Effects of ethanol and arachidonic acid pathway inhibitors on the effectiveness of gastric mucosa cytoprotection

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Cytoprotection in the stomach, consisting in the mucus secretion, mucous circulation intensification and bicarbonate secretion to the gastric lumen, is highly dependent on the products of arachidonic acid pathway and peroxidative-antioxidative balance. The aim of the paper was to examine the effects of selected inhibitors of arachidonic acid pathway on the natural protective system of the gastric mucosa exposed to 50\% ethanol. The results show that leukotrienes, thromboxane and oxygen reactive forms significantly impair the protective function of the gastric mucosa while prostaglandins and antioxidant enzymes act protectively.

key words: cytoprotection, ethanol, arachidonic acid pathway inhibitors, oxygen free radicals, antioxidants

INTRODUCTION

Numerous experimental studies and clinical observations indicate that the arachidonic acid (AA) pathway products are important elements of the natural protective system of the gastric mucosa. Many years ago, Robert showed the antiulcerous actions of prostaglandins, initiating the studies on cytoprotection defined as the protective actions of an organism against damaging factors. According to Robert, cytoprotection in the stomach consists in increased mucus secretion, intensified and modified mucous circulation and activated bicarbonate secretion to the stomach [33]. Prostaglandins, activating the mucus and bicarbonate secretion and dilating the vessels, intensify cytoprotection [28,32]. Moreover, cytoprotection is maintained due to the actions of antioxidants synthesised, among others, as a result of the generation of oxygen free radicals formed during inflammatory or haematogenic tissue injuries [31]. Leukotrienes, thromboxane and platelet activating factors — PAF, due to their vessel-constricting and inflammatory actions, contribute to ischaemic damage of the gastric mucosa [29,42]. In the healthy gastric mucosa, the balance between TXA2 and PGI2 as well as low quotient between the amounts of AA lipoxygenation and cyclooxygenation products are observed. The exposure to nonsteroid antiinflammatory drugs results in an increase in the factors mentioned above and is ulcerogenic [5].

The natural mucosal barrier is a complex protective biochemical and functional system characterised by the ability to maintain high ion concentration gradient between blood and gastric contents [39]. This system is composed of many elements. Firstly, there is a mucus layer — gel-like, viscous, elastic, selective and constantly regenerating — built of glycoprotein-protein-lipid complexes. Due to the bicarbonates it contains, it inactivates acids [34]. The next layer is composed of strictly connected epithelial cells which produce mucus and bicarbonates. Another
important protective factor is proper blood circulation in the mucosa, continuous prostaglandin and nitrogen oxide synthesis and innervation [37]. However, the non-ionised and fat-soluble substances, such as acetylsalicylic acid, ethanol or bile acids, easily penetrate through this barrier, resulting in transient or permanent disorders of its properties [26]. Moreover, ischaemia within mucosal and submucosal vessels, accompanied by the accumulation of oxygen free radicals, which cannot be inactivated by tissue scavengers, as well as the inhibited cyclooxygenase activity being the major cytoprotective factor, lead to mucus and epithelium loss, erosions and ulcerations of the mucous membrane, which may result in carcinogenesis.

The aim of the paper is to examine the effects of some arachidonic acid pathway inhibitors on the effectiveness of the natural protective system of the gastric mucosa exposed to ethanol.

**MATERIAL AND METHODS**

The study was performed on male Wistar rats. Animals were maintained in accordance with the guidelines of the Animal Ethical Research Committee of the Medical School in Lublin.

All experiments were carried out on 100 male Wistar rats weighing 190–200 g. The animals were starved for 24 hours before experiments, but they were allowed to drink 10% sucrose in 0.96% v/v NaCl solution, which was removed 1 hour before the investigation. The animals stayed in cages at temperature of 20–22 degrees C and humidity of about 70%. There was a 12 hour cycle of day light. All studies were carried out in the same room where the rats were kept. Rats were divided into five groups as follows:

**I group** — negative control group pretreated with physiological saline in the dose of 5 ml per kg b.w. per os.

**II group** — positive control group pretreated only with 50% w/v ethanol in the dose of 5 ml per kg b.w. per os.

**III group** — pretreated intraperitoneally with the compound AA 861 — lipooxygenase inhibitor (Chem. Pharm. Inst., Tokyo, Japan), in the dose of 100 μg per kg b.w.

