plasma level of interleukin-1 beta (IL-1 $\beta$ ) and tumor necrosis factor alpha (TNF $\alpha$ ) by ELISA technique (BioSource International, Camarillo, CA, USA). Details of this method were described previously (17,19,21).

#### *Measurement of lipid peroxydation*

For determination of lipid peroxydation in gastric mucosa of tested groups, tissue levels of malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE) were measured and used as indicators of lipid peroxidation. The procedure of MDA and 4-HNE determination was following: about 600 mg of gastric mucosa was excised, quickly washed in test tube and 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 sec. in pH 7.4. Then homogenate was centrifuged (3000 g at 4°C for 10 min) and 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 concentration. 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 nanomol per gram of tissue (nmol/g) according to our studies published previously (17,19).

#### *Determination of SOD activity*

For determination of activity of SOD, a sample of gastric mucosa was taken, 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. Results were expressed as units per gram of tissue (U/g) (17).

### *Measurement of glutathione level (GSH)*

To determine the level of reduced form of glutathione (GSH) a sample of gastric mucosa was taken, as described above. Then 5% aqueous solution of metaphosphoric acid was added in order to evoke protein precipitation. Then colorimetric assay for assessment of reduced glutathione concentration (Bioxytech, GSH-400, Oxis, Portland, USA) was used. The level of reduced glutathione was measured, with maximal absorbance at 400 nm, by spectrophotometer Marcel s300, Warsaw, Poland. Results were expressed as micromole per gram of tissue ( $\mu\text{mol/g}$ ) (19).

### *Statistical analysis*

Results are expressed as means  $\pm$ SEM. 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)*

*Fig. 2* shows the effect of administration of PTX, applied i.p., 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 PTX caused dose-dependent reduction of mean number of WRS lesions and significantly raised of the GBF. Maximal protective effect was exerted by PTX injected in a dose of 10 mg/kg (i.p.). Higher dose of 20 mg/kg failed to cause a significant enhancement in gastroprotection, above that observed with PTX given in a dose of 10 mg/kg (i.p.).

*Table 1* exhibits mean number of gastric lesions and GBF in rats exposed to 3.5 h of WRS with placebo, L-NNA (20 mg/kg i.p.) or L-arginine (50 mg/kg i.g.) without PTX treatment. We established that L-NNA applied prior WRS resulted in a significant increase of gastric damage and a significant attenuation of GBF, as compared to those recorded in placebo group. Pretreatment with L-arginine in dose of 50 mg/kg failed to influence significantly the mean lesion number and GBF, and therefore the dose of 50 mg/kg of L-arginine was employed in this study to elucidate the effects of the combination of L-arginine plus L-NNA on gastroprotection by PTX against WRS-induced gastric lesions.

As shown in *Fig.3* intact gastric mucosa did not exhibit any macroscopic lesions and the GBF in this mucosa averaged  $44 \pm 6$  ml/min/100 g of tissue, being accepted as the control value (100%). Following 3.5 h of WRS, numerous acute gastric mucosal lesions occurred and the mean number of these lesions was  $22 \pm 2$  while the GBF was reduced to  $60 \pm 3\%$  of control value. Pretreatment with PTX (10 mg/kg i.p.) resulted in a significant reduction of gastric lesion number and these effects were accompanied by an increase of GBF as compared to rats

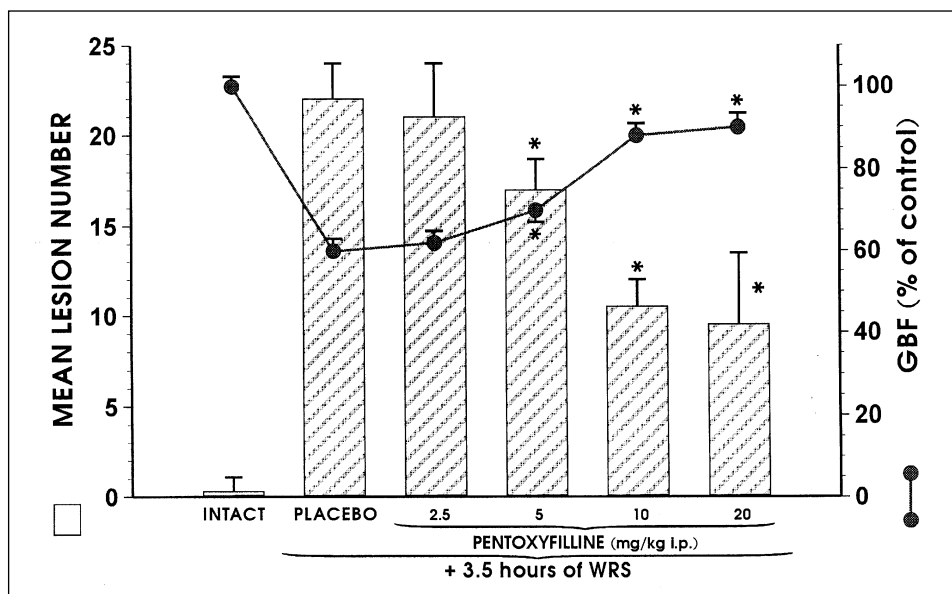


Fig. 2 Mean number of gastric lesions and gastric blood flow (GBF) in rats exposed to 3.5 h of water immersion restraint stress (WRS) without or with intraperitoneal (i.p.) pretreatment with pentoxifylline applied in graded doses. Results are mean  $\pm$ SEM of 8-10 rats. Asterisk (\*) indicates a significant change as compared to the values obtained in rats exposed to 3.5 h of WRS alone (placebo group).

