A. SZLACHCIC, R. BILSKI, A. DZIADUŚ-SOKOŁOWSKA, J. MICHALSKI, J. MROCZKA

THE EFFECT OF NITRIC OXIDE DONORS AND L-ARGININE ON THE GASTRIC ELECTROLYTE BARRIER

Department of Biophysics, Chair of Physiology, Collegium Medicum, Jagiellonian University, Kraków, Poland

The potential difference across the stomach wall (PD) is determined by the gastric mucosal barrier. The decrease in the PD evoked by "the barrier breakers", e.g. aspirin, ethanol or bile acids is believed as a sensitive index of the mucosal damage. The effect of glyceryl trinitrate (GTN), isosorbide dinitrate (IDN) and molsidomine (MOL) — all exogenous donors of nitric oxide (NO), as well as L-arginine (L-ARG), which is a substrate for NO-synthase and Nω-nitro-L-arginine (L-NNA), a non--selective NO synthase inhibitor on the gastric electrolyte barrier were studied against the gastric damage induced by ethanol. All NO donors given intragastrically alone caused only moderate, not significant changes in the PD and failed to affect the mucosal barrier, while L-NNA slightly decreased the PD. The NO donors and L-arginine applied as pretreatment prior to ethanol resulted in diminishing of its damaging action that was similar for all these drugs, while L-NNA intensified both the injury and the drop in the PD values caused by ethanol. In summary, our results showed the protective effect of endogenous nitric oxide from L-ARG and that originating from GTN, MOL and IDN on the gastric electrolyte barrier, supporting involvement of nitric oxide in the mechanism of gastric protection in the stomach.

Key words: *gastric electrolyte barrier, potential difference, nitric oxide, ethanol*

INTRODUCTION

The high concentration of hydrogen ions in the gastric juice is maintained by the electrolyte gastric mucosal barrier (1). The agents disrupting this barrier, such as ethanol, aspirin and bile acids cause the back-diffusion of hydrogen ions from the gastric lumen into the mucosa with coexisting shifts of other ions. Under normal physiological conditions ion concentration gradients originate an electrical potential difference (PD) across the stomach wall equal to 40—50 mV in humans with the lumen negatively charged. Both, the experimental studies in animals and the clinical gastroscopic investigations, have demonstrated that even a single ingestion of 100 ml of 40% ethyl alcohol

produces a fast drop in the absolute PD value followed by a transient mucosal damage, similar to that exhibited by the acute hemorrhagic gastritis (2, 3). Therefore the assessment of the decrease of the PD by various noxious agents, occurring very early upon damage, appears to be a sensitive index of the mucosal damage. The administration of the mucosal barrier disrupting agent for a long time may lead to the development of a chronic gastritis and even of peptic ulcers.

Our previous studies documented that various sorts of alcoholic beverages or intragastric application of ethanol that interacted with different chemical factors influenced the electrolyte barrier of the gastric mucosa and is reflected in the changes in the gastric potentials (4, 5). Release of free radicals was proposed as one of the essential mechanisms contributing to the damaging action of ethanol (6). Nitric oxide (NO) has attracted the considerable interest as a messenger molecule in many physiological reactions, including control of gastric motility and secretion, gastric blood flow and gastric mucosal defense mechanisms (7). Nitric oxide is expected to exert a protective mechanism in the gastric mucosa against the attack of oxygen free radicals released by alcohol. The aim of our study was to evaluate the action of NO released from NO-donors on the gastric mucosal barrier and the potential difference and its interaction with the barrier damaging effect of ethanol. We employed exogenous NO donors that are widely used in treatment of coronary insufficiency: glyceryl trinitrate (GTN), molsidomine (MOL) and isosorbide dinitrate (IDN). For comparison the effects of a substrate for NO-synthase L-arginine (L-ARG), and Nω-nitro-L-arginine (L-NNA) that was shown to suppress NO-synthase (8) were tested under the same experimental conditions.