**IV group** — pretreated intraperitoneally, with the compound BW 775C — 5-lipooxygenase cyclooxygenase dual inhibitor (Wellcome Res. Lab., Beckenham, England) in the dose of 100 μg/kg b.w.

**V group** — pretreated with CGS 13080 — TXA2 synthetase inhibitor (Ciba Geigy Summit USA) — 1 mg/kg b.w. s.c.

Each experimental group consisted of 20 animals. The animals from groups III, IV and V were administered the drugs described above and 30 minutes later treated orally with 50% ethanol solution (5 ml per kg of b.w.) using a stainless steel stomach tube. The ethyl alcohol solution was prepared by diluting 96% alcohol in 0.96% NaCl to achieve 50% concentration required, each time directly before use. All rats were killed by decapitation 2 hours after the ethanol administration. After decapitation their stomachs were resected, opened along the greater curvature and examined with a 2 x binocular magnifier for presence of erosions. The size of mucosal injury was estimated. Each injury was measured along its largest extensiveness and in the case of petechiae, all five haemorrhagic spots were treated as 1 mm of ulceration. Then the sum of the injury lengths was added up in each group and divided by the number of observed changes. The ulceration index achieved in this way was assumed as the measure of severity of mucosal injury [7,14].

The whole gastric mucosa was separated from the muscular thin layer and used for histological and biochemical examinations. A part of the mucosa was homogenised in three volumes of 0.1 m. Tris-HCl of pH 7.4. The homogenate was centrifuged at 3000 g for 10 minutes and in the supernatant the level of malonyl dialdehyde (MDA) was determined according to the method of Ledwożyw et al. and expressed in nM per 1 mg of protein [16]. The concentrations of hydroperoxides (HPETE) and conjugated dienes (CD) in the supernatant were determined according to the Buege and August method in Ward’s modification and expressed in OD (Optional Density) per 1 mg of protein [1,41].

The second part of the mucosa was homogenised in 10 volumes of 50 mM buffer Tris-HCl, pH 8.9 and then the homogenate was centrifuged at 2000 g for 10 minutes. The supernatant was used for enzymatic studies. Catalase (CAT) activity (EC1.11.1.7) was measured according to Cohen et al. [4], glutathione peroxidase (GPx) activity (EC1.11.1.9) according to Paglia and Valentine (25), peroxidase (POX) activity (EC1.11.1.7) according to Putter [30], glutathione (GR) reductase activity (EC1.6.4.2) according to Mizuno (22) and superoxide dismutase (SOD) activity (EC1.15.1.1) according to Misra and Fridovich (21). Mn-isoenzyme of superoxide dismutase was determined in the presence of 2 mM KCN in the incubation fluid.

The enzyme activity was expressed in units per 1 mg of protein. The protein was determined ac-
According to Lowry et al., using bovine albumin as a standard [19].

In histological studies we used HE, Alcian-blue (pH 3.0) and PAS methods to examine the secretion of gastric mucopolysaccharides and the severity of gastric mucosa destruction.

The results of the studies were analysed statistically by means of the Student’s test for independent variables.

**RESULTS**

**Quantity of lipid peroxidation products in rat mucosa** (Table 1, Fig. 1)

The concentration of conjugated dienes (CD) in mucosa of the rats pretreated with ethanol amounted to 330% of the control level. In each group pretreated with the arachidonic acid pathway inhibitors (AA 861, BW-755 C and CGS-13080) before the ethanol exposure, the levels of CD remained significantly higher than in the control groups but significantly lower than in the group pretreated only with ethanol.

The levels of hydroperoxides (HPETE) and malonyl dialdehyde (MDA) also significantly increased after ethanol in comparison with the control rats and were also higher in the groups pretreated with the drugs described above than the control values. The levels of HPETE and MDA significantly decreased in relation to the group pretreated only with ethanol.