Table 1 Mean number of gastric lesions and gastric blood flow (GBF) in rats exposed to 3.5 h of water immersion restraint stress (WRS) with placebo, L-NNA given intraperitoneally (i.p.) or L-arginine administered intragastrically (i.g.). Asterisk (\*) indicates a significant change as compared to the value obtained in group: placebo + WRS. Cross (+) indicates a significant change as compared to the value obtained in group: L-NNA 20 mg/kg (i.p.) + WRS.

Type of test	Mean lesion number	GBF (% control)
Placebo+WRS	22 $\pm$ 2	60 $\pm$ 3
L-NNA 20 mg/kg (i.p.) + WRS	26 $\pm$ 3*	51 $\pm$ 2 *
L-arginine 50 mg/kg (i.g.) + WRS	21 $\pm$ 4	69 $\pm$ 5
L-arginine 50 mg/kg (i.g.) + L-NNA 20 mg/kg (i.p.) + WRS	20 $\pm$ 3 <sup>+</sup>	62 $\pm$ 4 <sup>+</sup>

pretreated with placebo and exposed to 3.5 h of WRS. Combination L-NNA (20 mg/kg i.p.) with PTX (10 mg/kg i.p.) resulted in the significant increase in the number of gastric lesions and the fall in the GBF, as compared to those observed in PTX-treated rats without L-NNA administration. The addition of L-arginine (50 mg/kg i.g.) to L-NNA (20 mg/kg i.p.) restored the significant decrease in gastric lesion number and produced a significant increase in the GBF, in animals pretreated with PTX.

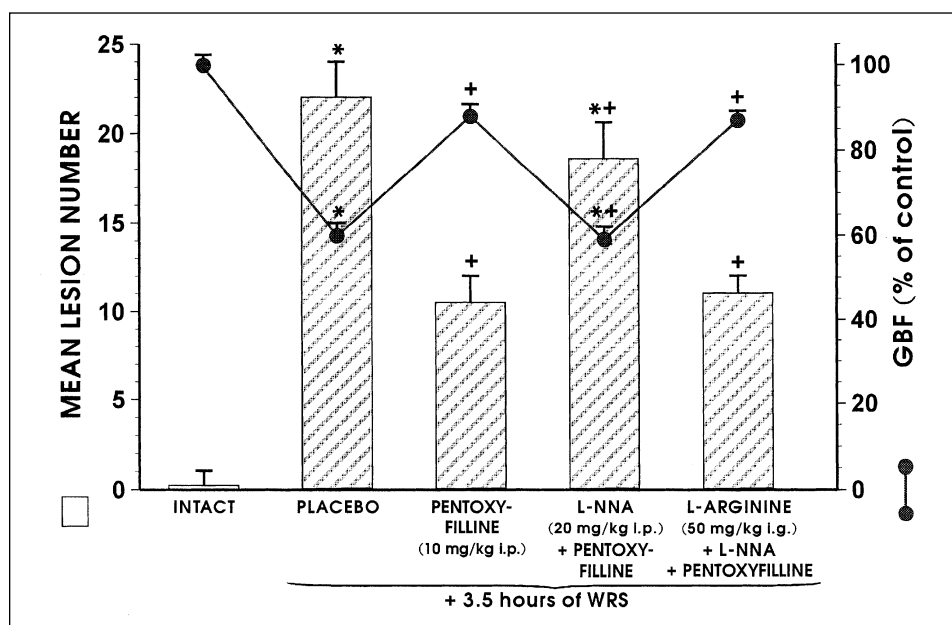


Fig. 3 Mean number of gastric lesions and gastric blood flow (GBF) in rats exposed to 3.5 h of water immersion restraint stress (WRS) without or with intraperitoneal (i.p.) pretreatment with pentoxifylline (10 mg/kg) applied alone or given in the combination with L-NNA (20 mg/kg i.p.) or with intragastric (i.g.) administration with L-arginine (50 mg/kg) added to L-NNA. Results are mean  $\pm$ SEM of 8-10 rats. Asterisk (\*) indicates a significant change as compared with the respective values obtained in intact gastric mucosa (control group). Cross (+) indicates a significant change as compared with the respective values obtained in rats exposed to 3.5 h of WRS alone (placebo group). Asterisk and cross (\*\*+) indicate a significant change as compared with the value obtained in rats pretreated with pentoxifylline.

