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Interactions between nitric oxide and hydrogen sulfide generating systems in gastric mucosa under condition of the combined action of stress and NDAIDs

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ABSTRACT

The metabolic relationship between H_2S and NO in gastric mucosa in norm and pathology is still poorly studied. Aim of this study was to determine mechanisms of interaction between NO and H_2S generating systems under conditions of the combined actions of NSAIDs and stress. Water restraint stress (WRS) was used to induce peptic lessions in rats; naproxen and ATB-346 were administered prior to WRS. *Nos2*, *Cbs* and *Ptgs2* gene expression level was determined by semiquantitative RT-PCR in gastric epitheliocytes. In the gastric mucosa were determined: alterations in H_2S and NO_x concentrations, changes in activity of myeloperoxidase. Both WRS and naproxen action prior to WRS cased a significant rise in myeloperoxidase activity. Administration of ATB-346 resulted in a considerable decrease of myeloperoxidase activity. Naproxen action caused the downregulation of *Nos2*. The level of *Cbs* expression in group pretreated with naproxen was much higher than in group of WRS alone. We suppose that it increases as a result of *Nos2* downregulation and the correspondent decrease of NO concentration. The relationship between NO and H_2S in the gastric mucosa is likely mediated through the regulation of genes expression. As a result of the released H_2S, ATB-346 administration decreased the severity of gastric mucosa lesions.

INTRODUCTION

The best characterized among numerous gaseous substance acting as signaling molecules are nitric oxide (NO) and hydrogen sulfide (H_2S) (Wallace *et al.*, 2015). Each has been shown to play important roles in many physiological and pathophysiological processes in the gastrointestinal system (Wallace, 2010). NO is produced from L-arginine by constitutive NO synthases (cNOS) and inducible (iNOS) isoenzyme (Lanas, 2008). cNOS are very important enzymes in synthesis of NO, which is involved in the control of the gastric blood flow and the regulation of gastric mucosal integrity (Brzozowski *et al.*, 2008).

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iNOS (Ca^{2+}-independent) isoenzyme is activated by pro-inflammatory stimuli such as cytokines and produces relatively large amounts of NO which contribute to injury and dysfunction of gastric mucosa (Nasadyuk and Sklyarov, 2013). The toxicity of NO has been attributed to the potent nitrating and oxidizing agent, peroxynitrite that affect proteins and DNA. Endogenous H_2S is produced from L-cysteine either via pyridoxal-5-phosphate (P5P)-dependent enzymes – cystathionine β-synthase (CBS) and cystathionine γ-lyase (CSE), or the more recently described, cysteine aminotransferase (CAT; also P5P-dependent) and -3-mercaptopyruvatesulfurtransferase (3-MST) pathway (Chan, Wallace, 2013). At physiological conditions H_2S is produced by gastric mucosa and contributes to gastric ability to resist damage (Yan and Li, 2014), and like NO, it plays a role in modulating gastric inflammatory responses (Aboubakr *et al.*, 2013). Both NO and H_2S can exert pro- or anti-inflammatory effects depending on their concentrations; both are important mediators of gastric

mucosal defense and as well contribute significantly to repair of damage and resolution of inflammation (Wallace *et al.*, 2015). Moreover, there are close relationships between NO and H_2S generating systems.

Nonsteroidal anti-inflammatory drugs (NSAIDs) and acute stress are considered to be the main risk factors for the development of gastrointestinal pathology. On the other hand, they can affect metabolism of NO and H_2S. Adverse effects of NSAIDs are associated with the inhibition of cyclooxygenase (COX) and the deficiency of prostaglandins (PGs) (Takeuchi, 2012). Stress is well known to be associated with the formation of peptic ulcers. Ulcers frequently developed as a result of many stressful events including surgery, trauma, shock, sepsis and burns (Azlina *et al.*, 2015). The pathological basis of stress is connected to the action of hormones such as epinephrine and glucocorticoids which result in oxidative damage, reduced gastric blood flow and inhibition of gastric mucosal prostaglandins synthesis (Fomenko *et al.*, 2014). Both NSAIDs and stress increase the formation of reactive oxygen species (ROS), lipid peroxidation and cause a reduction of antioxidant status of the gastric mucosa (Konturek *et al.*, 2011). Moreover, NSAID- or stress-induced damage causes an inhibition of cNOS and a deficiency of H_2S that can result in disturbances of gastrointestinal motility, blood flow, secretion, etc (Wallace *et al.*, 2012).