MATERIALS AND METHODS

The experiments were carried out in Wistar rats of either sex weighing 250—300 g anaesthetized with α-chloralose (0.06 g/kg) plus ethyl urethane (0.6 g/kg) applied i.m. Each of the twelve series consisted of 8—12 rats, which results were included into consideration after elimination of the animals with the PD value lower than 20 mV. The PD across the stomach wall was measured with high resistance digital multimeter using method described previously (9) and modified by our group (10). The electrical circuit consisted of two calomel electrodes and agar-agar bridges (polyethylene drainage tubes filled with 3% agar-agar gel in saturated KCl solution). The potentials of the mucosal side of the stomach was measured by the KCl-agar-agar bridge introduced into the stomach through esophagus and of serosal side by measuring equivalent potential of the blood (in these experiments a cut tail dipped in saturated KCl solution), according to the method described elsewhere (4).

The substances tested were given by the polyethylene tube introduced into the gastric lumen. Immediately after the initial PD measurement at the moment t = 0, denoted as PD_0, 1 ml of water or 1 ml of 40% ethanol (ETH) were given intragastrically. All NO donors and NO synthase inhibitor were administered intragastrically in aqueous solutions (0.5 ml) as pretreatment, 15 minutes before t=0, in the following doses: GTN — 0.04 mg/kg; MOL — 0.16 mg/kg; IDN

— 0.57 mg/kg; L-ARG — 20 mg/kg; L-NNA — 10 mg/kg. The applied doses corresponded with ca. daily human doses recalculated for rat body weight. The changes in the PD after administration of the solution tested were compared with initial values (PD_0) and expressed in percentages. The statistical significance of the obtained results was assessed with the Student's "t" test for comparing results in the group and χ^2-test for comparison between groups.

RESULTS

Table 1 shows the effect of administration of different drugs modifying the level of NO in the gastric mucosa prior to the 1 ml of water introduction as compared to animals without drug pretreatment (control).

Table 1. The effect of the drugs modifying level of NO given alone on the intact gastric mucosa compared to the control experiment. The drugs were applied intragastrically as pretreatment 15 min prior to 1 ml of water introduction. There are compared the absolute PD values ± standard errors of the mean (S.E.M.) measured at the following moments: 1/ initial, immediately before water introduction (PD_0), 2/ when the PD was maximally changed (PD_m) and 3/ the final, 60 min after water introduction (PD_{60}). Asterisks indicate the statistical significance as compared to the respective PD_0 values [(*) — $p < 0.05$].

Pretreatment:	PD_0 [mV]	PD_m [mV]	PD_{60} [mV]
control without any drug pretreatment	29.9 ± 1.4	31.2 ± 1.8	29.8 ± 1.8
glyceryl trinitrate [GTN]	34.7 ± 1.3	36.9 ± 2.2	35.9 ± 1.8
molsidomine [MOL]	34.6 ± 2.0	33.7 ± 1.8	35.1 ± 1.7
isosorbide dinitrate [IDN]	36.0 ± 1.9	34.8 ± 1.8	33.7 ± 1.8
L-arginine [L-ARG]	33.2 ± 1.4	34.1 ± 1.6	33.7 ± 1.8
Nω-nitro-L-arginine [L-NNA]	30.8 ± 1.1	27.4 ± 1.2 (*)	29.7 ± 0.9

The major aim of these investigations was to determine the role of nitric oxide in intact stomach. Results are presented as the absolute values of the electrical potential difference of the stomach mucosa (PD) and were expressed in millivolts. Three measurements were compared: 1/ initial (PD_0) immediately before water introduction, 2/ when the PD was maximally changed (PD_m) and 3/ at 60 min after water introduction (PD_{60}). The statistical significance of the PD in comparison to the respective initial PD_0 values are marked if occurred with asterisks: (*) — $p < 0.05$.

In *Table 2* the effect of pretreatment with the equipotent doses of drugs modifying the level of NO in the gastric mucosa prior to 1 ml ethanol 40% introduction was compared against the effect of 40% ethanol given alone.