#### Plasma levels of IL-1 $\beta$ and TNF $\alpha$

As shown in Fig. 4 the plasma concentration of IL-1 $\beta$  in intact (control) animals averaged  $4 \pm 1$  pg/ml. After 3.5 h of WRS, a significant rise in IL-1 $\beta$  level was noticed, averaging  $40 \pm 5$  pg/ml. Application of PTX (10 mg/kg i.p.), prior to WRS, led to a significant decrease in plasma IL-1 $\beta$  levels, as compared to that measured in the 3.5 h of WRS rats pretreated with placebo (saline). The combined treatment with L-NNA (20 mg/kg i.p.) and PTX (10 mg/kg i.p.), restored the plasma IL-1 $\beta$  to the level observed in placebo pretreated rats, exposed to WRS. The addition of L-arginine (50 mg/kg i.g.) to L-NNA (20 mg/kg i.p.) in rats treated with PTX (10 mg/kg i.p.) resulted in similar decrease of IL-1 $\beta$  level to that recorded in rats given PTX alone without L-arginine administration (Fig. 4).

In intact rats the concentration of TNF $\alpha$  in the plasma remained at low level ( $1.6 \pm 0.5$  pg/ml) (Fig.5). Following 3.5 h of WRS, there was a remarkable rise in of TNF $\alpha$  concentration, reaching the value of about 10 pg/ml. Pretreatment

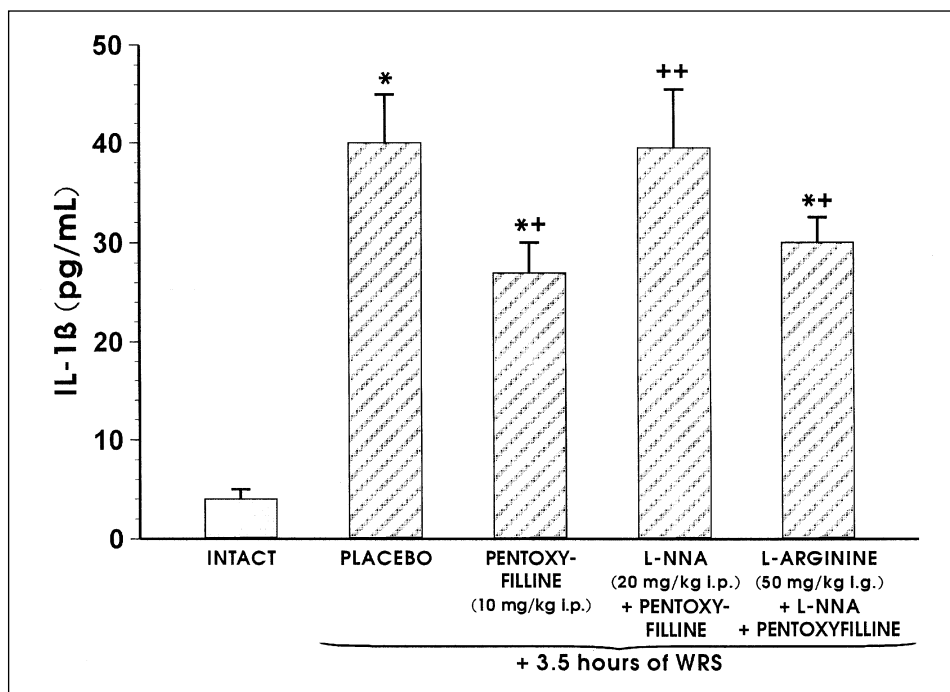


Fig. 4 Plasma level of interleukin-1 beta (IL-1 $\beta$ ) in rats exposed to 3.5 h of water immersion restraint stress (WRS) without or with pentoxifylline (10 mg/kg i.p.) applied alone or given in the combination with L-NNA (20 mg/kg i.p.) or with L-arginine (50 mg/kg i.g.) added to L-NNA. Results are mean  $\pm$ SEM of 8-10 rats. Asterisk (\*) indicates a significant change as compared with the value obtained in intact gastric mucosa (control group). Asterisk and cross (\*+) indicates a significant change as compared with the value obtained in rats exposed to 3.5 h of WRS alone (placebo group). Double cross (++) indicates a significant change as compared with the value obtained in rats with pentoxifylline pretreatment.

with PTX resulted in a significant decrease in plasma TNF $\alpha$  level, as compared with that subjected to 3.5 h of WRS alone. L-NNA, added to PTX, caused a small but not significant increase in the plasma TNF $\alpha$  and this effect was attenuated by addition of L-arginine to L-NNA in PTX-treated animals (Fig. 5).