However, the combined action of NSAIDs and stress is still poorly studied, as well as mechanisms of the metabolic relationship between H_2S and NO in the gastric mucosa. Thus, the purpose of this study was to determine the possible mechanisms of interaction between NO- and H_2S-generating systems under conditions of the combined actions of NSAIDs and stress.

MATERIALS AND METHODS

Animals

The structure of this study and the experimental procedures performed on the animals were approved by the Ethical Committee of L'viv National Medical University in accordance with the norms of the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes (1986). The experimental procedures were carried out in accordance with international guidelines for the use and care of laboratory animals. Male, outbred rats weighing 200-220 g were used. They were grouped and housed under conditions of controlled temperature (21-22^{o}C), humidity (65-75%) and light cycle and fed with standard rat chow and water *ad libitum*.

Study protocol

The rats were randomly divided into 4 groups (n=5-8 in each). Group 1 consisted of rats treated only with vehicle. In group 2, rats were exposed to 5 h of WRS to induce gastric lesions. For this reason, they were restrained in wire cages and immersed up to the depth of the xiphoid process in a water bath (23°C) for 5 hours to induce gastric mucosal lesions, as described by (Takagi *et al.* 1964). In group 3, naproxen, a non-selective cyclooxygenase (COX) inhibitor, (Sigma-Aldrich, Milwaukee) was administered prior to WRS. In group 4, an H_2S-releasing derivative of naproxen, ATB-346 (Antibe Therapeutics Inc., Toronto, Canada), was administered. Before administration, naproxen and ATB-346 were dissolved in dimethylsulfoxide (DMSO)/1% carboxymethylcellulose (CMC); 5:95 ratio. Both naproxen and ATB-346 were administered intra-gastrically (via an orally introduced polyethylene tube) at a single dose (10 mg·kg^{-1}; volume of 1 ml) 30 min prior to WRS. This dose was selected because it produced significant and comparable anti-inflammatory activity in reducing swelling in experimental arthritis (Blackler *et al.*, 2012). Rats were anesthetized with 1 mL of urethane at a dose of 1.1 mg/kg injected intraperitoneally and killed by cervical dislocation. Gastric mucosal samples from the whole mucosa were collected and homogenized in saline (1:4), centrifuged at 2,000 *g* and the supernatant was used for measurement of biochemical parameters. Mucosal specimens for RNA isolation were immediately snap-frozen in liquid nitrogen and stored at –80°C until analysis.

Histological Evaluation of Gastric Lesion

Specimens of the gastric walls from each rat were cut into pieces and fixed with 10% buffered formalin. The processed tissues were then infiltrated with paraffin to produce tissue-paraffin embedding block. Sectioning of the stomach was accomplished by microtome at a thickness of 5 μm. The section was mounted on individual microscope slide. Then, tissues were stained with haematoxylin and eosin for histological evaluation.

Semiquantitative RT-PCR

Expression of mRNA for COX-2, iNOS and CBS was determined using reverse-transcriptase polymerase chain reaction (RT-PCR). RNA was isolated following (Chomczynski and Sacchi, 1987). cDNA was synthesized in 20 µl of reaction mix containing 2 µg of RNA, 1 mM dNTP, 200 U of reverse transcriptase "Thermo Scintific RevertAid Reverse Transcriptase", corresponding buffer, 20 U of ribonuclease inhibitor "Thermo Scintific RiboLock RNase Inhibitor" ("Thermo Scientific", Lithuania), 20 pmol (1,0 µM) of reverse primer. Synthesis was carried out in the following conditions: 65° C – 5 min, 45° C – 1 hour. Polymerase chain reaction was performed in 30 µl of reaction mix containing 3 µl of cDNA, PCR buffer, 200 µM of each dNTP, 30 pmol (1,0 µM) of each primer, 2,5 mM of $MgCl_2$ and 1 U of Taq DNA polymerase ("Taq DNA Polymerase (recombinant)", "Thermo Scientific", Lithuania).

