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## ASYMMETRIC DIMETHYLARGININE, AN ENDOGENOUS INHIBITOR OF NITRIC OXIDE SYNTHASE, INTERACTS WITH GASTRIC OXIDATIVE METABOLISM AND ENHANCES STRESS-INDUCED GASTRIC LESIONS

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Asymmetric dimethylarginine (ADMA) is an endogenous competitive inhibitor of nitric oxide (NO) synthase known to exert vasoconstriction of vascular bed. The elevation of ADMA has been considered as the cardiovascular risk factor associated with hyperlipidemia, hypercholesterolemia and metabolic syndrome. ADMA is produced by the action of dimethylarginine dimethylaminohydrolase (DDAH), which hydrolyzes ADMA to L-citrulline and dimethylamine. Previous studies have shown that endogenous NO plays an important role in the mechanism of gastric mucosal defense, but the role of ADMA in the pathogenesis of serious clinical entity, such as the acute gastric mucosal injury induced by stress has been little studied. In present study, we determined the effect of intragastric (i.g.) pretreatment with ADMA applied in graded doses ranging from 0.1 up to 20 mg/kg on gastric mucosal lesions induced by 3.5 h of water immersion and restraint stress (WRS). The number of gastric lesions was determined by planimetry and the gastric blood flow (GBF) was assessed by laser Doppler technique. The malondialdehyde and 4-hydroxynonenal (MDA+4-HNE) concentration, as an index of oxygen radical-lipid peroxidation was assessed in the gastric mucosa in rats exposed to WRS with or without ADMA administration. Proinflammatory cytokines IL-1 $\beta$ , TNF- $\alpha$ , superoxide dismutase (SOD) and glutathione peroxidase (GPx) mRNAs in the gastric mucosa and plasma levels of ADMA, IL-1 $\beta$  and TNF- $\alpha$  were analyzed by RT-PCR and ELISA, respectively. The exposure of rats to WRS for 3.5 h produced acute gastric lesions accompanied by a significant rise in the plasma ADMA levels and a significant fall in the GBF, an increase in MDA+4-HNE concentrations and the significant increase in the expression and release of IL-1 $\beta$  and TNF- $\alpha$ . The pretreatment with ADMA, applied i.g. 30 min before WRS dose-dependently, aggravated WRS damage and this effect was accompanied by a further significant fall in the GBF. The ADMA induced exacerbation of WRS lesions and the accompanying rise in the plasma ADMA levels and the fall in GBF were significantly attenuated by concurrent treatment with glyceryl trinitrate (GTN) (10 mg/kg i.g.) in the presence of ADMA. Administration of ADMA resulted in a significant decrease in the expression of SOD and GPx mRNAs and the up-regulation of mRNA for IL-1 $\beta$  and TNF- $\alpha$  followed by an increase in these plasma cytokine levels as compared to respective values observed in vehicle-pretreated animals. We conclude that 1) ADMA could be implicated in the mechanism of WRS-induced ulcerogenesis, 2) ADMA exacerbates WRS-induced gastric lesions due to enhancement in neutrophil dependent lipid peroxidation and overexpression and release of proinflammatory cytokines IL-1 $\beta$  and TNF- $\alpha$  and a potent depletion of antioxidative enzymes SOD and GPx expression and activity.

**Key words:** *asymmetric dimethylarginine, malonylaldehyde, superoxide dismutase, glutathione peroxidase, interleukin-1 $\beta$ , tumor necrosis factor- $\alpha$ , oxidative stress, water immersion restraint stress*

### INTRODUCTION

The maintenance of gastric mucosal integrity depends upon a variety of factors and physiological mechanisms, such as in particular the maintenance of microcirculation, mucus-alkaline secretion, the high concentrations of sulfhydryls and activity of antioxidizing factors, such as superoxide dismutase (SOD), glutathione peroxidase (GPx) and the reduced form of glutathione (GSH) (1). These factors were also implicated in the

gastroprotection against exogenous and endogenous irritants originating from lumen of stomach (2, 3). Previous studies revealed that stress-induced disturbances of blood perfusion of gastric mucosa may result in the formation of mucosal erosions and gastric ulcers (4-6). Nitric oxide (NO) is widely accepted as a potent vasorelaxant involved in control of the gastric blood flow (GBF) and the gaseous mediator contributing to the maintenance of gastric mucosal integrity (7-9). NO is produced due to expression and activity of cNOS and iNOS enzymatic pathways. Inhibition of

NO-synthase (NOS), that causes decrease in local NO production, impairs gastric microcirculation and aggravates gastric lesions induced by noxious agents (8, 9). Under physiological conditions, NO is produced by NOS from its substrate L-arginine, which is metabolized to amino acid, L-citrulline. Under pathological conditions, L-arginine may be involved in another metabolic pathway catalyzed by protein arginine methyltransferase (PRMT) (see Fig. 1). The activity of PRMT, in the presence of proteins containing methylated arginine residues, leads to formation of asymmetric dimethylarginine (ADMA) and symmetric methyl arginine (MMA) (10). Previous studies documented that ADMA acts as the endogenous NOS inhibitor and the inhibition of this enzyme results in a decrease of NO production (11, 12) (Fig. 1). The excessive accumulation of ADMA can decrease NO bioavailability in many cells causing an impairment of multiple systems including gastrointestinal tract (13). Apart from NO synthase inhibition, ADMA can directly induce oxidative stress and cell apoptosis, and participate in the inflammatory reactions (14-16). The importance of ADMA was well documented in a wide range of cardiovascular disorders. ADMA impairs endothelial functions, thus leading to hypertension, atherosclerosis, coronary heart disease, diabetes mellitus, pulmonary hypertension and renal failure (17, 18). Depletion of NO leads to a multifactorial consequences, such as decrease in the organ blood flow, and has been implicated in the pathogenesis of numerous diseases, such as hypertension, arteriosclerosis, heart failure, chronic kidney disease, diabetes mellitus (18-20). ADMA can be metabolized to L-citrulline by enzyme called dimethylarginine dimethylaminohydrolase (DDAH) (10) (Fig. 1). The status of DDAH activity seems to play an important role in the mechanism of gastric defense, because the acute effects of ethanol resulting in gastric lesions, followed by an increase in ADMA content in the gastric juice and the decreased DDAH activity in gastric mucosa, were reversed by pretreatment with BTM-0512 (100 mg/kg), a novel analog of resveratrol or L-arginine, a substrate for NO-synthase (21). Improvement of DDAH activity by this compound afforded protection of the gastric mucosa against ethanol-induced injury and diminished the ADMA content (21).

Previous studies revealed that the NOS inhibitor, N-nitro-L-arginine (L-NNA) delayed ulcer healing and the accompanying increase in GBF at ulcer margin and documented, that these effects can be reversed by application of L-arginine, a substrate for NOS activity (2, 6, 8). However, little information is available regarding the role of ADMA in clinically relevant model of experimental gastric injury with respect of the interaction between ADMA and oxidative metabolism in gastric mucosal cells exposed to stress. Recently, ADMA was implicated in the lesions induced by ethanol, indomethacin and cold stress by demonstration, that the gastric lesions induced by these gastric barrier breakers and levels of this NO synthase inhibitor in the blood were both aggravated in animals administered with ADMA, while the DDAH activity was markedly inhibited in injured mucosa (13, 22). Moreover, the incubation of gastric epithelial cell line (GES-1) with *Helicobacter pylori* (*H. pylori*) *in vitro* increased levels of proinflammatory cytokine TNF- $\alpha$  and plasma ADMA levels, while decreasing the activity of DDAH (22). Thus, ADMA could be considered as pathogenic factor playing an important role in facilitating gastric mucosal injury due to an inhibition of NO synthesis and promotion of inflammatory reaction.