#### Lipid peroxidation products

Concentration of MDA and 4-HNE in intact mucosa (control group) was relatively very low, almost at the level of analytical limit of detection and averaged  $5.5 \pm 0.8$  nmol/g. After 3.5 h of WRS the MDA and 4-HNE concentration showed almost three fold increase, reaching the value of  $16 \pm 1$  nmol/g. Application of PTX (10 mg/kg i.p.) resulted in a significant decrease of lipid peroxidation products to the level not significantly different from that recorded in intact mucosa (control group). Pretreatment with L-NNA (20 mg/kg



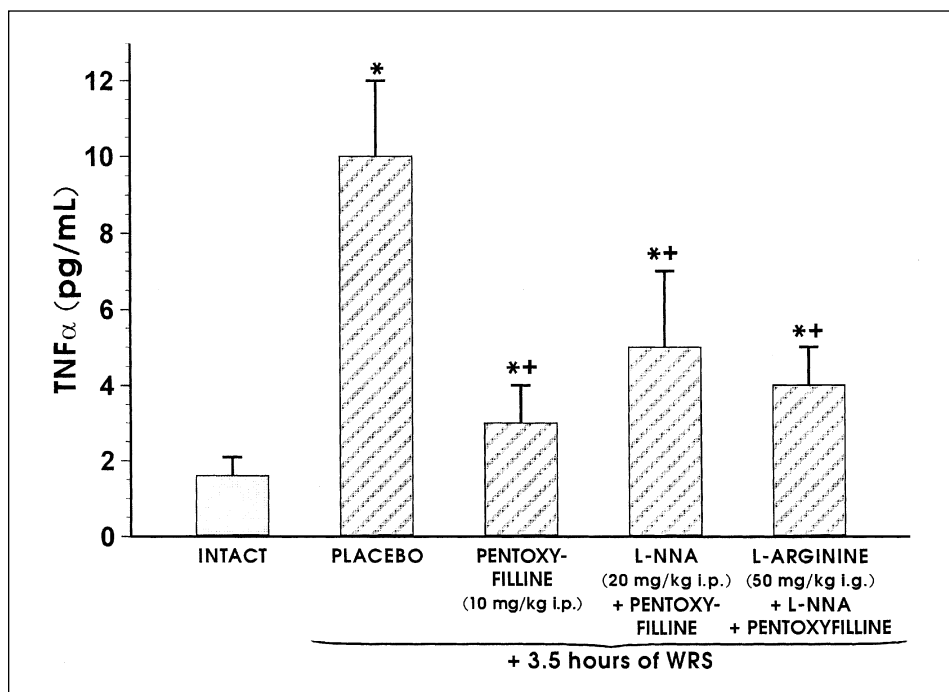
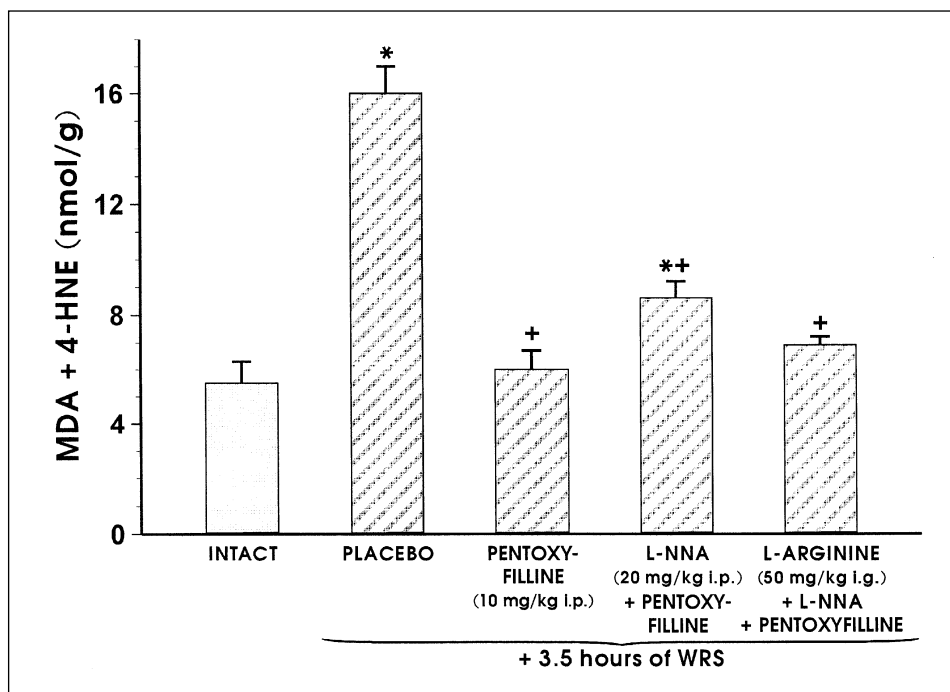


Fig. 5 Plasma level of tumor necrosis factor alpha (TNF $\alpha$ ) in rats exposed to 3.5 h of water immersion restraint stress (WRS) without or with pentoxifylline (10 mg/kg i.p.) applied alone or given in the combination with L-NNA (20 mg/kg i.p.) or with L-arginine (50 mg/kg i.g.) added to L-NNA. Results are mean  $\pm$ SEM of 8-10 rats. Asterisk (\*) indicates a significant change as compared with the value obtained in intact gastric mucosa (control group). Asterisk and cross (\*\*+) indicate a significant change as compared with the values obtained in rats exposed to 3.5 h of WRS pretreated with placebo.

i.p.) produced a small but significant increment in MDA and 4-HNE level, as compared with that recorded in PTX-treated animals and addition of L-arginine (50 mg/kg i.g.) to L-NNA (20 mg/kg i.p.) significantly restored the contents of lipid peroxidation products to the values detected in PTX-treated rats (Fig. 6).