Table 2. The effect of the drugs that modify level of NO given intragastrically in combination with ethanol compared to ethanol alone. The drugs were applied as pretreatment 15 min prior to 1ml of 40 % ethanol introduction. There are compared the absolute PD values $\pm$ standard errors of the mean (S.E.M.) measured at the following moments: 1/ initial, immediately before ethanol introduction (PD_0); 2/ when the PD was maximally decreased (PD_m) and 3/ the final, measured 60 min after ethanol introduction (PD_{60}). Asterisks indicate statistical significance as compared to the respective PD_0 values [(***) — $p < 0.001$; (**) — $p < 0.01$; (*) — $p < 0.05$].

Pretreatment:	PD_0 [mV]	PD_m [mV]	PD_{60} [mV]
ethanol without any drug pretreatment [ETH]	29.9 ± 1.4	18.4 ± 1.2 (***)	29.8 ± 1.8
glyceryl trinitrate [GTN]	33.2 ± 1.8	36.9 ± 2.2 (*)	34.1 ± 1.6
molsidomine [MOL]	35.2 ± 2.0	26.8 ± 2.1 (*)	32.6 ± 1.9
isosorbide dinitrate [IDN]	34.0 ± 1.7	24.1 ± 2.7 (***)	34.1 ± 2.1
L-arginine [L-ARG]	30.2 ± 1.2	21.3 ± 1.5 (**)	30.1 ± 1.8
Nω-nitro-L-arginine [L-NNA]	32.0 ± 1.5	17.4 ± 1.7 (***)	29.2 ± 1.2

Similarly as in previous *Table 1*, the results are presented as the absolute values of the PD expressed in millivolts and measured in three moments: 1/ initial (PD_0) immediately before ethanol introduction, 2/ when the PD was maximally decreased (PD_m) and finally 3/ at 60 min after ethanol introduction (PD_{60}). The statistical significances of the PD in comparison to the respective initial PD_0 values are marked if occurred with asterisks: (***) — $p < 0.001$; (**) — $p < 0.01$; (*) — $p < 0.05$.

For better recognizing the effect of investigated drugs the time course of the PD relative values were evaluated. The relative values expressed in percentages of the respective initial PD_0 values were proposed instead of the absolute ones for better compatibility of results, because the average initial absolute PD_0 values were different in the each experimental set of animals.

Figure 1 shows the time courses of the relative PD values in experiments with 1 ml of water introduction 15 minutes after the administration of each drug applied alone on the intact mucosa. For comparison the plot of the PD in the control experiment with water introduction without pretreatment is presented.
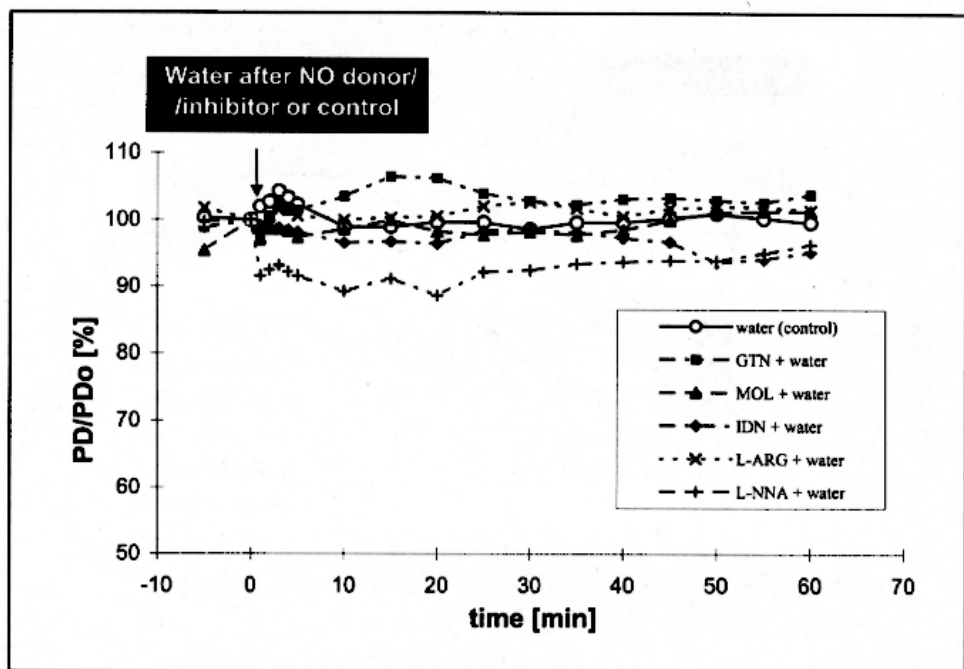
Fig. 1. The time courses of the relative PD expressed in percentages of the respective initial PD_0 values in all series of experiments with the intragastric administration of water after pretreatment with NO exogenous donors (GTN, MOL, IDN), NO synthase substrate (L-ARG) and NO synthase inhibitor (L-NNA) in comparison to the introduction of water alone in the control experiments.