PCR amplifications consisted of an initial denaturing step of 95ºC for 3 min, followed by 35 (28 for *Actb* - gene used as internal control of reaction due to its constitutive expression; 30 for *Cbs*) cycles of 95ºC for 1 min, the annealing step (with optimal annealing temperature): *Ptgs2* (123 b.p., 53ºC – 45 s), *Cbs* (125 b. p., 55ºC – 40 s), *Nos2* (440 b. p., 52º C – 45 s) and *Actb* (521 b. p., 49º C – 40 s); the extending step at 72º C for 1 min 15 s (for *Ptgs2*, *Nos2*) or 1 min (for *Actb* and *Cbs*). Final extension step was performed upon 72º C for 5 min.

Such primer sequences were used in reactions: for *Nos2* – forward - GTGTTCCACCAGGAGATGTTG and reverse - CTCCTGCCCACTGACTTCGTC; for *Ptgs2* – forward - TGCTGTTCCAACCCATGTCA and reverse - TGTCAGAAACTCAGGCGTAGT; for *Cbs* – forward - ACACGATCATTGAGCCAACTTC and reverse - CAGCACATCCACCTTCTCCATA; for *Actb* – forward - TGGGACGATATGGAGAAGAT and reverse – ATTGCCGATAGTGATGACCT.

Reproducibility of the amplification results was evaluated in parallel experiments by the repetition of the PCR reactions with all animals and each primer at least three times. Separation of PCR products was performed electrophoretically in 1,6 % agarose gel with 0,5 x TBE buffer following (Sambrook *et al.*, 2000). For semi-quantitative analysis of amplicons expression based on densitometry the ImageJ 1.45s program was used. Indices of mRNA expression were calculated for each sample following (Konturek *et al.*, 1998).

Measurement of myeloperoxidase (MPO) activity

Gastric mucosal MPO was assayed spectro-photometrically by the method of (Bradley *et al.*, 1982) with some modifications. The MPO activity was analyzed spectro-photometrically as follows: 1 ml of homogenate was added to 2.9 ml of 0.1 M K_3PO_4 buffer (pH 6.0) involving O-dianisidine dihydrochloride (0.167 mg/ml) and 0.005% hydrogen peroxide of the resection mixture was recorded at a wave length of 450 nm. One unit (U) of activity was defined as that degrading 1 □mole of peroxide/mg of protein.

Determination of nitrite/nitrate (NO_2/NO_3)

NO*x* (nitrite/nitrate) concentration in gastric mucosal tissues was assayed by the Griess reaction-dependent method of (Green *et al.*, 1992)]. In order to determine total (NO_2/NO_3) concentration to deproteinized homogenates (1:100) of zinc for reduction of nitrate to nitrite or manganese sulfate for measurement of nitrate-anion where added. Naftil-ethylenediamine was used to perform Griess reaction. The absorbance was read in a Statfax at 520-560 (550) nm. Concentration of stable products of NO was expressed as nitrite+nitrate (mmol/g).

Determination of H_2S concentration in gastric mucosa

1 ml of homogenates of gastric mucosa were incubated for 60 min at 37°C with 1 ml of solution containing 4 mmol/l pyridoxal 5-phosphate, 20 mmol/l L-cysteine and Tris-HCL buffer (pH 8.5). Thereafter, 0.5 ml of 1 % zinc acetate, 0.5 ml of 20 mmol N,N-dimethyl-p-phenylenediamine in 7.2 M HCl were added and 0.4 ml of 0.4 mol FeCl3 were added and incubated for 20 min. Then, 0.5 ml of 20% trichloroacetic acid was added to precipitate any protein that might be present in the media and centrifugation (10,000 g) was performed. Absorbance (670 nm) of aliquots from the resulting supernatant was determined (Wilinski *et al.*, 2011). The calibration curve of absorbance vs. H_2S concentration was obtained by using NaHS solution.