#### SOD activity

In intact gastric mucosa (control group) SOD activity averaged  $375 \pm 20$  U/g. Following exposure of rats to 3.5 h of WRS, a significant decrease of SOD activity (by about 30%) was observed. Administration of PTX (10 mg/kg i.p.) caused a marked and significant rise of SOD activity, above that observed in the 3.5 h of WRS exposed rats (placebo group). Pretreatment with L-NNA (20 mg/kg i.p.) prior to PTX (10 mg/kg i.p.) produced a small but significant decrease in SOD activity, as compared to PTX-alone and addition of L-arginine (50 mg/kg i.g.) to L-NNA (20 mg/kg i.p.), prior to intraperitoneal PTX application, resulted



*Fig. 6* Concentration of malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE) in the gastric mucosa of rats exposed to 3.5 h of water immersion restraint stress (WRS) without or with pentoxifylline (10 mg/kg i.p.) applied alone or given in the combination with L-NNA (20 mg/kg i.p.) or with L-arginine (50 mg/kg i.g.) added to L-NNA. Results are mean  $\pm$ SEM of 8-10 rats. Asterisk (\*) indicates a significant change as compared with the value obtained in intact gastric mucosa (control group). Cross (+) indicates a significant change as compared with the value obtained in rats exposed to 3.5 h of WRS. Asterisk and cross (\*\*+) indicate a significant change as compared to the value obtained in rats pretreated with pentoxifylline.

in the increment of SOD activity similar to that observed in the PTX-treated rats without L-NNA pretreatment (*Fig. 7*).

#### *Concentration of glutathione*

*Fig. 8* shows the results with the concentration of reduced form of glutathione (GSH) in intact gastric mucosa as well as in that pretreated with placebo (saline) and exposed to WRS or in the gastric mucosa of rats treated with PTX with or without L-NNA and L-arginine pretreatment. Mucosal concentration of GSH in intact gastric mucosa reached the highest value and averaged  $0.9 \pm 0.06$   $\mu$ mol/g. A significant decrease of GSH to the value of  $0.63 \pm 0.02$   $\mu$ mol/g was measured in rats exposed to 3.5 h of WRS alone (placebo group), when compared with that in intact mucosa. Administration of PTX (10 mg/kg i.p.), before the WRS, resulted in a significant increase of GSH level, as compared with that recorded

