Investigation of gastroprotective compounds at subcellular level in isolated gastric mucosal cells

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Nagy, Lajos, Romeo E. Morales, Martin Beinborn, Peter Vattay, and Sandor Szabo. Investigation of gastroprotective compounds at subcellular level in isolated gastric mucosal cells. Am J Physiol Gastrointest Liver Physiol 279: G1201–G1208, 2000.—We tested the hypothesis that recognized gastroprotective agents exert direct protection against ethanol-induced injury in isolated rat gastric mucosal cells in vitro. If protection exists, we also wanted to identify subcellular targets in the reversible and/or irreversible stages of cell injury. Ethanol-induced cell injury was quantified by measuring plasma membrane leakage (trypan blue exclusion and lactate dehydrogenase release), mitochondrial integrity (succinic dehydrogenase), and nuclear damage (ethidium bromide-DNA fluorescence). Initial cell viability and responsiveness were estimated by the effects of carbachol, carbachol + atropine, or 16,16-dimethyl-PGE, or on chief cell peptic mucosa contains numerous cell types, data are lacking concerning the possible direct protection of diminished by PG and SH derivatives in cultured and transformed epithelial surface cells. Because the gastric mucosa contains numerous cell types, data are lacking concerning the possible direct protection of parietal, chief, and neuroendocrine cells by cytoprotective agents. Thus we tested the hypothesis that old and new cytoprotective compounds might exert direct protection on a mixed population of gastric mucosal cells (GMC). We also wanted to identify subcellular targets in the interaction of gastroprotective agents and ethanol. For this purpose, a method was adapted in our laboratory for the isolation of a mixed population of rat GMC with long viability and preserved membrane receptor sensitivity using minimal amounts of pronase and calcium-binding EGTA (13, 25). The new method not only includes the measurement of plasma membrane damage by the usual dye exclusion tests, e.g., by trypan blue (TB) exclusion, leakage of lactate dehydrogenase (LDH), or total protein from cytosol, but we...
can also assess mitochondrial integrity by measuring the activity of mitochondrial succinic dehydrogenase (SDH) and nuclear damage by fluorescence induced by ethidium bromide (EB)-DNA binding (25).

The main purpose of this study was to evaluate the possible direct cellular effects of established and newly synthesized gastroprotective compounds in isolated mixed rat GMC at the levels of the plasma membrane, mitochondria, or nuclei alone or against a moderate uniform cell injury after a short incubation with ethanol.

**MATERIALS AND METHODS**

Reagents and Chemicals

The following reagents were purchased from Sigma Chemical (St. Louis, MO): N-acetyl-l-cysteine (NAC), l-alanine, d-arginine, l-arginine, atropine sulfate, brilliant cresyl blue (BCB), BSA, bovine hemoglobin, carbamoylcholine chloride (carbachol), DMSO, EB, EGTA, Folin and Ciocalteau’s phenol reagent, fumaric acid, D-glucose, L-glutamine, glycine (carbachol), DMSO, EB, EGTA, Folin and Ciocalteau’s phenol reagent spectrophoto metrically (A = 578 nm) with a Gildorf 2400–2 spectrophotometer, calculated from a bell (Merek, Rahway, NJ) dose-response curve and expressed as micrometions of pepsin per minute per 5 x 10⁶ GMC.

**Preparation of Mixed GMC**

GMC from one or two nonfasted Sprague-Dawley rats (180–210 g; Taconic Farms, Germantown, NY) were obtained by sequential incubation with a low concentration of pronase E in calcium-free medium (EGTA) as we previously described (13, 25). Cells were counted in an improved Neubauer counting chamber (Hemocytometer; Fisher Scientific), dispersed, and kept in warm (37°C) HEPES-buffered salt solution (0.159 M, pH 7.4) produced fresh in our laboratory with the following ingredients (in mM): 98.0 NaCl, 5.8 KCl, 2.54 NaH₂PO₄, 5.1 Na pyruvate, 6.9 Na fumarate, 2.0 l-glutamine, 24.5 HEPES-Na, 1.0 Tris base, 11.1 d-glucose, and 1.0 CaCl₂ with 2.0 mg/ml (wt/vol) BSA. The volume of GMC suspension was adjusted to a density of 1.5 x 10⁷ cells/ml for incubations, TB, and biochemical assays. The numbers of viable and nonviable cells indicating initial cell viability (CV) were also counted by TB exclusion test (% of unstained cells) indicating initial cell viability (CV) were also counted by TB exclusion test (% of unstained cells).