It is known, that adverse effects of systemic or local stress could be attributed to the microbleedings and therefore, this issue represents important clinical problem because of the risk for acute hemorrhagic gastric mucosal lesions, that appear mainly in fundic gastric mucosa in humans and experimental animals in response to variety of stressors (23). Animal models serve suitable in studying of various aspects of stress in gastrointestinal tract, including the pathomechanism of acute gastric damage. The model of water

immersion and restraint stress (WRS), proposed by Takagi *et al.* (24) seems to be most useful in testing of various physiological and pharmacological factors involved in the formation of gastric damage and those known to exert protection against these lesions. It has been documented, that endogenous prostaglandins, NO, superoxide dismutase (SOD) and sulfhydryls, for example, as functional residue in reduced form of glutathione, protect the gastric mucosa from the mucosal damage induced by stress (3, 25).

Therefore, we were particularly interested to determine influence of ADMA on antioxidative potential of the gastric mucosa exposed stress and to examine the role of reactive oxygen metabolites and lipid peroxidation products in the effect of ADMA on stress-induced gastric lesions. Two parameters are usually useful for assessment of biological effects of reactive oxygen species: the levels of malondialdehyde (MDA) plus 4-hydroxynonenal (4-HNE) and the activity of SOD. Gastric tissue levels of MDA and 4-HNE are considered as indicators of lipid peroxidation, while SOD activity reflects the antioxidative properties of various tissues including gastric mucosa (23, 25). We attempted to determine the contribution of plasma ADMA levels and lipid peroxidation reflecting the mucosal content of MDA+4-HNE during onset of WRS in rats with or without ADMA administration. In addition, the expression of proinflammatory cytokines IL-1 $\beta$  and TNF- $\alpha$  and their plasma levels as well as the alterations in expression of SOD and GPx mRNAs in gastric mucosa exposed to WRS without and with concurrent treatment with ADMA were determined.

## MATERIAL AND METHODS

Experiments were carried out on 120 male Wistar rats, weighing 200–240 g and fasted for 24 hours before all studies. Studies were approved by the Ethic Committee for Animal Research of Jagiellonian University and run according to protocol proposed by Helsinki Declaration.

### *Production of gastric lesions and ADMA treatments*

The animals were divided into 6 groups each consisting of 6-8 animals. In group 1, rats received vehicle (1 ml of saline i.g.) and 30 min later they underwent 3.5 hours of water immersion and restraint stress (WRS) in water temperature of 23°C. This method was originally proposed by Takagi *et al.* (24) and employed in our previous studies (2, 3, 8). In group 2, ADMA was applied i.g. in graded doses ranging from 0.1 mg/kg to 20 mg/kg, prior to 3.5 hours of WRS. Animals of group 3 received the pretreatment of glyceryl trinitrate (GTN, 10 mg/kg i.g.), a NO donor that was administered 15 min before vehicle (1 ml saline i.g.) or ADMA (20 mg/kg i.g.) and 30 min later these rats were subsequently exposed to 3.5 hours of WRS. The effect of ADMA on WRS-induced gastric lesions and the accompanying changes in GBF were compared with those evoked by symmetric dimethylarginine (SMDA) which is not a NO-synthase inhibitor. For this purpose the SMDA (Group 5) was administered 30 min before the onset of WRS analogically as that presented for ADMA or vehicle given before WRS. Group 6 of intact animals (N=3) served as a control group and did not undergo any procedures.

### *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 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, 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 was determined by computerized planimetry (Morphomat, Carl Zeiss, Berlin, Germany) after stomach of each group being photographed as described previously (4).

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

Immediately after the termination of experiments, a venous blood sample (about 3 ml) was withdrawn from the vena cava of rats with or without vehicle or ADMA and transferred into EDTA containing vials later used for the determination of plasma IL-1 $\beta$  and TNF- $\alpha$  by a solid phase sandwich ELISA (BioSource International Inc. Camarillo, CA, USA) according to the manufacturer's instructions. For comparison, intact rats fasted overnight and given i.p. only vehicle (saline) were also anaesthetized with ether and the blood samples were collected for the determination of control values of plasma cytokines IL-1 $\beta$  and TNF- $\alpha$  concentration. Briefly, each sample (50  $\mu$ l) was incubated with biotinylated antibodies specific for rat IL-1 $\beta$  and TNF- $\alpha$  and washed three times with an assay buffer and finally conjugated with streptavidin peroxidase to form a complex with stabilized chromogen as described previously (3).

For determination of plasma ADMA levels, the blood samples collected in heparin coated polypropylene tubes were centrifuged at 3000 rpm for 20 minutes at 4°C, and the supernatant clear plasma was then stored at -80°C until the measurement. The concentration of ADMA in the blood was measured using ADMA direct rat ELISA kit manufactured by Immunodiagnostik AG (Enzo Life Sciences GMBH, Lorrach, Germany) according to the procedure recommended by a manufacturer. Briefly, the aliquots (50  $\mu$ l) of the pretreated standards, controls and samples were pipetted into wells of the microtiter plate and the antiserum solution (ADMA antibody, 50  $\mu$ l) was added to each well. The plate was incubated for 15 h at 2–8°C and washed four times with wash buffer. Subsequently, the enzyme conjugate solution (100  $\mu$ l) was added to each well and then the microtitre plate was incubated for 1 hour at room temperature. The wells were again washed four times and the substrate solution (100  $\mu$ l) was pipetted into the wells and the plate was incubated for 25 min. Then the reaction was stopped with stopping solution and the optical density was read at 450 nm using microtitre plate reader. The limit of assay sensitivity was 3 pg per tube; the intra-assay variation was less than 7% and the interassay variation less than 4%.

#### *Measurement of lipid peroxidation*

For determination of lipid peroxidation in gastric mucosa of tested groups, the gastric mucosal tissue levels of malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE) were measured and used as indicators of lipid peroxidation (5). 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  $\mu$ l 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 homogenate was centrifuged (3000 g at 4°C for 10 min) and obtained clear supernatant that was immediately 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, then subsequently analyzed by spectrophotometry (Marcel s300 Instrument, Warsaw, Poland). Results were expressed as nanomol per gram of tissue (nmol/g) according to our studies described in detail previously (1-3).

#### *Determination of SOD and GPx transcripts by reverse-transcriptase polymerase chain reaction*

The expression of SOD and GPx was determined by RT-PCR in the gastric mucosa of investigated animals. Samples of gastric mucosa, weighing about 500 mg, were scraped off on ice, using glass slides, then immediately frozen in liquid nitrogen and stored in temperature -80°C. Total RNA was isolated from the mucosa according to method of Chomczynski and Sacchi (26), using a rapid guanidium isothiocyanate/phenol chloroform single step extraction (Heidelberg, Germany). After precipitation, the RNA was resuspended in RNase-free Tris EDTA buffer and the concentration was estimated by absorbance at 260 nm wavelength. Samples were frozen at -80°C until analysis.