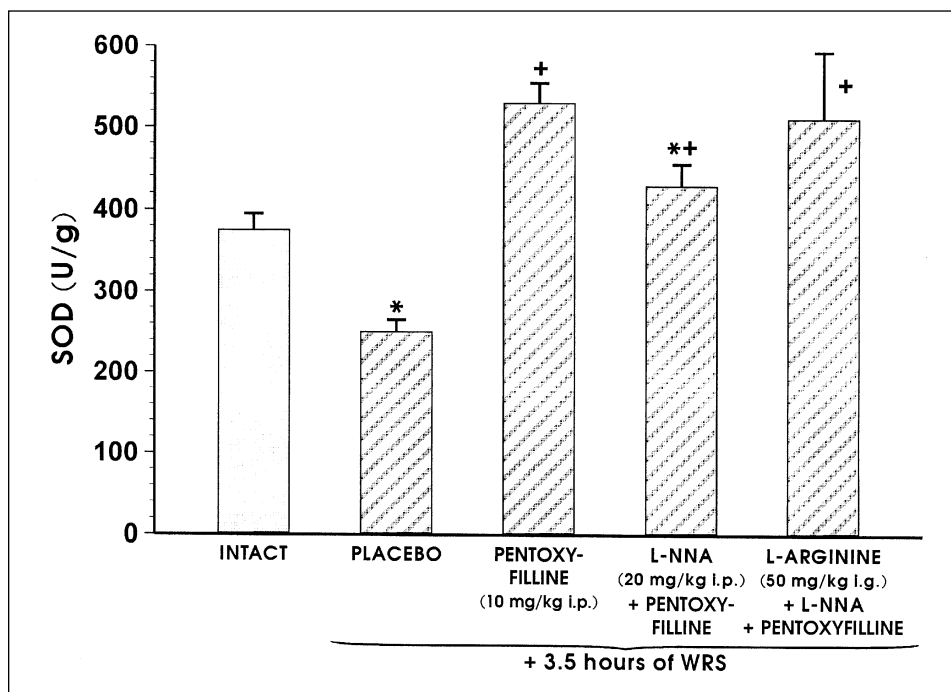


Fig. 7. Superoxide dismutase (SOD) activity in the gastric mucosa of rats exposed to 3.5 h of water immersion restraint stress (WRS) without or with pentoxifylline (10 mg/kg i.p.) applied alone or given in the combination with L-NNA (20 mg/kg i.p.) or with L-arginine (50 mg/kg i.g.) added to L-NNA. Results are mean  $\pm$ SEM of 8-10 rats. Asterisk (\*) indicates a significant change as compared with the respective value obtained in intact gastric mucosa (control group). Cross (+) indicates a significant change as compared with the value obtained in rats pretreated with placebo and exposed to 3.5 h of WRS. Asterisk and cross (\*\*+) indicate a significant change as compared to the value obtained in rats pretreated with pentoxifylline.

in rats exposed to 3.5 h of WRS. Pretreatment with L-NNA (20 mg/kg i.p.) significantly decreased the GSH concentration, when compared to that recorded in placebo or PTX-pretreated rats, but addition of L-arginine (50 mg/kg i.g.) to L-NNA (20 mg/kg i.p.) markedly increased the mucosal GSH level, as compared with the value observed in L-NNA treated rats without L-arginine pretreatment (Fig. 8).

#### DISCUSSION

The animal WRS model seems to be especially suitable for examination of both local and systemic consequences of damaging action of stress on the gastric mucosa. WRS results in attenuation of microcirculation due to increase of

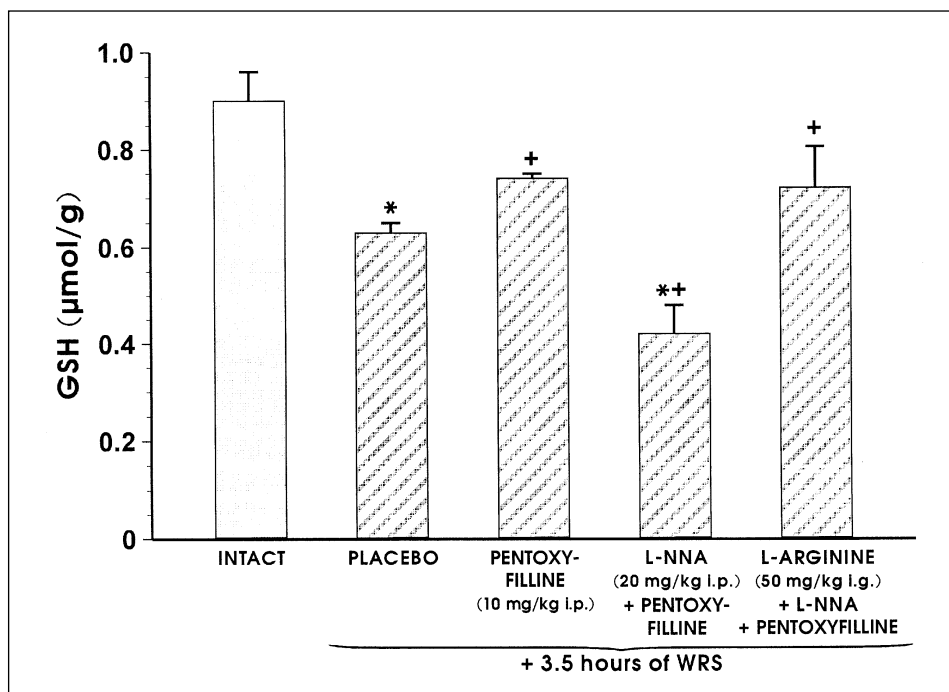


Fig. 8. Concentration of reduced glutathione (GSH) in the gastric mucosa of rats exposed to 3.5 h of water immersion restraint stress (WRS) without or with pentoxifylline (10 mg/kg i.p.) applied alone or given in the combination with L-NNA (20 mg/kg i.p.) or with L-arginine (50 mg/kg i.g.) combined with L-NNA. Results are mean  $\pm$ SEM of 8-10 rats. Asterisk (\*) indicates a significant change as compared with the value obtained in intact gastric mucosa (control group). Cross (+) indicates a significant change as compared with the value obtained in placebo pretreated rats exposed to 3.5 h of WRS. Asterisk and cross (\*\*+) indicate a significant change as compared to the value obtained in rats pretreated with pentoxifylline.