**The activity of antioxidant enzymes in rat mucosa** (Table 1, Fig. 2)

The activity of each examined enzymatic free radical scavenger in rat mucosa significantly increased when ethanol was given. The activity of Cu-Zn SOD amounted to about 170% of the control value in the ethanol pretreated group. After the pretreatment with arachidonic acid pathway inhibitors, Cu-Zn SOD significantly decreased in comparison with the ethanol group but was still higher than the control value. The activity of Mn-SOD amounted to about 250% of the control activity after ethanol and decreased after the inhibitor pretreatment just as Cu-Zn SOD. The activity of CAT was about 50% higher after ethanol in relation to the activity of the control group and signifi-

| Table 1. The influence of some arachidonic acid pathway inhibitors on the activity of antioxidant enzymes, levels of malonyl dialdehyde (nM/g tissue), conjugated dienes (OD 233 nm) and hydroperoxides (OD 353 nm) and ulcer index (mm) in rat stomach mucosa |
|----------------------------------|---------|--------|--------|--------|--------|
|                                | Control| 50% ethanol| AA-861 | BW-755C| CGS-13080|
| -------- | -------- | -------- | -------- | -------- | -------- |-------- |
| **Cu-Zn SOD** | 1600 ± 145 | 2400 ± 210 | 2200 ± 190 | 2300 ± 221 | 2350 ± 221 | **/** |
| **Mn-SOD** | 160 ± 7 | 407 ± 30 | 368 ± 22 | 360 ± 22 | 395 ± 23 | **/** |
| **CAT** | 605 ± 40 | 916 ± 58 | 850 ± 41 | 820 ± 41 | 840 ± 39 | **/** |
| **GPx** | 110 ± 16 | 300 ± 20 | 305 ± 15 | 290 ± 17 | 285 ± 14 | **/** |
| **GR** | 45 ± 3 | 115 ± 7 | 110 ± 8 | 117 ± 8 | 108 ± 7 | **/** |
| **CD** | 245 ± 17 | 810 ± 73 | 333 ± 29 | 337 ± 27 | 333 ± 28 | **/** |
| **HPETE** | 0.04 ± 0.03 | 0.43 ± 0.039 | 0.08 ± 0.007 | 0.12 ± 0.01 | 0.07 ± 0.005 | **/** |
| **MDA** | 3.05 ± 0.21 | 9.5 ± 0.83 | 4.6 ± 0.25 | 4.5 ± 0.22 | 3.2 ± 0.26 | **/** |
| **ULCER INDEX** | 4.0 ± 0.5 | 22.5 ± 15 | 5.0 ± 2.8 | 14.3 ± 4.99 | 10.8 ± 4.1 | **/** |

The enzyme activities are expressed in units per mg of protein: SOD (superoxide dismutase) — 1u. = 50% inhibition of pyrogallol autooxidation, GPx (glutathione peroxidase) — 1u. = nM NADPH x min⁻¹ x mg protein⁻¹, GR (glutathione reductase) — 1u. = nM NADPH x min⁻¹ x mg protein⁻¹, CAT (catalase) — 1u. = 1mM H₂O₂ metabolised x min⁻¹ x mg protein⁻¹, POX — 1u. = μM of guaiacol x mg protein⁻¹. The results are expressed as arithmetical means ± standard deviations (X ± SD). Student’s t-test for unpaired data was used for statistical analysis. p < 0.05 was considered statistically significant.

* — a statistically significant difference between the saline and the ethanol groups and between the saline and each of the groups pre-treated with AA pathway inhibitors or with the drugs - free radical scavengers.

**/** — a statistically significant difference between the ethanol group and each of the groups pre-treated with AA pathway inhibitors or free radical scavengers.

* p < 0.05, ** p < 0.01, *** p < 0.001. **/** p < 0.05, **/**/** p < 0.01, **/**/**/** p < 0.001.
Significantly decreased when AA inhibitors were given but it was still higher than the control one. The activity of GPx increased to about 300% after ethanol and was also higher than the control value after the AA-inhibitor pretreatment. The activity of GR also increased in the ethanol group. We did not notice any significant decrease in GR activities after the pretreatment with drugs in comparison with the ethanol group.