First strand cDNA was synthesized from total cellular RNA (5  $\mu$ g), using 200 U StrataScript RT (Stratagene). After the reverse transcription, the transcriptase activity was destroyed by heating, and then the cDNA was stored at -20°C until PCR.

A 201-bp fragment of SOD and 324-bp fragment of GPx were amplified from single-stranded DNA by PCR, by means of two oligonucleotide primers to SOD and GPx, respectively. The SOD sense primer was 5'CGAGTTATGGCGACGAAG3' and antisense primer was 5'GTCAGCAAGTCACATTGCC3'. The GPx sense primer was 5'CACCGTGTATGCCTTCTC3' and antisense primer was 5'GCCTTCTCACCATTACC3', respectively. The sequence of primers for IL-1 $\beta$ , TNF- $\alpha$  and  $\beta$ -actin were identical to those published by our group previously (27). All primers were synthesized by Biometria (Gottingen, Germany). Concomitantly, amplification of control rat  $\beta$ -actin (Clon Tech, Palo Alto, California, USA; 764 bp) was performed on the same samples to verify RNA integrity.

DNA amplification was carried out under the following conditions: denaturation at 94°C for 1 min., annealing at 60°C for 45 s. The number of amplification cycles was 29 for SOD and IL- $\beta$  and 33 for GPx and TNF- $\alpha$ . Each PCR product (8  $\mu$ l) was electrophoresed on 1.5% agarose gel, stained with ethidium bromide, and then visualized under UV light. Localization of predicted PCR was confirmed by means of 100-base pair ladder (Gibco BRL/life Technologies, Eggenstein, Germany), as standard marker.

The intensity of bands was quantified in semi-quantitative manner, using densitometry (LKB, Ultrascan, Pharmacia, Sweden). The gel was photographed under UV transillumination. The intensity of PCR products was measured by means of video image analysis system (Kodak Digital Science). The SOD and GPx mRNA signals were standardized against the  $\beta$ -actin mRNA signal for each sample and results were expressed as SOD or GPx mRNA/ $\beta$ -actin mRNA ratio (25). The signals of IL-1 $\beta$  and TNF- $\alpha$  mRNAs were also normalized to the  $\beta$ -actin mRNA signal and expressed as IL-1 $\beta$  and TNF- $\alpha$  mRNA over the  $\beta$ -actin mRNA ratio (25, 27).

#### *Statistical analysis*

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

## RESULTS

*Effect of ADMA administration on water immersion restraint stress-induced gastric lesions and the alterations in the plasma ADMA levels and gastric blood flow. Comparison with symmetric dimethylarginine (SDMA).*

Fig. 2 shows the effect of administration of ADMA, applied i.g., in graded doses, ranging from 0.1 mg/kg up to 20 mg/kg, compared with that SDMA (20 mg/kg), on the mean lesion number and accompanying changes in the plasma ADMA levels and GBF induced by WRS. Plasma levels of ADMA were negligible in intact animals not exposed to WRS and no microscopical lesions were observed in these animals (data not shown). Exposure of animals to 3.5 hours of WRS resulted in formation of numerous hemorrhagic mucosal erosions accompanied by the fall in the GBF by about 30% and a significant rise in the plasma concentration of ADMA (Fig. 2). The pretreatment with ADMA, beginning at the dose of 1 mg/kg administered 30 min before the onset of WRS, caused a dose-dependent increase of mean number of WRS-induced lesions and a significant reduction of the GBF and a further increase in the plasma ADMA levels, as compared to vehicle-treated animals. Maximal increase in mean lesion number was observed with ADMA applied i.g. in a dose of 20 mg/kg. When ADMA was administered i.g., in a small dose of 0.1 mg/kg, no significant change in the GBF and plasma ADMA levels was

observed comparing to those observed in vehicle-control rats exposed to WRS. With increased doses of ADMA the significant fall in GBF and the significant increase in plasma ADMA levels were observed being the most pronounced in animals treated with the dose of 20 mg/kg of this compound. In contrast, pretreatment with SDMA (20 mg/kg i.g.) applied 30 min before the WRS failed to significantly alter the mean lesion number and the accompanying changes in GBF and plasma ADMA levels, as compared to vehicle-controls (Fig. 2).

*Effect of ADMA administration on the plasma levels of IL-1 $\beta$  and TNF- $\alpha$  in rats exposed to WRS*

As shown in Fig. 3, the plasma concentration of IL-1 $\beta$  and TNF- $\alpha$  in intact animals averaged 10 $\pm$ 2.5 pg/ml and 2.2 $\pm$ 0.3 pg/ml, respectively. In rats exposed to 3.5 hours of WRS, a significant rise in plasma IL-1 $\beta$  and TNF- $\alpha$  levels was noticed reaching the value of 28 $\pm$ 4 pg/ml and 16 $\pm$ 2.5 pg/ml, respectively. The application of ADMA (1 mg/kg i.g.) prior to WRS, resulted in a significant increase in plasma IL-1 $\beta$  and TNF- $\alpha$  levels, as compared to those recorded WRS rats pretreated with vehicle (saline). The pretreatment with ADMA, administered in higher doses 10 mg/kg and 20 mg/kg, produced a dose-dependent increment of plasma IL-1 $\beta$  and TNF- $\alpha$  levels reaching the maximal increase at the dose of 20 mg/kg of this NO synthase inhibitor compared to the respective values of these cytokines in intact rats and those pretreated with vehicle and exposed 30 min later to WRS (Fig. 3).