catecholamines production by suprarenal glands, what leads to induction of inflammatory phenomena (20,22,23). This animal model mimics clinical acute gastric ulcerations, caused by major trauma, surgery or sepsis (24), being widely accepted for studying the mechanism stress-induced gastric lesions (25). An acute inflammation in gastric mucosa contributes to pathomechanism of WRS ulcerogenesis. This inflammation is accompanied by increase of expression of several proinflammatory cytokines, including IL-1 $\beta$  and TNF $\alpha$ , that seem to be responsible for the enhanced permeability of blood vessels to neutrophils (26). It is known that neutrophils are equipped in the NADPH oxidase in their membrane, which appears to be the principal source of a superoxide radical anion (O<sub>2</sub><sup>•-</sup>) (27,28). It is not excluded that IL-1 $\beta$  and TNF $\alpha$  contribute to the enhancement of the production of reactive oxygen species (ROS) in the gastric mucosa exposed to ischemia-reperfusion. Konturek et al. (29) showed the interaction between the

level of proinflammatory cytokines and ROS generation in the model of gastric ischemia-reperfusion and concluded that a decrease of ROS production caused the inhibition of adhesion molecules expression, leads to the decrease of mucosal neutrophil infiltration. These results are in keeping with our present results, since we found the close relationship between oxidative stress parameters and concentration of IL-1 $\beta$  and TNF $\alpha$ . The rise in plasma proinflammatory cytokine levels coincided with the increase of WRS-induced gastric lesions, the augmentation of ROS-induced lipid peroxidation (expressed as increase of MDA+4-HNE concentration) and accompanied by suppression of antioxidative mechanisms, particularly, the reduction in mucosal SOD activity and mucosal GSH content.

Our previous studies (30) documented that ROS are involved in the formation of WRS-induced gastric mucosal damage due to an enhancement of lipid peroxidation and attenuation of mucosal antioxidative mechanisms. Our present observations are in keeping with our previous findings (17,30,31) indicating that the production of free radical, enhanced lipid peroxidation and an impairment of antioxidizing enzyme activity, and the increased generation of cytokines, such as IL-1 $\beta$  and TNF $\alpha$ , contribute to the pathomechanisms of stress-induced gastric lesions. This is supported by our present observation that PTX, which is capable of inhibiting plasma TNF $\alpha$  and IL-1 $\beta$  levels and ameliorated WRS-induced gastric lesions with concomitant and accompanying fall in the GBF.

Relationship between IL-1 $\beta$  and NO production, as well as ROS generation, was investigated by Ishikawa et al (32) in gastric isolated cells *in vitro*. They found that IL-1 $\beta$  expression of inducible NO synthase (iNOS) resulted in excessive NO release with the augmentation of ROS production. Inhibition of excessive NO synthesis caused the decrease in IL-1 $\beta$  - dependent ROS generation. This data suggested an important role of IL-1 $\beta$  in the up-regulation of NO expression and release as well as ROS generation.

Besides, both IL-1 $\beta$  and TNF $\alpha$  have an ability to induce iNOS expression. To elucidate more details of this process, further investigations on iNOS and cNOS expression are necessary.

Results of above-mentioned experiments were related to effects of high, cytotoxic concentrations of NO. Ishii et al. (33) documented that acute gastric lesions in rats, induced by ischemia followed by reperfusion (I/R) can be attenuated by antioxidant enzymes, such as SOD, catalase and by NO-synthase inhibitors, suggesting that ROS may cooperate with NO in I/R-induced gastric damage and that reduction in ROS and NO toxicity is required to achieve satisfactory protection against gastric lesions induced by I/R.

However, previous studies documented that NO may be also considered as a gastroprotective factor, when released from gastric epithelium in smaller amounts and that this NO contributes to the reduction of the area and number of gastric lesions induced by variety of noxious agents (7,8,10,30,34). This was

supported by aggravation of WRS-induced damage with the attenuation of tissue release of NO, probably by constitutive NOS (cNOS) inhibitors, resulting in fall in tissue generation of NO and impairment of the gastric microcirculation (35,36). In this paper, PTX-induced gastroprotection and accompanying increase in the GBF were attenuated by L-NNA, that nonspecifically inhibits both, cNOS and iNOS forms, indicating that NO, produced by these enzymes in smaller amounts, plays an important role in the protective and hyperemic effects of PTX, a TNF $\alpha$  inhibitor. Moreover, we showed in our present study that stress-induced ulcerogenesis could be mediated by the imbalance between ROS-dependent lipid peroxidation, NO and cytokines, as proposed recently (17,19,30,37).

Importance of NO in the mechanism of gastric integrity is supported by our previous observations (31,37,38) that NO-releasing derivatives of aspirin, such as NO-aspirin (NO-ASA) in contrast to native ASA, exerted gastroprotective activity and failed to delay of chronic gastric ulcer healing and these effects of NO-ASA were accompanied by an attenuation of lipid peroxidation. These results emphasized the protective role of NO in the action of NO-ASA on gastric mucosa due to suppression of ROS and decreased expression by tissue concentration of MDA and 4-HNE (30).

Our present investigations attempted to determine the involvement of NO in gastroprotective action of PTX and for this purpose the substrate of NO biosynthesis (L-arginine), the inhibitor of NO synthase (L-NNA) or both were used (33,39-41). The dose of 50 mg/kg of L-arginine was selected because L-arginine applied in this dose by itself failed to affect the WRS-induced gastric lesions (*Table 1*).