Determination of proteins concentration in gastric mucosa

The concentration of proteins was determined by microbiuret method (Ruth *et al.*, 1964). Benedict's reagent was added to the homogenates of gastric mucosa in an alkaline environment and the change of color from blue to purple was observed.

Statistical Analyses

Statistical processing of experimental data was performed using GraphPad Prism 4.03 (“GraphPad Software Inc.”, USA). The normal Gaussian distribution of the data was verified by the Shapiro-Wilk normality test. Non-parametric Kruskal-Wallis test and Dunn’s post test were performed on obtained data. Statistical significance was set at $p \leq 0.05$. The data are expressed as means and standard deviations (SD).

RESULTS

The histological results showed that WRS induced mucosal congestion and disruption of surface epithelial cells with severe inflammatory cell infiltration at the base of mucosa (fig 1-B). Pre-treatment with naproxen potentiated the development of gastric lesions caused by WRS and showed a large hemorrhagic ulcerated area with significant leucocyte infiltration, edema, and disruption of deep mucosa (fig. 1-C). The pre-treatment of rats with ATB-346 prior to WRS significantly reduced the disruption of surface epithelial cells (fig. 1-D). The infiltration of the mucosa with leucocytes was significantly decreased proving the fact that H_2S can reduce leucocyte migration to the sites of injury (Zanardo *et al.*, 2006).

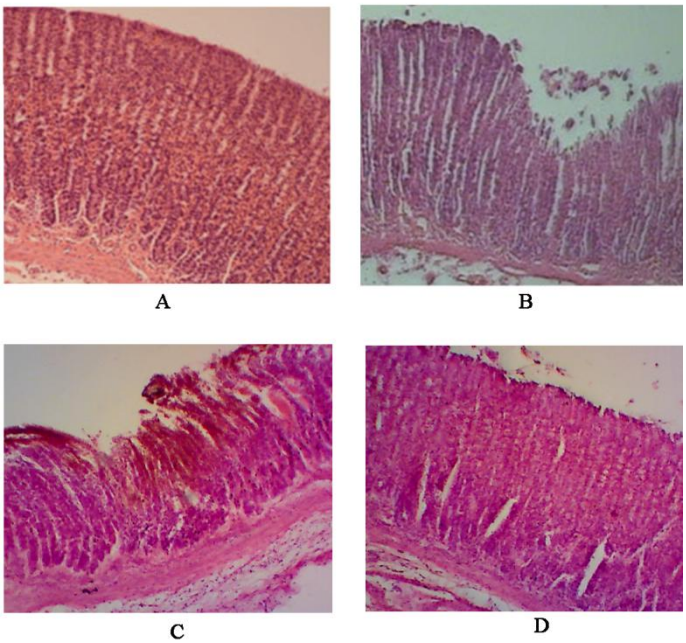

Fig. 1: Histological Status of gastric mucosa of vehicle-treated rats (A), rats subjected to WRS (B), pretreated with naproxen (C) and ATB-346 (D) prior to WRS. Magnification of 1:300.