**Ulcer index** (Table 1, Fig. 3)

The ulcer index significantly increased in the ethanol group in relation to the control one. After the pretreatment with AA pathway inhibitors it was still higher than the control value but lower in comparison with the ethanol group. A decrease in the ulcer index value was most effective after the AA-861 administration and less significant after the remaining arachidonic acid pathway inhibitors.

**Histological results**

In the saline group, the haematoxylin and eosin (HE) staining showed regular, tubular, branched gastric glands composed mostly of high basophilic cells (Fig. 4A). The intensive PAS (periodic acid Schiff) reaction was observed on the mucosal surface, also visible in the necks (Fig. 4B). The external parts of glands are especially rich in acid mucopolysaccharides, visible during AB staining (Fig. 4C). The posi-
Figure 2. The influence of some arachidonic acid pathway inhibitors on the activities of enzymes: Cu-Zn SOD and Mn-SOD (superoxide dismutase) \([1 u = 50\%\text{ inhibition of pyrogallol autooxidation}]\), GPx (glutathione peroxidase) \([1 u = \text{nM NADPH} \times \text{min}^{-1} \times \text{mg protein}^{-1}]\), GR (glutathione reductase) \([1 u = \text{nM NADPH} \times \text{min}^{-1} \times \text{mg protein}^{-1}]\), CAT (catalase) \([1 u = 1 \text{ } \mu\text{mol H}_2\text{O}_2 \text{ metabolised} \times \text{min}^{-1} \times \text{mg protein}^{-1}]\), POX \([1 u = \text{M. of guaiacol} \times \text{mg protein}^{-1}]\) in the rat gastric mucosa injured by ethanol.

Figure 3. The influence of some free radical scavengers and arachidonic acid pathway inhibitors on the ulcer index in rat gastric mucosa.

Figure 4. The morphological picture of the rat gastric mucosa after saline treatment, \(\times 100\). A) The section stained with HE. B) The section stained with PAS. C) The section stained with AB.

cyte infiltrations are observed on the mucosal surface. The picture of gland structures is blurred. The epithelia of the upper mucosa exfoliate. The figure shows the surface defect of mucosa. The PAS reaction results in slightly decreased intensity of staining and flattening of secretory cells compared to the previous group (Fig. 5B). AB staining reveals a decrease in acid mucopolysaccharide secretion (Fig. 5C). The gland cells are filled with blue granules, which mainly concerns the external parts of glands and mucosal surface.

The gastric mucosa of rats pretreated with AA 861 before the ethanol administration, after HE staining, does not differ morphologically from the mucosa in saline groups (Fig. 6A). The picture of the glands is regular, no mucosal defects are found.
The PAS reaction is intensive especially in the mucosal surface. After the PAS reaction, the specimens show abundant vascularisation of the mucosa (thick basilar membranes of vessels) (Fig. 6). AB staining shows significantly increased intensity of the reaction to acid mucopolysaccharides (Fig. 6C). This concerns the gland necks and mucosal surface.

In group IV (BW + ethanol) HE staining reveals significant mucosal destruction, extensive blurring of the glandular structure, inflammatory infiltrations, oedema, focal liquefactive necrosis (Fig. 7A). The PAS reaction in less injured places of the tissue is quite intensive (Fig. 7B). However, the degree of AB staining is significantly smaller in comparison with the previous group and the control one (Fig. 7C). Only single and focally preserved glands of the mucosal surface show the presence of acid mucopolysaccharides. In the remaining parts of the mucosa the reaction is diffuse in character.

In group V (CGS 13080 + ethanol) after HE staining the mucosal destruction is observed: blurred picture of the glands, abundant inflammatory infiltrations, interstitial tissue oedema (Fig. 8A). After AB staining the reaction is weakened in the full thickness of the mucosa (Fig. 8C) while the specimens stained with PAS show the diffuse reaction in the tissue and clear staining of the mucosal surface (Fig. 8B).
DISCUSSION

The pathogenesis of gastric ulcer disease is complex and has not been fully explained yet. The development of chronic as well as acute ulcer is a result of mucosa barrier damage and a secondary decrease in gastric mucosa resistance, which enables reflux diffusion of hydrochloric acid leading to erosions and ulcerations.