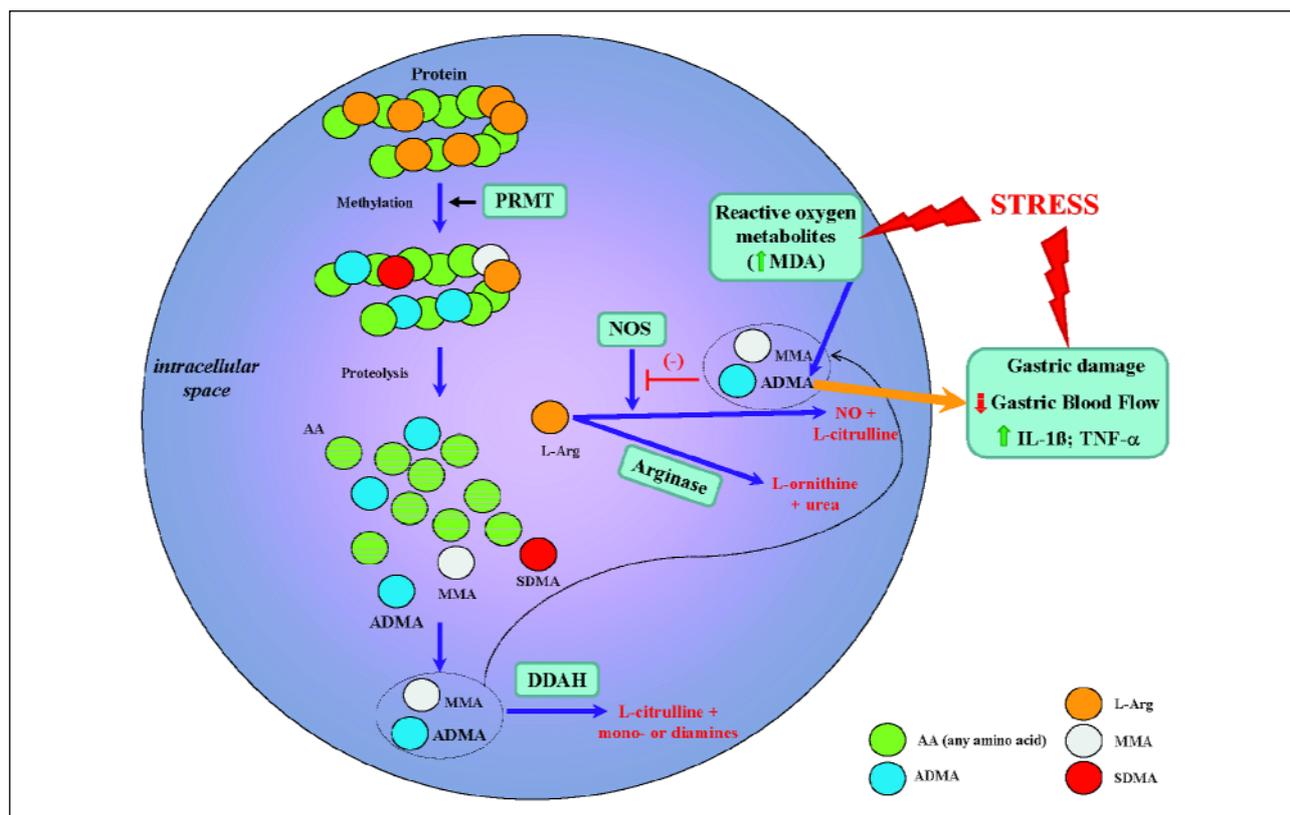


Fig. 1. The scheme presenting formation of asymmetric dimethylarginine (ADMA) via subsequent steps including activity of protein arginine methyltransferases (PRMT), the methylation of L-arginine (L-Arg) residues and the formation of monomethylarginine (L-MMA), ADMA or symmetric dimethylarginine (SDMA). MMA and ADMA, but not SDMA can be converted to L-citrulline and mono- or diamines by an enzyme dimethylarginine dimethylaminohydrolase (DDAH). Free L-Arg can be metabolized by nitric oxide synthases (NOS) to NO and L-citrulline. ADMA and MMA, but not SDMA, act as potent endogenous inhibitors of NOS enzymes. Stress can generate reactive oxygen metabolites and enhance the gastric mucosal and plasma levels of ADMA. Exogenous ADMA exacerbates gastric damage induced by stress. ADMA can contribute to the pathogenesis of stress-induced gastric damage by the impairment in the gastric blood microcirculation and the increase in the expression and release of proinflammatory cytokines IL-1 $\beta$  and TNF- $\alpha$ .

*Effect of vehicle and ADMA administration on the mucosal content of lipid peroxidation products in rats exposed to ADMA and WRS*

Concentrations of MDA and 4-HNE in the intact mucosa were relatively low, almost at the level of analytical limit of detection and averaged  $5.9 \pm 0.2$  nmol/g. After the end of 3.5 hours of WRS, the MDA and 4-HNE concentration was increased by about 2 folds, reaching the value of  $14.3 \pm 0.9$  nmol/g, comparing to the respective value in intact gastric mucosa. Administration of ADMA in dose of 1 mg/kg i.g. failed to affect significantly the MDA and 4-HNE level as compared to that measured in vehicle-control animals. With the increase of the dose of ADMA up to 10 mg/kg i.g., a significant increase of these lipid peroxidation products to the value of  $16.8 \pm 0.2$  nmol/g ( $p < 0.05$ ) was observed and this increase was statistically significantly higher, comparing to that obtained with the dose of 1 mg/kg of this NOS inhibitor

(Fig. 4). Maximal intensification of lipid peroxidation products in the gastric mucosa was observed when ADMA was administered i.g. in a dose of 20 mg/kg ( $p < 0.05$  vs. ADMA at the dose of 1 mg/kg) (Fig. 4).

*Effect of concurrent treatment with glyceryl trinitrate on the mean lesion number and the changes in the plasma ADMA levels and gastric blood flow induced by WRS*

Pretreatment with glyceryl trinitrate (GTN) significantly decreased the mean number of WRS-induced gastric lesions and this effect was accompanied by the significant rise in the GBF and a significant decline in the plasma ADMA concentration (Fig. 5). The administration of ADMA (20 mg/kg i.g.) resulted in a significant increase in the mean number of WRS lesions, the significant increase in the plasma ADMA levels ( $p < 0.05$ ) and the significant fall in the GBF, all values being similar to those

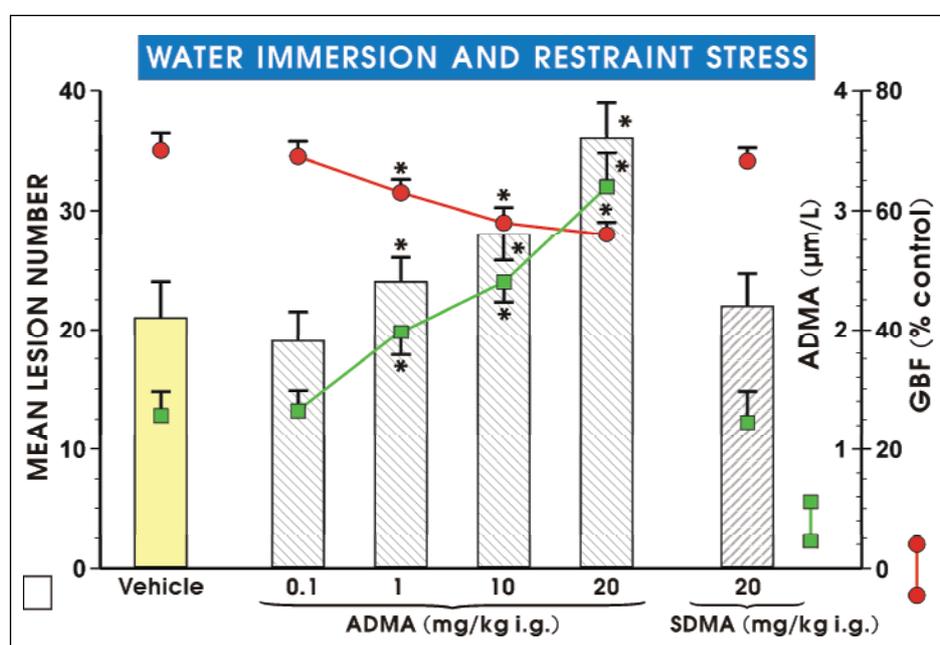


Fig. 2. Mean number of gastric lesions and the accompanying changes in the plasma ADMA levels and gastric blood flow (GBF) in rats exposed to 3.5 hours of water immersion restraint stress (WRS) with or without vehicle (saline) or ADMA given intragastrically (i.g.) in graded doses ranging from 0.1 mg/kg up to 20 mg/kg. Results are mean  $\pm$  S.E.M. of 6-8 rats per each group. Asterisk (\*) indicates a significant change ( $p < 0.05$ ) as compared to the value obtained in vehicle-control group.