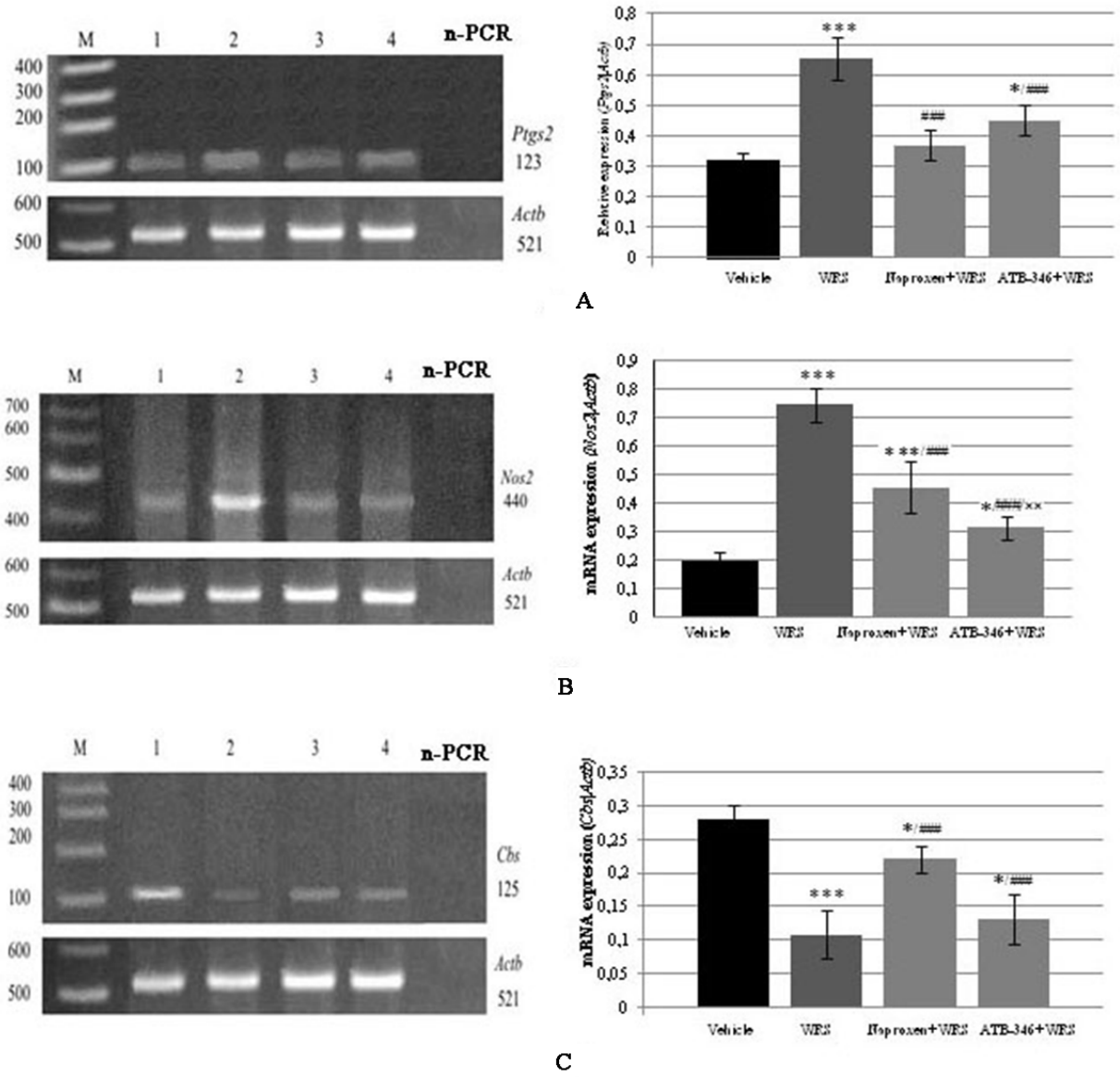

Fig. 2: Expression of mRNA for *Ptgs2* (A), *Nos2* (B) and *Cbs* (C) genes in gastric mucosa of vehicle-treated rats, rats subjected to WRS, pretreated with naproxen or ATB-346 prior to WRS. Mean ± SD. n=5 in each group of animals. * – p≤0.05, *** – p≤0.001, in relation to vehicle-treated animals; $^{\#\#}$ – p≤0.01, $^{\#\#\#}$ – p≤0.001, as compared to the rats subjected to WRS; $^{\times\times}$ – p≤0.01, $^{\times\times\times}$ – p≤0.01, as compared to the animals pre-treated with naproxen prior to WRS.