It is clearly visible that three slightly different types of the PD time dependence occur. Introduction of water (control) and pretreatment with MOL, IDN and L-ARG alone prior to water failed to influence the PD value. GTN application increased very slightly the PD by about 8% in 15th min after water introduction; this increase was however not significant. In contrast, L-NNA produced a significant ($p < 0.05$) decrease of the PD at 20th min by 10.7% as compared to the PD_0 value. This drop was also more persistent. No significance however occurred between the different series compared with χ^2-test. All of the drugs used alone failed to induce macroscopically visible gastric lesions.

In *Figure 2* the time courses of the relative PD show significant differences between effect of pretreatment with the drugs modifying a level of nitric oxide in the gastric mucosa on the action of 40% ethanol in comparison to the effect of ethanol alone and to the control experiments.
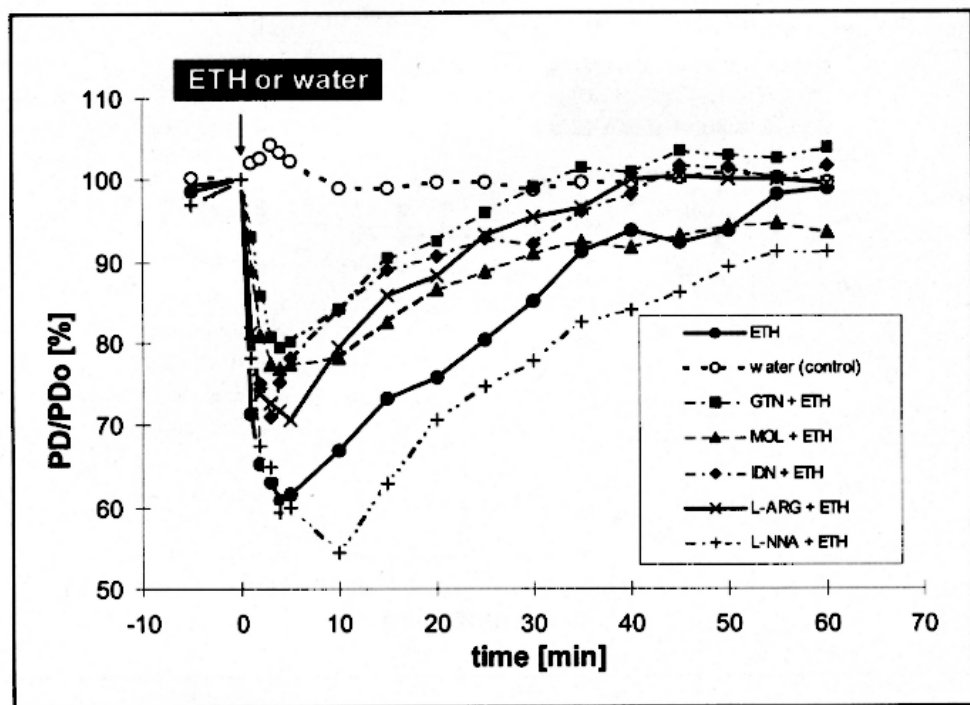
4*

Fig. 2. The time courses of the relative PD expressed in percentages of the respective initial PD_0 values in all series of experiments with the intragastric administration of ETH alone and ETH after pretreatment with NO exogenous donors (GTN, MOL, IDN), NO synthase substrate (L-ARG) and NO synthase inhibitor (L-NNA) in comparison to the introduction of water alone (the control experiments).