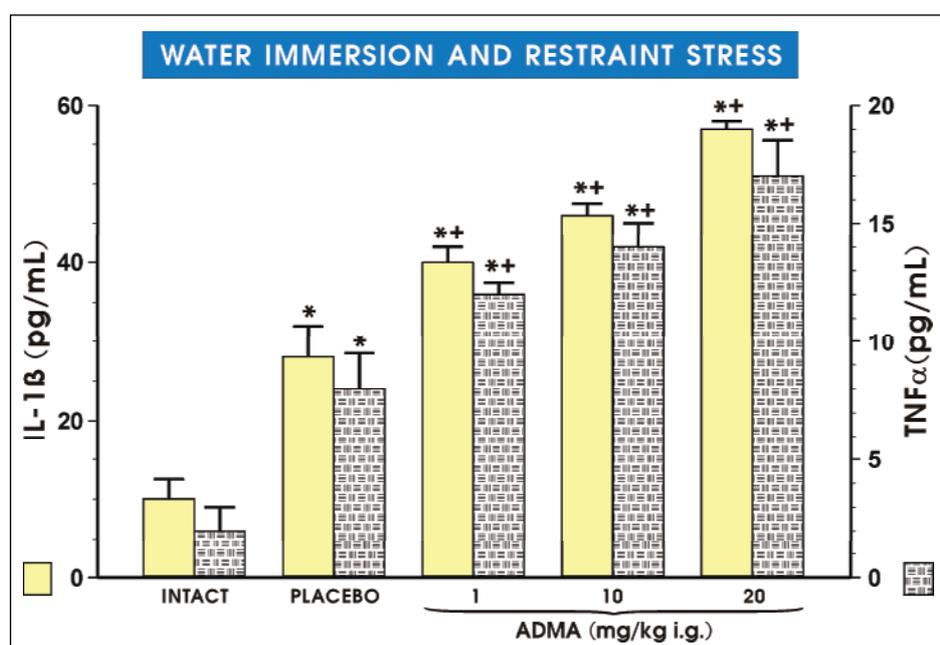


Fig. 3. Plasma level of interleukin-1 beta (IL-1 $\beta$ ) and tumor necrosis factor-alpha (TNF- $\alpha$ ) in rats exposed to 3.5 hours of water immersion restraint stress (WRS) with or without vehicle or ADMA given intragastrically (i.g.) in graded doses ranging from 0.1 mg/kg up to 20 mg/kg. Results are mean  $\pm$  S.E.M. of 6-8 rats per each group. Asterisk (\*) indicates a significant change ( $p < 0.05$ ) as compared to the value obtained in intact gastric mucosa. Asterisk and cross (\*\*+) indicate a significant change ( $p < 0.05$ ) as compared with the value obtained in rats pretreated with vehicle and exposed to 3.5 hours of WRS.

presented in Fig. 2. The concurrent treatment with GTN (10 mg/kg i.g.) significantly reduced the number of gastric lesions and significantly attenuated the accompanying rise in plasma ADMA levels and the decrease in the gastric blood flow, evoked by ADMA in rats exposed to WRS ( $p < 0.05$ ) (Fig. 5).

*Expression of superoxide dismutase and glutathione peroxidase mRNA and proinflammatory cytokines IL-1 $\beta$  and TNF- $\alpha$  by reverse-transcriptase polymerase chain reaction in gastric mucosa without or with ADMA pretreatment*

Fig. 6 presents the RT-PCR expression of  $\beta$ -actin, SOD and GPx mRNA in the gastric mucosa of intact rats and those pretreated with vehicle or ADMA administered i.g. in graded doses ranging from 1 mg/kg up to 20 mg/kg, at 30 min before the

WRS. The expression of  $\beta$ -actin mRNA was well preserved in the mucosal biopsy samples taken both from intact rats and those treated with vehicle or ADMA in graded doses and then subjected to WRS (Fig. 6, left panel). The SOD and GPx mRNA was detectable in the intact gastric mucosa and in the mucosa exposed to WRS without or with ADMA pretreatment (Fig. 6, left panel, lanes 1-5). Semi quantitative analysis of the SOD/ $\beta$ -actin mRNA ratio and GPx/ $\beta$ -actin mRNA ratio revealed that the expression of mRNAs for SOD and GPx was significantly lower in WRS animals pretreated with ADMA, comparing to those in vehicle-controls, especially when this NOS inhibitor was administered at the highest dose of 20 mg/kg before the onset of WRS (Fig. 6, right panel).

Fig. 7 shows the RT-PCR expression of mRNAs for  $\beta$ -actin, IL-1 $\beta$  and TNF- $\alpha$  in the gastric mucosa of intact rats and those

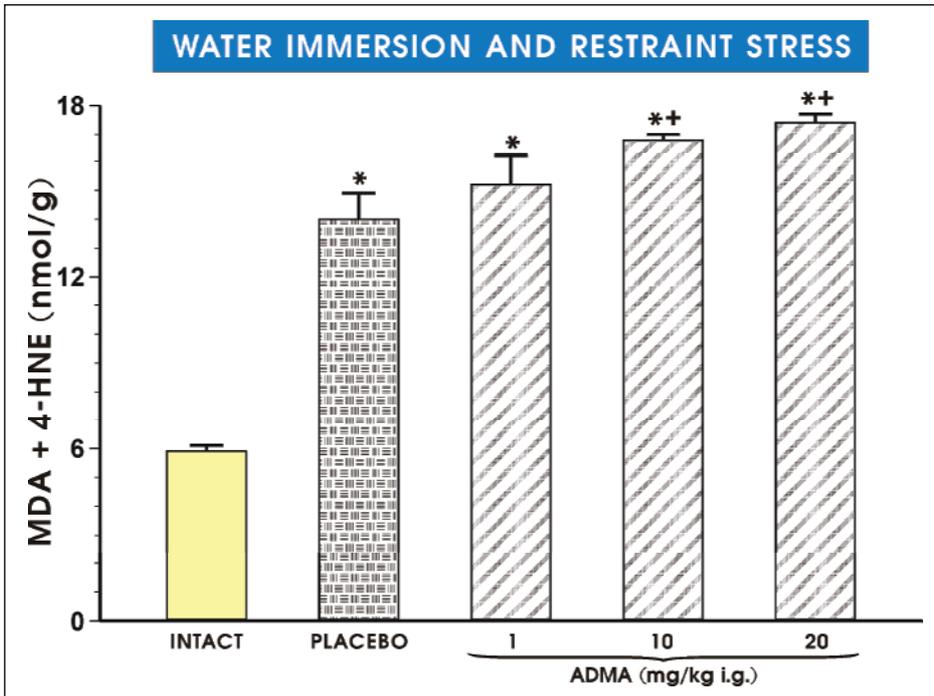


Fig. 4. Concentration of malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE) in the gastric mucosa of rats exposed to 3.5 hours of water immersion restraint stress (WRS) with or without placebo or ADMA given intragastrically (i.g.) in graded doses ranging from 0.1 mg/kg up to 20 mg/kg. Results are mean  $\pm$  S.E.M. of 6-8 rats per each group. Asterisk (\*) indicates a significant change as compared to the value obtained in intact gastric mucosa. Asterisk and cross (+) indicate a significant change ( $p < 0.05$ ) as compared with the value obtained in rats pretreated with vehicle and exposed to 3.5 hours of WRS.