COX is the enzyme responsible for PG production, and exists in two isozymes, the constitutively expressed COX-1 and the inducible one COX-2 (Kargman *et al.*, 1996; Wallace, 2008). COX-2 plays an important role in the maintenance of mucosal defense in digestive tract, as well as in modulating mucosal inflammation (Wallace, 2008). There is considerable evidence for interactions between gasotransmitters and COX (Salvemini *et al.*, 1993). In our research, WRS caused the activation of adaptive cytoprotection manifested by the upregulation by 2.1-fold (p≤0.001) of *Ptgs2* gene expression (Fig. 2 – A), responsible for coding of COX-2 protein. Naproxen non-selectively inhibits activities of both COX-1 and COX-2. When administered 30 min prior WRS, it significantly down-regulated *Ptgs2* expression as compared to WRS alone, practically to the normal level. ATB-346 showed the similar effect on COX-2 gene expression to its parent drug.

Both WRS and nonselective COX inhibition by naproxen prior to WRS caused a significant rise in MPO activity (Fig. 3 – A), which indicates the enhancement of neutrophil migration in the gastric mucosa. As a result, neutrophils are involved in the damage process. Administration of ATB-346 prior to WRS resulted in a considerable decrease of MPO activity. This explains one of the aspects of H_2S-releasing NSAID's reduced gastrotoxicity.

We previously reported that subjecting rats to WRS resulted in a considerable rise of iNOS activity, while the administration of naproxen prior to WRS resulted in its decrease in the gastric mucosa (Fomenko *et al.*, 2014). In this research we have measured an expression of mRNA of *Nos-2*, the gene

responsible for coding of iNOS protein. *Nos2* expression was increased 3.7-fold ($p \leq 0.001$) under conditions of WRS. Application of naproxen caused the down-regulation of iNOS gene expression, which explains the decrease of iNOS activity. ATB-346 decreased *Nos2* expression 1.7-fold ($p \leq 0.001$) more than naproxen. This may have been attributable to the actions of H_2S released from ATB-346, which can regulate iNOS gene expression. However, the concentration of stabile metabolite of NO was very similar in groups of rats pretreated with either of the NSAIDs (Fig.3-B).