The relevant elements of the pathogenesis of the gastric mucosa damage are oxygen free radicals. These extremely reactive atoms and molecules with an unpaired electron, initiate chain reactions of the destruction of membrane phospholipids, structural and enzymatic proteins, saccharides and nucleic acids. Beside direct injuries and despite the chain reaction quenching, free radicals may induce carcinogenesis and apoptosis which, according to the literature data, is intensified in the gastric mucosa with ulcerations [13,35].

Numerous experimental data also suggest the role of oxygen free radicals in the ethanol-induced gastric injury. Mutoh et al. [24] showed that cultured rat gastric mucosal cells, exposed to ethanol, produced superoxide anions directly proportionally to the ethanol dose and SOD and CAT enzymatic activities were maintained in the presence of 15% ethanol. They also showed that ethanol damage of the gastric cells is closely linked with the intensity of superoxide anion production. Hirokawa et al. [9], in-

Figure 7. The morphological picture of the gastric mucosa from rats which were given 50% ethanol and BW 775C-lipoxygenase-cyclooxygenase dual inhibitor, × 50. A) The section stained with HE. B) The section stained with PAS. C) The section stained with AB.

Figure 8. The morphological picture of the gastric mucosa from rats which were given 50% ethanol and CGS 13080 — TXA2 synthetase inhibitor, × 50. A) The section stained with HE. B) The section stained with PAS. C) The section stained with AB.
vestigating the details of ethanol-induced gastric mucosal damage, showed that ethanol administration induced intracellular oxidative stress and produced mitochondrial permeability transition and mitochondrial depolarisation, which resulted in cell death in the mucosa. They also noticed that glutathione — an intracellular antioxidant had a protective action against ethanol. Suzuki et al. [36] noticed in their investigations that oral administration of ethanol caused an increase in the content of thiobarbituric acid-reactive substances of the injured mucosa in rats. The pretreatment with compounds scavenging both superoxide anions and hydroxyl radicals (quercitin, alpha-tocopherol, nifedipine and tetracycline) markedly prevented the ethanol gastric mucosal injury and an increase in the MDA level. Cho et al. [3] and Moghadasian et al. [23] showed that ethanol-induced injury of an antioxidant status in the gastric mucosa was dependent on the concentration of alcohol. Cho showed that oral administration of absolute ethanol caused an increase in the CAT-activity and did not influence the SOD activity. Moghadasian noticed that 8% ethanol increased the activity of glutathione peroxide dismutase in the gastrointestinal tract but undiluted alcohol only increased the glutathione peroxide activity in the gastric mucosa.

The excessive production of oxygen reactive forms following the ethanol exposure may also result from the changes of ATP degradation products to uric acid due to xanthine oxidase in ischaemia and the superoxide anion radical synthesis resulting from reperfusion, oxygen release of phagocytes, oesinophils and marophages accumulated in the mucosa due to leukotriene chemotactic actions and IL-1 effects and from oxidation reactions in the arachidonic acid pathway [3]. Our studies indicate that the reaction of lipid peroxidation is intensified by the administration of ethyl alcohol and leukocytic infiltrations are observed in the histological specimens. Using gross and microscoping scoring, Ligumsky et al. [18] showed that radical scavengers such as Mn2+, glycine, carotenes, catalase and dimethylhydroxyurea (DMTU) given simultaneously with ethanol, caused gastroprotection while allopurinol did not. Matsumoto et al. also noticed that gastric lesions induced by ethanol were not reduced in mice treated with allopurinol. Matsumoto concluded that the main sources of oxygen free radicals after ethanol exposure were neutrophils.

The results of our studies show that leukotrienes and thromboxane play an important role in the disorders of gastric mucosa protective functions while prostaglandins act protectively. In the ethanol-induced gastric ulcer, the pathogenetic elements are, among others, oxygen reactive forms produced excessively in ischaemia during oxygen release of phagocytes and arachidonic acid pathway [2,6,10]. Oxygen free radicals, acting as secondary cell transmitters, activate genes to produce enzymatic antioxidants, interleukins, interleukin receptors and adhensive molecules. However, the antioxidative cytoprotection is insufficient to protect the gastric mucosa exposed to ethanol despite high enzyme activities [12]. The concentration of ethanol administered in our studies resulted in significant damage of the gastric mucosa, intensified secretion, mainly in the mucosal cells and on their surface, and inflammatory infiltrations from neutrophils. The concentration of lipid peroxidation products increased and despite significantly increased activities of antioxidative enzymes, the ulcer index was 5.5 times higher than the control values.