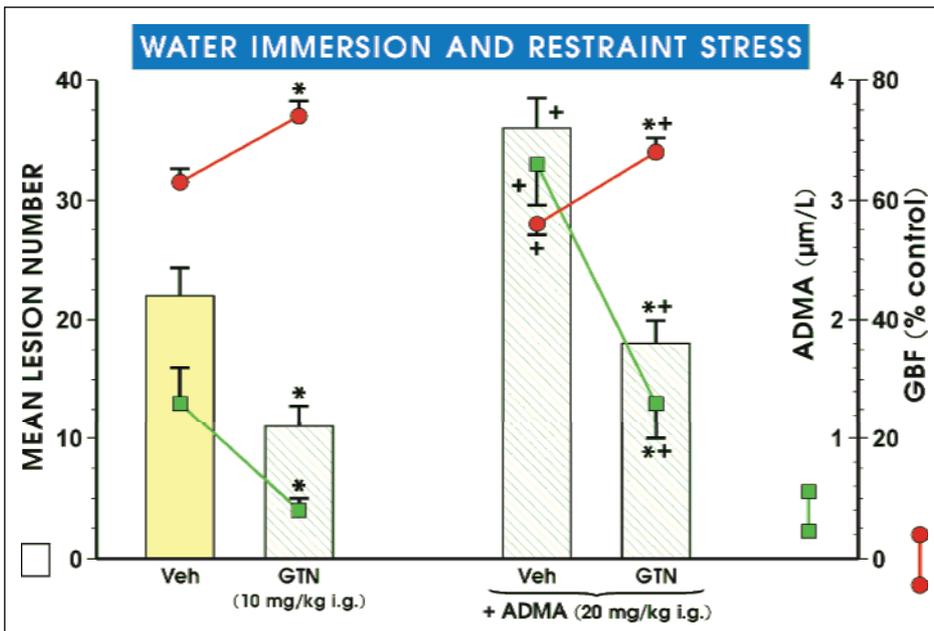


Fig. 5. The effect of pretreatment with vehicle or glyceryl trinitrate (GTN, 10 mg/kg i.g.) on mean lesion number of water immersion restraint stress (WRS) induced gastric lesions and the accompanying changes in the plasma ADMA levels and GBF. Results are mean  $\pm$  S.E.M. of 6-8 rats per each group. Asterisk (\*) indicates a significant change ( $p < 0.05$ ) as compared to the values obtained in vehicle-control gastric mucosa. Cross (+) indicates a significant change as compared with the values obtained in rats without ADMA administration. Asterisk and cross (+) indicate a significant change ( $p < 0.05$ ) as compared with the value obtained in rats pretreated with ADMA and exposed to 3.5 hours of WRS.

exposed to 3.5 hours of WRS, pretreated with either vehicle or ADMA applied i.g. in graded doses, ranging from 1 mg/kg up to 20 mg/kg. The expression of  $\beta$ -actin mRNA was well preserved in the mucosal biopsy samples taken from intact rats and those pretreated with vehicle or ADMA in graded doses and then subjected to WRS (Fig. 6, left panel). The IL-1 $\beta$  and TNF- $\alpha$  mRNAs were weakly detectable in the intact gastric mucosa, but were overexpressed in the mucosa exposed to WRS with or without the pretreatment with ADMA (Fig. 7, left panel, lanes 1-5). Semi quantitative analysis of the ratio of IL-1 $\beta$ / $\beta$ -actin mRNA and TNF- $\alpha$ / $\beta$ -actin revealed, that the expression of both, IL-1 $\beta$  and TNF- $\alpha$  mRNAs in intact gastric mucosa was almost negligible but it significantly rose in animals exposed to WRS. Elevation of IL-1 $\beta$ - and TNF- $\alpha$  mRNA expression was observed in the animals pretreated with graded doses of ADMA administered before WRS, especially when this compound was applied at the high dose of 20 mg/kg (Fig. 7, right panel).

## DISCUSSION

The animal model of water immersion and cold stress seems to be in particular suitable for examination of both local and systemic consequences of damaging action of stress on the gastric mucosa. This model mimics clinical acute gastric lesions, that may

appear in the gastric mucosa as a consequence of major trauma, surgery or sepsis, being widely accepted for studying the mechanism of stress-induced gastric lesions (1, 8). An acute inflammation of gastric mucosa plays an important role in the pathogenesis of stress-induced ulcerogenesis, being responsible for the enhanced permeability of blood vessels to activated neutrophils resulting in an excessive infiltration of gastric mucosal tissue (2, 4, 7). We confirmed that the overexpression and subsequent release of proinflammatory cytokines IL-1 $\beta$  and TNF- $\alpha$  play an essential role in pathomechanism of WRS-induced gastric damage, which could explain an enhanced permeability of blood vessels to neutrophils as reported before (2). It is known, that the membrane of neutrophils exhibit the activity of NADPH oxidase, a key enzyme in the formation of a superoxide radical anion ( $O_2^{\cdot-}$ ) (1-3). Thus, the enhanced expression and release of IL-1 $\beta$  and TNF- $\alpha$  contribute to the increased generation reactive oxygen species (ROS) in the gastric mucosa. Superoxide radical anion reacts with cellular membrane lipids, leading to the formation of lipid peroxides and giving rise to malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE). Gastric mucosa possesses an effective enzymatic system capable of scavenging ROS and preventing the cell damage and the injury to the gastric mucosa (3-5). Superoxide dismutase (SOD) is the major antioxidative enzyme in gastric mucosa that catalyzes the dismutation of superoxide radical anion ( $O_2^{\cdot-}$ ) into less noxious