After the intragastric administration of GTN, MOL, IDN and L-ARG prior to ETH, the reductions in the PD by 20.5% ($p < 0.05$), 23.7% ($p < 0.05$), 29.2% ($p < 0.001$) and 29.5% ($p < 0.01$), respectively, were observed, while ETH alone decreased the PD in significant manner ($p < 0.001$) by about 39%. All these drops of PD were calculated in relation to the PD initial values (PD_0) in each particular experimental group. The PD value reached the highest decline at 3rd — 4th min after ETH administration for NO donors and at about 5th min for L-ARG. Following time-course of the recovery of the PD was similar in all groups tested, excluding MOL, and finally the PD values returned to the initial ones within 60 min upon ethanol administration. Pretreatment with MOL caused slower reincrease of the PD value to about 93% of its initial value within 60 min.

The pretreatment with each of the NO donors before ETH, as well as with L-ARG, a substrate for NO synthase applied 15 min prior to ETH evoked a significantly smaller drop in the PD as compared to that produced by ETH

alone. Calculated with the χ^2-test the respective significance were as followed: $p < 0.01$ for GTN + ETH and for IDN + ETH, $p < 0.05$ for MOL + ETH and for L-ARG + ETH. The attenuation of the drop in the PD was however similar for each of the NO donor tested and the differences between experiments with the different drugs given prior to ethanol were not significant for any combination examined.

ETH given after pretreatment with L-NNA caused a reduction of the initial PD_0 by 45.7% ($p < 0.001$). The minimum in the PD value was reached later than in the NO donors' pretreatment, about 10 min after ETH administration. Further time course of the PD recovery was similar to that measured after ETH alone, however it reached within 60 min about 92% of the PD_0 value only. The difference between ETH alone versus L-NNA combined with ETH failed to show statistically significant difference. The differences between the all NO donors tested or L-ARG combined with ETH versus L-NNA combined with ETH were equally significant ($p < 0.01$).

DISCUSSION

This study demonstrates that glyceryl trinitrate, molsidomine and isosorbide dinitrate known as donors of NO and L-arginine, a substrate for NO synthase administered intragastrically failed by themselves to alter significantly the normal gastric mucosal barrier as reflected by minor influence of these compounds on the PD value. Glyceryl trinitrate evoked slight increase in the PD value, but molsidomine, isosorbide dinitrate, and L-arginine did not exert any appreciable effects on the PD. These results confirmed our preliminary observations (11) that NO derived from NO-donors may influence other functions in the stomach but remained without effect on the PD.

It is known that ethanol produces a significant drop in the gastric PD and that this effect depends upon the concentration of ethanol employed (5). The 40% ethanol solution applied in the present study is relevant to the concentration of this agent in many alcoholic beverages (4). According to our study, this concentration markedly decreased the PD, an index of the damage of the gastric mucosal barrier.

One of the mechanisms responsible for the destructive action of ethanol on the gastric mucosa could be the generation of oxygen free radicals (ROS) that follows the oxidative stress. Antioxidants such as vitamins E and A decrease post-alcohol peroxidation of lipids in liver and brain and attenuate the suppression of glutathione activity induced by ethanol in liver (6). Alcohol is metabolized with few enzymes; the most important are alcohol dehydrogenase and, however quantitatively less important, enzymes in microsomal cytochrome P-450, especially isoenzyme CYP2E1. Ethanol induces the biosynthesis of

this latter isoenzyme (6). In our previous studies the damaging effect of strong alcohol beverages was markedly diminished by the earlier ingestion of the low concentration alcohol solution (5). At least a part of this protection exhibited by, so called, "mild irritants" could be attributed to improved antioxidant activity of certain enzymes, including alcohol dehydrogenase and CYP2E1.