PTX has an ability to suppress the production of proinflammatory cytokines, through intracellular accumulation of cAMP. Since PTX causes relaxation of smooth muscle mediated by cAMP, it is easily speculated that this agent increases mucosal blood flow by directly dilating vascular smooth muscle. However, in the present study, the increased blood flow response to PTX was attenuated by the NO synthase inhibitor. These results indicate that PTX causes increment of GBF also through NO-mediated way. NO-mediated way probably plays main role, comparing to direct dilating action, in case of our experimental procedures.

Goeking et al. (42) showed that inhibition of NO synthesis abolished reepitelization of intestinal epithelium, while application of L-arginine enhanced restitution of surface epithelium. In our present paper we observed similar beneficial effects of L-arginine administration, because its addition to L-NNA, an inhibitor of NOS, before PTX restored the protective and hyperemic effects of PTX against WRS damage.

Oxygen-derived radical species were implicated in the pathogenesis of necrotizing enterocolitis (NEC). Akisu et al. (43) examined the protective action of L-arginine, a NO-synthase substrate, against hypoxia-reoxygenation-induced

NEC in mice. They concluded, that dietary supplementation with L-arginine ameliorated intestinal injury and decreased lipid peroxidation in hypoxia-reoxygenation-induced bowel injury (43). We also observed that L-arginine added to inhibitor of NO production, L-NNA, ameliorated parameters of oxidative stress and significantly attenuated WRS-induced gastric damage.

Brzozowski et al. (37) examined influence of new derivative of NSAID releasing NO (NO-ASA), on healing of WRS-induced gastric ulcers. They documented beneficial effect of NO-ASA, as manifested by a decrease of gastric lesions, suppression of proinflammatory cytokines (IL-1 $\beta$  and TNF $\alpha$ ), with concomitant diminution of lipid peroxidation and producing an increase in SOD activity. Our results are in keeping with this finding by demonstrating that the inhibition of NOS by L-NNA resulted in reversal of gastroprotective and hyperemic effects of PTX and these effects were accompanied by increase of plasma IL-1 $\beta$  level and the significant rise in lipid peroxidation. This supports our notion that NO is involved in beneficial action of PTX against WRS-induced gastric lesions. Further study is required to determine whether PTX affects the expression of NOS or its activity in the gastric mucosa.

PTX was reported to act as antagonist of TNF $\alpha$  and found to be effective in the treatment of ischemic disorders. PTX exhibits gastroprotective action due to increase of microcirculation in the stomach (36,44). Recently, we have demonstrated that administration of PTX reduced ASA-induced gastric damage via mechanism involving the attenuation in lipid peroxidation (45).

In our present study the administration of PTX exerted beneficial effects against WRS-induced gastric lesions, followed by an increase of gastric blood flow, and finally accompanied by the fall in lipid peroxidation products such as MDA plus 4-HNE concentration and by a potent increase in SOD activity, as well as decrease of plasma IL-1 $\beta$  and TNF $\alpha$  levels.

This indicates that PTX-induced gastroprotection involves attenuation of lipid peroxidation and preservation of antioxidative enzyme activity in the mucosa exposed to stress. Further studies are needed to answer the question why the pretreatment with L-NNA, failed to affect the suppression of TNF $\alpha$  level in rats treated with PTX. One of possible explanation could be that PTX exerts direct potent inhibitory action on TNF $\alpha$  production that is independent of NO produced by NOS. Another surprising observation was that pretreatment with L-NNA prior PTX produced rather slight inhibition of SOD activity, which remained still at quite high level despite suppression of NOS. Therefore we examined next antioxidative parameter, i.e. gastric tissue level of reduced glutathione (GSH) and we found that L-NNA, indeed, significantly diminished the GSH content. The preserved activity of SOD in PTX-treated animals despite L-NNA pretreatment require further studies but it is possible that elevated activity of SOD led to increase of hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) production, which in turn, resulted in utilization of GSH.

GSH is considered to provide cellular protection against oxidative damage. The antioxidant function of this tripeptide is related to its ability to become oxidized in thiol group of its cysteine residue with formation of a disulfide (46). It is of interest that the application of PTX produced a marked increase in gastric mucosal GSH content. On the contrary, the inhibition of NOS by L-NNA led to depletion of GSH pool and this was accompanied by increase of gastric lesions and the significant fall in the GBF in PTX-treated stressed animals. These effects were further restored by the concurrent addition of L-arginine added to L-NNA.

We conclude, that oxidative metabolism plays an important role in pathogenesis of WRS-induced gastric lesions. PTX affords a potent gastroprotection against these lesions, an effect probably associated with the suppression of proinflammatory cytokines, reduction of lipid peroxidation as well as increase of the antioxidative enzymes, including the SOD activity and increased GSH mucosal concentration. Inhibition of NO-synthase counteracted the PTX-induced gastroprotection and hyperemia and these effects were restored after administration of substrate for NO-synthase, L-arginine, suggesting that NO contributes to the beneficial effect of PTX in rat stomach.

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Received: March 2, 2004

Accepted: May 7, 2004

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