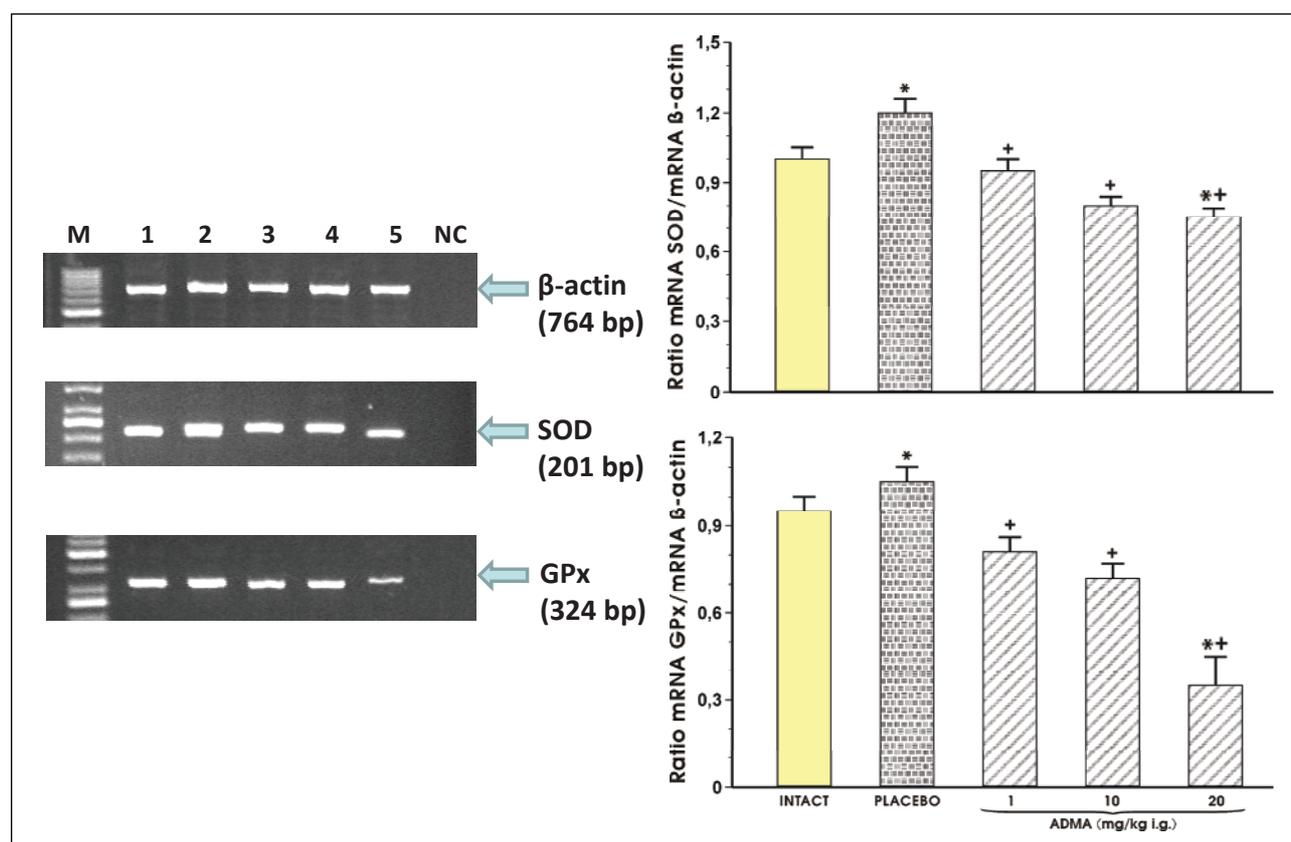


Fig. 6. Messenger RNA expression for  $\beta$ -actin, superoxide dismutase (SOD) and glutathione peroxidase (GPx) in the intact gastric mucosa (lane 1) and in the rats pretreated with vehicle (lane 2) as well as in those pretreated with ADMA given intragastrically (i.g.) in graded doses of 1 mg/kg (lane 3), 10 mg/kg (lane 4) and 20 mg/kg (lane 5) and 30 min later exposed to 3.5 hours of water immersion restraint stress (WRS) (left panel), NC - negative control (water), M - size marker DNA. Arrow shows expected PCR product. Semi quantitative assessment of the ratio of SOD mRNA/ $\beta$ -actin mRNA and the ratio GPx mRNA/ $\beta$ -actin mRNA expression in intact- and WRS rats pretreated with vehicle or ADMA (right panel). Results are mean  $\pm$  S.E.M. of 4 determinations. Asterisk (\*) indicates a significant change ( $p < 0.05$ ) as compared to the value obtained in intact gastric mucosa. Cross (+) indicates a significant change ( $p < 0.05$ ) as compared with the value obtained in rats pretreated with vehicle and exposed to 3.5 hours of WRS. Asterisk and cross (\*\*+) indicate a significant change ( $p < 0.05$ ) as compared with the value obtained in rats pretreated with ADMA at the dose of 1 mg/kg.