It is known that the direct action of alcohol, among others, results in disorders of production and quality of the secreted gastric mucus as ethanol significantly decreases the N-acyl-glucoseamine building into gastric glicoproteins and inhibits the synthesis of galactoamines inhibiting the activity of galactoamine synthetase [5]. The studies of numerous authors suggest an important role of leukotrienes in the pathogenesis of gastric mucosa ulcers. Peskar [28,29] showed that in rats ethanol induced the synthesis of LTC4 in the stomach and that 5-lipoxygenase inhibitor administered after ethanol was protective in action. Wallace [39,40] claims that oral ethanol administration results in vasoconstriction dependent on the action of cysteine leukotrienes LTC4 and LTD4.

Apart from being vasocontrictors, cysteine leukotrienes have also immunomodulatory actions. LTD4 increases the II-1 generation by monocytes, which is an interleukin of wide-range inflammatory actions [11]. LTC4 and LTD4 stimulate the proliferation of glomerular endothelial cells and fibroblasts, the former being chemoattractive for eosinophils, the latter for eosinophils and neutrophils [17].

Our findings show that 5-lipoxygenase inhibitor, AA-861, used during ethanol exposure acts cytoprotectively, which is visible in the increased secretion of mucopolysaccharides and simultaneously decreased neutral mucopolysaccharide pool in mucus. Moreover, it significantly decreases the ulcer index.

Free arachidonic acid from the membrane phospholipids may be more extensively used for the prostaglandin synthesis in the cyclooxygenase pathway.
In the gastric mucosa prostaglandins counteract the damaging actions of leukotrienes. The main prostaglandin synthesised in the gastric mucosa is PGI₂. Its production is several times higher than the synthesis of PGE₂. PGI₂ increases the mucus and bicarbonate secretion and dilates blood vessels preventing the mucosal injury caused by ischaemia [38]. Aspirin decreases the gastric mucus synthesis inhibiting the synthesis of prostaglandins. Halter [8] found out that the inhibition of cyclooxygenase activities resulted in reduced mucus secretion and its bicarbonate content and changed the life cycle of gastric mucosa cells. The cyclooxygenase inhibition intensifies the production of leukotrienes from arachidonic acid and this also contributes to the development of ulcerations.

The double 5-lipoxygenase/cyclooxygenase inhibitor, BW-775C, used in our studies slightly decreased the ulcer index during ethanol exposure. However, this rat group showed significantly damaged gastric mucosa with necrotic changes and decreased secretion of neutral mucopolysaccharides.

TXA₂ is formed from arachidonic acid being initially a substrate for cyclooxygenase and facilitates the gastric mucosa injury in the course of ischaemia [42]. The inhibition of TXA₂ synthetase results in the conversion of all cyclic endoperoxides into prostaglandins which act protectively. However, it does not affect the leukotriene synthesis and thus the injury of the gastric mucosa exposed to ethanol and treated with CGS-13080 remains significant. Compared to BW-775C, the ulcer index value was more reduced after the administration of the thromboxane inhibitor CGS-13080.

It seems that the production of oxygen reactive forms exceeds the antioxidant abilities of the gastric mucosa and despite increased activities of antioxidative enzymes, the ethanol exposure results in a significant increase in ulcer index values.

Our histopathological findings show that the administration of ethanol and inhibitors of arachidonic acid pathway significantly changes the synthesis, secretion and composition of mucopolysaccharides in the gastric mucosa. The intensified peroxidation of lipids observed after the administration of ethanol and arachidonic acid pathway inhibitors indicates, among others, the degradation of lipids bound to mucous glycoprotein polymers and the injury of the gastric cell membranes. It may be supposed that alcohol first damages the gel and glycoprotein-lipid structure of the mucous layer. The injury of gastric epithelial cells under the mucus results in the disorders of mucus secretion, which leads to ulcerations.

REFERENCES