Pretreatment with the nitric oxide donors was proposed to check whether these compounds affect gastric electrolyte barrier. The NO molecule is well-known antioxidant involved in many biochemical processes in living organism. It was shown that NO enhanced blood flow in the gastric microcirculation and had a potent influence on the gastric acid secretion with overproduction of the gastrin (7, 12). Other authors revealed that moderate amount of lipopolysaccharides originated from *Helicobacter pylori* protected gastric mucosa against the damage provoked by ethanol with accompanying increase of gastric blood flow and these effects were mediated by NO; it was proposed that low concentrations of endotoxin could induce gastric adaptation due to overexpression of iNOS mRNA and excessive NO production (13).

The importance of the NO in this protection was additionally supported by the fact that suppression of NOS by Nω-nitro-L-arginine methyl ester (L-NAME) abolished the protective effect of lipopolysaccharide (14). Other mechanism of NO action includes effect of this molecule on the gastric mucus secretion. It was shown that NO donors increase gastric mucus gel thickness in rats and stimulate mucus secretion by gastric epithelial cells *in vitro* (15). In other *in vitro* experiments the hydroperoxide-induced lipid peroxidation in intestinal epithelial cells was attenuated by NO (16). The protective role of NO derived from cNOS is well documented against damage induced by indomethacin, but the activation of iNOS leading to excessive release of NO was considered to act as proulcerogenic (17).

The pretreatment with exogenous NO donors GTN, MOL and IDN or the NO- synthase substrate L-ARG led to the enhancement in NO concentration, diminished significantly the injuring effect of ethanol on the mucosal barrier and caused smaller fall in the PD value as compared to those treated with ethanol alone. The acceleration of the recovery process after ethanol damage in rats pretreated with NO donors or L-arginine was also significantly improved. There was no difference in the protective activity on gastric mucosal barrier between exogenous NO donors and L-arginine in the doses applied.

In order to examine the protective role of endogenous nitric oxide in the mechanism of electrolyte barrier in the mucosa exposed to ethanol the additional experiments with administration of the NO synthase inhibitor Nω -nitro-L-arginine (L-NNA) were performed. L-NNA alone given intragastrically evoked small injuring action on the mucosal barrier and slightly, but significantly diminished the PD value. Significance was not confirmed however in comparison to control experiments. This notion is supported by the

observation that L-NNA intensified the PD decrease evoked by ethanol potently below that recorded in mucosa exposed to ethanol without L-NNA pretreatment. Both these effects may be due to the suppression of NO-synthase activity and failure of scavenging action of this molecule on the generation of free radicals.

It is likely that these effects occurred only in the limited time of vasodilation caused by increasing amount of NO due to short half life of this molecule, thus limiting the protective action of NO-donors on gastric mucosal barrier in these experiments. In contrast to exogenous NO donors that donated NO, application of L-arginine was expected to increase the biosynthesis of NO. The intragastric application of L-arginine as pretreatment enhanced this mechanism, however to the small extent. This minor effect can explain the lack of statistically significant difference between the action of L-ARG on the PD in comparison to the effect of the exogenous NO donors applied in experiments as well with as without ethanol introduction.

In summary, our results show the protective effect of endogenous nitric oxide from L-arginine and that originating from glyceryl trinitrate, molsidomine and isosorbide dinitrate on the gastric electrolyte barrier, supporting the notion that NO plays an important role in the mechanism of gastric protection and in maintenance of mucosal integrity (7, 18). The time courses of the electrical potential difference confirmed also that after intragastric application the nitric oxide donors exerted the protective activity on gastric electrolyte barrier by simple donating of NO molecules rather than due to more complicated biochemical mechanisms.