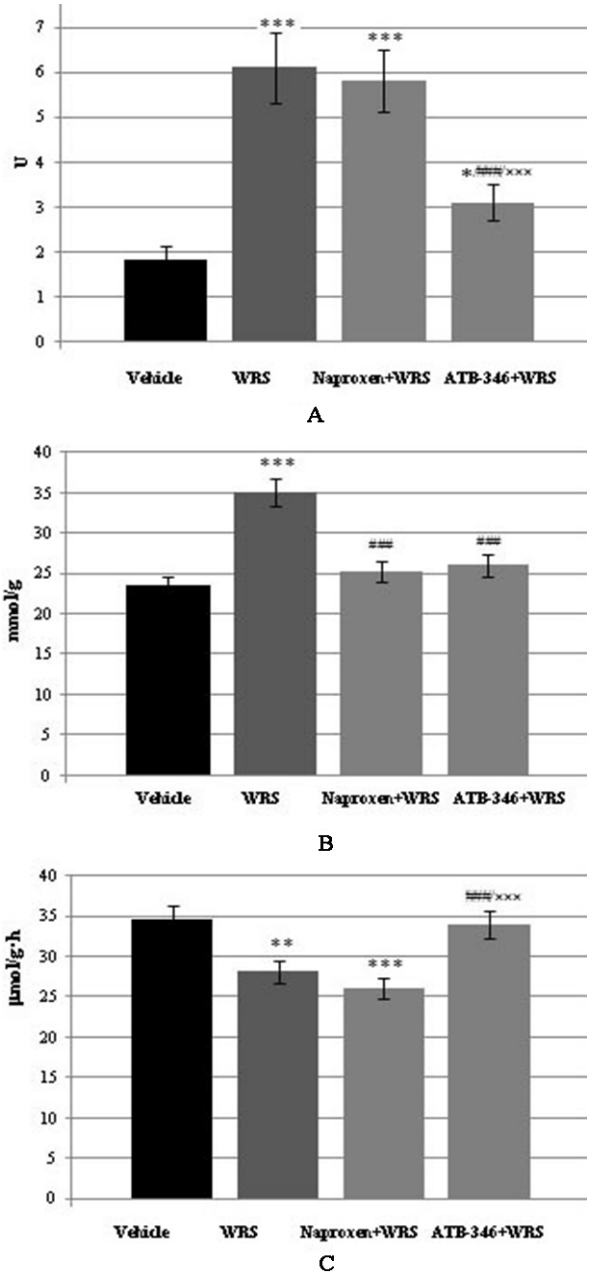

Fig. 3: Activity of myeloperoxidase (MPO) (A), concentrations of stable products of NO (B) and H_2S (C) in gastric mucosa of vehicle-treated rats, rats subjected to WRS, pretreated with naproxen orATB-346 prior to WRS. Mean ± SD, n=8 in each group of animals. * – $p \leq 0.05$, *** – $p \leq 0.001$, in relation to vehicle-treated animals; $^{\#\#}$ – $p \leq 0.01$, $^{\#\#\#}$ – $p \leq 0.001$, as compared to the rats subjected to WRS; $^{\times\times}$ – $p \leq 0.01$, $^{\times\times\times}$ – $p \leq 0.01$, as compared to the animals pre-treated with naproxen prior to WRS

It was previously shown that NO metabolites can regulate activity of CBS but not CSE (Taoka and Banerjee, 2001). In this research we estimated the level of *Cbs* mRNA expression, WRS caused a decline of *Cbs* expression, it was 2.6-fold lower than in the control group ($p \leq 0.001$), likely a consequence of the down-regulation of CBS gene expression, H_2S concentration in gastric mucosa declined by 25% ($p \leq 0.001$). Unexpectedly, the level of *Cbs* expression in rats pretreated with naproxen prior to WRS was much higher than with WRS alone. It is possible that it increased as a result of *Nos2* downregulation and the corresponding decrease of NO concentration. We speculate that NO can influence not only CBS activity but also *Cbs* expression. However, the H_2S concentration in gastric mucosa of rats subjected to the combined action of WRS and naproxen was 25% lower ($p \leq 0.01$) than in control group. This suggests a key role of other enzymes involved in the generation of the intracellular H_2S pool, such as CSE or pyridoxal-5-phosphate independent enzymes.

In the gastric mucosa of rats which were treated with ATB-346 prior to WRS, the H_2S concentration was practically normal, buth there was still a down-regulation of *Cbs* expression similar to that observed in the group treated with WRS alone.

DISCUSSION

In this study we have examined the combined action of two types of NSAIDs: conventional (naproxen) and an H_2S-releasing derivative (ATB-346), as well as WRS, on the metabolism of gaseous mediators (NO and H_2S) in the gastric mucosa. Both NO and H_2S dose-dependently contribute to many physiological and pathological processes, including the maintenance of gastrointestinal integrity and the mechanism of gastroduodenal protection. Nevertheless, if the synthesis of NO and H_2S is overwhelmed by injurious factors, such an acute stress or use of NSAIDs, a gastric mucosal lesion may develop (Wallace, 2010).

The exposure to stress is one of the most important risk factors in the pathogenesis of various diseases of gastrointestinal system (Huerta-Franco *et al.*, 2013). Stress affects different functions of the digestive system including gastric secretion, gut motility, mucosal permeability, mucosal blood flow and the gastrointestinal microflora (Konturek *et al.*, 2011). In our study, rats subjected to WRS developed ulcerative and hemorrhagic gastric lesions with corresponding histological changes.