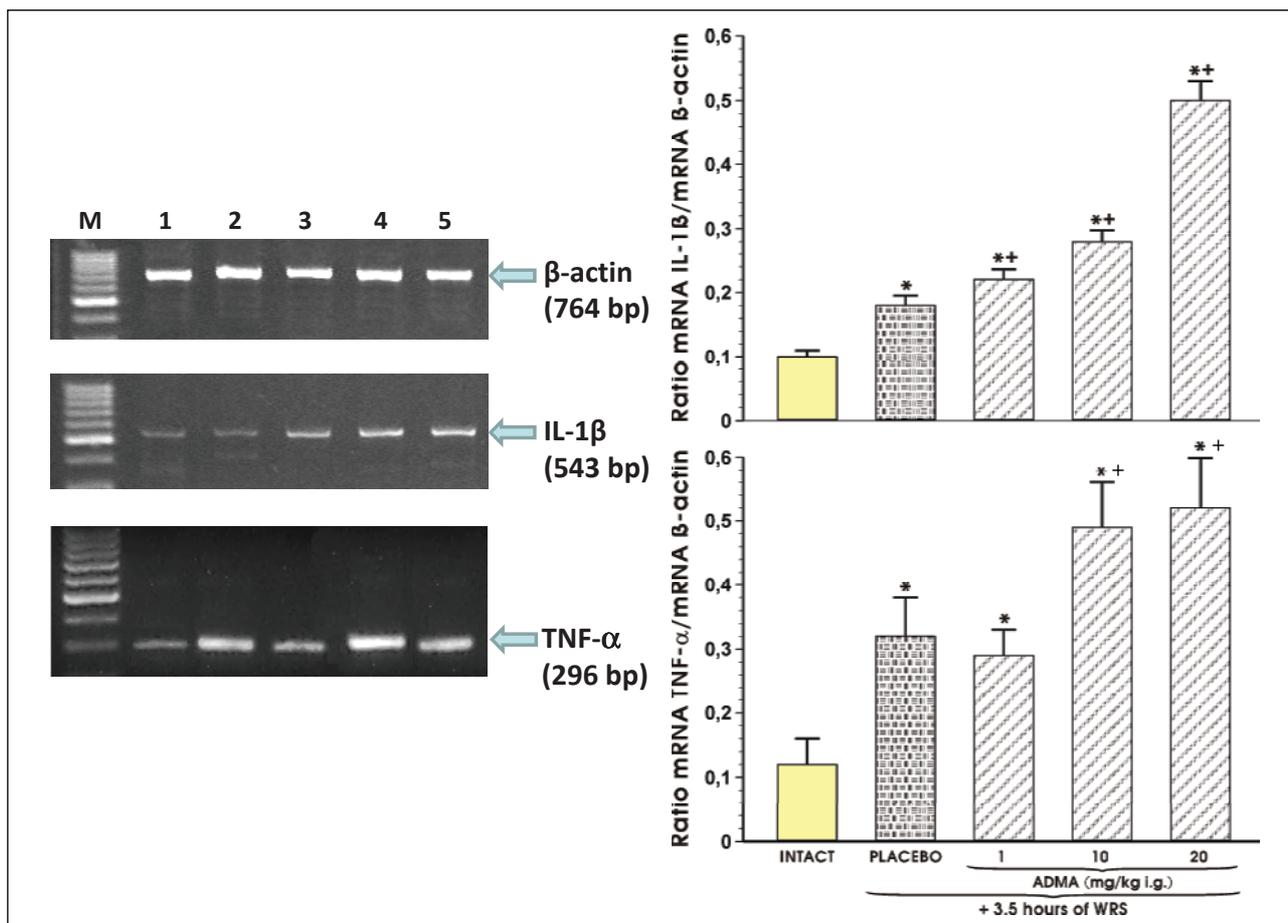


Fig. 7. Messenger RNA expression for  $\beta$ -actin, interleukin-1 beta (IL-1 $\beta$ ) and tumor necrosis factor-alpha (TNF- $\alpha$ ) in the intact gastric mucosa (lane 1) and in the rats pretreated with vehicle (lane 2) as well as in those pretreated with ADMA given intragastrically (i.g.) in graded doses of 1 mg/kg (lane 3), 10 mg/kg (lane 4) and 20 mg/kg (lane 5) and 30 min later exposed to 3.5 hours of water immersion restraint stress (WRS) (left panel), NC - negative control (water), M - size marker DNA. Arrow shows expected PCR product. Semi quantitative assessment of the ratio of IL-1 $\beta$  mRNA/ $\beta$ -actin mRNA and the ratio TNF- $\alpha$  mRNA/ $\beta$ -actin mRNA expression in intact- and WRS rats pretreated with vehicle or ADMA (right panel). Results are mean  $\pm$ S.E.M. of 4 determinations. Asterisk (\*) indicates a significant change ( $p < 0.05$ ) as compared to the value obtained in intact gastric mucosa. Asterisk and cross (\*+) indicate a significant change ( $p < 0.05$ ) as compared with the value obtained in rats pretreated with vehicle and exposed to 3.5 hours of WRS.

product, hydrogen peroxide ( $H_2O_2$ ), that undergoes further inactivation due to enzymatic activity of glutathione peroxidase (GPx) (6). The reduction of  $H_2O_2$  into water by GPx is accompanied by the conversion of glutathione from reduced form (GSH) into oxidized form (GSSG). As shown in our present study, the WRS-induced gastric damage is associated with augmented ROS-induced lipid peroxidation manifested by an increase of MDA+4-HNE concentration. These effects were accompanied by an enhancement of antioxidative mechanisms, particularly, an increase in the mucosal expression of SOD and GPx as shown in this study and the fall in the mucosal GSH content as reported before (2, 3).

Our previous studies documented that ROS are involved in the formation of WRS-induced gastric mucosal damage due to the production of free radicals and an enhancement of lipid peroxidation, the attenuation of mucosal antioxidative mechanisms and an impairment of antioxidizing enzyme activity and decrease of GSH tissue level (1, 2, 23). This depletion of GSH pool in gastric mucosa exposed to WRS could explain the apparent diminution of antioxidative potential of gastric mucosal barrier (2, 3). This fact may be explained by the mechanism of GSH-4HNE conjugates production, as an effective mechanism of elimination of toxic products of lipid peroxidation by reduced

form of glutathione (25). Moreover, Miyamoto *et al.* (28) documented, that an excess of lipid peroxide was neutralized by phospholipase  $A_2$  and then further reduced by glutathione peroxidase. They proposed that the glutathione peroxidase required large amounts of GSH for its function during ulcerogenesis leading to the depletion of cellular store of GSH. This was confirmed by Altinkaynak *et al.* (29) who showed that administration of rofecoxib caused a decrease in the GSH pool in rats with indomethacin-induced gastric lesions. However, in our present study, the major mechanism of GSH depletion could be attributed to the enhancement in expression of GPx, because under stress conditions gastric mucosal GPx was overexpressed compared to that in the intact mucosa.

Our present study provided for the first time an evidence that the administration of ADMA dose-dependently enhanced the number of WRS-induced gastric lesions and that this action of ADMA was accompanied by the marked rise in the plasma ADMA levels and the potent fall in gastric blood flow. These effects could be attributed to the inhibition of NOS activity and NO production by ADMA. The similar effects on number of WRS accompanied with fall in GBF were observed after inhibition of NOS by L-NNA, the nonspecific NOS inhibitor, and the supplementation of rats treated with L-NNA with donors of

NO such as L-arginine or GTN reversed these effects in L-NNA-treated animals (7, 9). In our present study, GTN, a known donor of NO, ameliorated the ADMA-induced increase in gastric lesions, the accompanying rise in plasma levels of ADMA and the marked fall in GBF. Moreover, Das and coworkers (18) evidenced that augmentation of NO production, by administration of NO substrate (L-arginine), counteracted the detrimental action of ADMA in hypertension and diabetes. Leiper *et al.* (30) also suggested, that the inhibition of NO synthesis by ADMA could be the major pathogenic mechanism involved in cardiovascular diseases. Recent evidence indicates that the gastric tissue contents of ADMA were significantly increased in *H. pylori*-positive volunteers, the effect being decreased, by *H. pylori* eradication (31). This observation indicates ADMA could contribute to the mechanism of pathologic changes associated with *H. pylori* infection (31). Moreover, ADMA can inhibit crucial element of gastric mucosal defense such as bicarbonate secretion and this could be explanatory for the adverse effect of this NO synthase inhibitor in the *H. pylori*-infected stomach (31). In keeping with this notion, the exogenous administration with ADMA inhibited the mucosal alkaline response to acid exposure. In fact, both *H. pylori* and water extract of *H. pylori* inhibited the acid-induced duodenal mucosal alkaline secretion concomitantly with an elevation of ADMA suggesting ADMA could be involved in the pathogenesis of gastric mucosal injury (31, 32).

We evidenced here that pretreatment with ADMA in rats exposed to WRS resulted in enhancement of oxidative stress as indicated by the increased level of lipid peroxidation products. This is in keeping with previous observations confirming that ADMA may trigger oxidative stress (10-12). Our results are corroborative with the observations by Wang *et al.* (13) and Zhang and coworkers (22) that ADMA may contribute to the pathogenesis of gastric mucosal injury induced by stress and possibly other damaging agents through an induction of oxidative stress, the aggravation of mucosal inflammatory response and the promotion of apoptosis. In our present study, the role of inflammatory processes exacerbated by ADMA during stress ulcerogenesis was confirmed by an elevation of plasma levels of proinflammatory cytokines IL-1 $\beta$  and TNF- $\alpha$  as well as activation of gene expression for these cytokines after ADMA application to rats exposed to WRS. ADMA administration during stress led to an impairment of antioxidative, protective mechanisms in gastric mucosa, as evidenced in our study by the decrease in the mucosal expression of SOD and GPx and the prominent rise in content of lipid peroxidation products MDA and 4-HNE.

In summary, we conclude that ADMA plays an important role in the pathomechanism of stress-induced gastric damage, due to suppression of NO generation, increased expression and release of proinflammatory cytokines IL-1 $\beta$  and TNF- $\alpha$ , the increment of lipid peroxidation, and downregulation of gene expression for antioxidative enzymes SOD and GPx. These results seem to also shed a little more light into pathomechanism of ADMA under clinical conditions associated with stress since ADMA has recently been implicated as causative factor in the pathogenesis of various system disorders including those evoked in gastrointestinal tract.

Conflict of interests: None declared.

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