REFERENCES

1. Świerczek J, Konturek S. Effect of gastric mucosal barrier breakers on canine alkaline secretion and transmucosal potential difference. *Adv Physiol Sci* 1980; 12: 221—225.
2. Bode JC. Alcohol and the gastrointestinal tract. *Adv Inter Med and Ped* 1980; 45: 1—75.
3. Tarnawski A, Hollander D, Stachura J, Klimczyk B, Mach T, Bogdał J. Alcohol injury to the normal human gastric mucosa: endoscopic, histologic and functional assessment. *Clinical and Investigative Medicine* 1987; 10: 259—263.
4. Dziaduś-Sokołowska A, Bilski R, Szlachcic A, Mroczka J, Michalski J. Comparison of the effects of some alcohols on the gastric electrical potentials. *Current Topics in Biophysics* 1993; 17: 30—34.
5. Dziaduś-Sokołowska A, Bilski R, Bieleń M, Mroczka J, Michalski J, Szlachcic A. Evaluation of the injuring action of various ethanol concentrations and of some kinds of alcoholic beverages on the gastric mucosal barrier (in Polish). *Alkoholizm i narkomania* 1991; L: 67—83.
6. Bartosz G. The Second Oxygen Face (in Polish: Druga twarz tlenu), Warszawa, Wydawnictwo Naukowe PWN, 1995.
7. Konturek S, Konturek P. Role of nitric oxide in the digestive system. *Digestion* 1995; 56: 1—13.

8. Ivata F, Joh T, Yokoyama Y, Itoh M. Role of endogenous nitric oxide in ischaemia-reperfusion injury of rat gastric mucosa. *J Gastroenterol Hepatol* 1998; 13(10): 997—1001.
9. Tarnawski A, Ivey K. Transmucosal potential difference profile in rat upper gastrointestinal tract. A simple model for testing gastric effects of pharmacologic agents. *Canad J Physiol Pharm* 1978; 56: 471—473.
10. Mroczka J, Bilski R, Dziaduś-Sokołowska A, Szlachcic A, Sieppel-Maciejewska K, Michalski J. Interference of psychotropic drugs with the injuring action of ethanol on gastric mucosal barrier. *Pol J Pharmacol* 1993; 45, 67—74.
11. Bilski R, Szlachcic A, Dziaduś-Sokołowska A, Mroczka J, Michalski J. Nitric oxide and the gastric mucosal barrier. *VIII Biennial Meeting Intern. Soc. For Free Radical Research, Barcelona* 1996; Abstr. Book, Symp. 13: 282.
12. Bilski J, Konturek S, Cieszkowski M, Czarnobilski K, Pawlik W. Endogenous nitric oxide in the regulation of gastric acid secretion, gastrin release and blood flow. *Biomed Res* 1994; 15 (Suppl.2): 63—67.
13. Brzozowski T, Konturek PC, Sliwowski Z *et al.* Lipopolisacccharide of *Helicobacter pylori* protects gastric mucosa via generation of nitric oxide. *J Physiol Pharmacol* 1997; 48: 699—717.
14. Konturek SJ, Brzozowski T, Majka J, Czarnobilski K. Role of nitric oxide and prostaglandines in sucralfate-induced gastroprotection. *Eur J Pharmacol* 1992; 211: 277—279.
15. Brown JF, Hanson PJ, Whittle BJR. Nitric oxide donors increase mucus gel thickness in rat stomach. *Eur J Pharmacol* 1992; 223: 103—104.
16. Chamulitrat W. Nitric oxide inhibited peroxyl and alkoxyl radical formation with concomitant protection against oxidant injury in intestinal epithelial cells. USDE*Arch Biochem Biophys* 1998; 355: 206—214.
17. Tanaka A, Kunikata K, Mizoguchi H, Kato S, Takeuchi K. Dual action of nitric oxide in pathogenesis of indomethacin-induced small intestinal ulceration in rats. *J Physiol Pharmacol* 1999; 50, 405—417.
18. Brzozowski T, Drozdowicz D, Szlachcic A, Pytko-Polończyk J, Majka J, Konturek S. Role of nitric oxide and prostaglandins in gastroprotection induced by capsaicine and papaverine. *Digestion* 1993; 54, 21—24.

Author's address: Dr. A. Szlachcic, Department of Biophysics, Chair of Physiology, Collegium Medicum, Jagiellonian University, Krakow, Poland