As reported previously (Nie *et al.*, 2004), up-regulation of the *Ptgs2* and *Nos2* genes, which are responsible for coding of pro-inflammatory enzymes COX-2 and iNOS, respectively, was detected in gastric mucosa of rats subjected to WRS. It was previously shown that there was an up-regulation of COX-2 mRNA post-stress which, may be important in the production of PGE_2 during the healing of ulcers (Nie *et al.*, 2004). We had previously showed that considerable activation of iNOS takes place as a result of acute stress (Azlina *et al.*, 2015). In the present study, we showed that the concentration of NO_x increased as a result of *Nos2* up-regulation and iNOS activation. Oxidative stress

developed as a consequence of vasoconstriction in the WRS model. The rise of NOx concentration created grounds for the peroxynitrite generation, which is one of the most toxic derivatives of NO (Lundberg and Weitzberg, 2013). Thus, our research confirmed the suggestion that NO production from iNOS plays a key role in stress-induced gastric injuries (Azlina *et al.*, 2015).

Inflammation of the gastric mucosa developed as a result of stress, which causes enhanced permeability of blood vessels, activation of neutrophils, and their excessive infiltration of the gastric mucosa (Azlina *et al.*, 2015).As a result, we detected the considerable increase in the activity of MPO in the WRS group. Concentrations of another gaseous mediator, H_2S, decreased under the stress conditions as a result of *Cbs* gene down-regulation. It created additional conditions for the development of oxidative stress, because the gastric mucosa loses the antioxidant potential of H_2S.

A range of different NSAIDs are widely used for the treatment of different pathologies. Their use is often accompanied by acute or chronic stress. NSAID administration is associated with both topical and systemic effects leading to the formation of gastric lesions (Wallace, 2008). Endogenous H_2S generation under these conditions is often inhibited and thus increases the susceptibility of the mucosa to damage induced by NSAIDs (Fomenko *et al.*, 2014). In our study, e pretreatment of rats with a non-selective COX inhibitor (naproxen) under conditions of an acute stress considerably worsened the histological status of the gastric mucosa. The H_2S-releasing derivative of naproxen (ATB-346) exhibited reduced gastro-toxicity as compared to its parent drug. This phenomenon was previously described and it was associated with the gastroprotective action of H_2S (Fomenko *et al.*, 2014). We previously reported that the reduced gastrotoxicity of ATB-346 under conditions of acute stress was not connected to the antioxidant role of H_2S, because MDA concentrations in gastric mucosa were not different from those of rats subjected to simultaneous action of naproxen and stress (Fomenko *et al.*, 2014). In the present study we showed that ATB-346 considerably inhibited MPO activity in correspondence to the previously shown data (Palinkas *et al.*, 2015). Thus, we can conclude that the main cytoprotective effect of H_2S released from ATB-346 given at a single dose 10 $mg \cdot kg^{-1}$ is likely due to its inhibitory effect on infiltration of neutrophils to gastric mucosa.

Taking into account that both NO and H_2S are actively involved in the mechanisms of gastroprotection and ulcerogenesis, and ATB-346 showed the reduced gastrotoxicity under condition of WRS as compared to its parent drug naproxen, the question of potent mechanisms of interaction between gasotransmitters is very important. It should be pointed out that stress caused a sharp u-pregulation in *Nos2* expression and activity of iNOS while naproxen and ATB-346 administered prior the stress considerably decreased them. This was possibly mediated by the COX-1 inhibition by these NSAIDs and the interaction between COX and NOS (Mollace *et al.*, 2005). The up-regulation of *Nos2* under condition of stress was accompanied by a corresponding increase in NO_x concentrations and led to *Cbs* down-regulation and a lack of H_2S synthesis. Administration of either naproxen or ATB-346 prior the stress caused considerable up-regulation of *Cbs* mRNA. Still, the influence of ATB-346 on the level of *Nos2* expression was more significant than that of naproxen, suggesting that H_2S released from ATB-346 may decrease the generation of NO. Thus, the relationship between NO and H_2S in the gastric mucosa under conditions of stress and NSAIDs is likely mediated through the regulation of genes expression. As a result of the released H_2S, ATB-346 administration decreased the severity of gastric mucosa lesions.

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Conflict of Interests: There are no conflicts of interest.

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