**Secretory Studies**

The responsiveness of rat mixed GMC and the membrane receptor sensitivity of both chief and parietal cells were also measured in separate experiments using secretory agents.

**Pepsinogen secretion.** Freshly isolated and dispersed (1 ml) rat GMC (5 x 10⁶ cells/ml, CV = 94 ± 8%; chief cells = 42 ± 6%) were incubated with carbachol (10⁻⁴ - 10⁻⁵ M) or dmPGE₂ (10⁻⁴ - 10⁻⁵ M) alone as well as in combination with carbachol (10⁻⁵ M) + atropine (10⁻⁴ M) for 15 min at 37°C. Pepsinogen was determined by a modified Berstad method at pH 2.0 (HCl-KCl buffer, 0.1 M) using 3.0 M urea-denaturated bovine hemoglobin (1.0%) as substrate and incubated for 10 min at 37°C (5). The enzymic products were quantified with freshly diluted Folin and Ciocalteau’s phenol reagent spectrophotometrically (A = 578 nm) with a Gildorf 2400–2 spectrophotometer, calculated from a bell (Merek, Rahway, NJ) dose-response curve and expressed as micrometions of pepsin per minute per 5 x 10⁶ GMC.

**Enrichment of parietal cells and measurement of [¹⁴C]aminopyrine accumulation.** Freshly isolated and dispersed rat GMC (1–2 x 10⁶) were separated in a Beckman 5.0 elutriation system (600 rpm, 10°C) in Hanks’ balanced salt solution supplemented with 0.1% (wt/vol) BSA. Parietal cells (60–65% of total GMC) were collected by Percoll density gradient centrifugation at a density of 1.06 mg/ml (15). The implication of HCl production in enriched rat parietal cells is based on accumulation of [¹⁴C]-labeled aminopyrine ([¹⁴C]AP) in acidic tubulovesicles of parietal cells on stimulation (3, 33, 34). Parietal cells (10⁶/ml) were suspended in Hanks’ balanced salt solution (0.15% albumin) and incubated at 37°C in an orbital shaker (100 oscillations/min). Incubations were carried out in a total volume of 1.0 ml with 0.05 Ci of [¹⁴C]AP in the absence or presence of receptor stimuli such as carbachol (10⁻⁴ M), histamine (10⁻⁴ M), IBMX (10⁻⁴ M), or their combination. The washed cells were dissolved in 1.0 M NaOH, and neutralized aliquots were used for liquid scintillation counting.

**Treatment of Rat Isolated GMC**

All compounds for incubation were freshly dissolved and diluted in HEPES-buffered salt solution containing albumin (0.2% wt/vol). The pH was adjusted to 7.4, and the agents were used immediately. Initially, 30% ethanol was freshly diluted and subsequently diluted in the reaction tube containing GMC and test substances. The final concentration was 15% ethanol (25).

GMC (1.5 x 10⁷ cells/tube) were tested for direct cytoprotection by 60-min incubation (1.0 ml of cell suspension plus 1.0 ml of drug solution) before addition of 2.0 ml of 30% ethanol for 5 min. The tested compounds were also used in the parallel tubes without ethanol treatment. The following gastroprotective compounds were tested for direct cytoprotection: glycine (10⁻³–10⁻⁶ M), l-arginine (10⁻³–10⁻⁶ M), l-arginine (10⁻⁵–10⁻⁶ M), l-alanine (10⁻⁵–10⁻⁶ M), dmPGE₂ (10⁻⁴–10⁻⁶ M), cysteamine (10⁻²–10⁻⁴ M), NAC (10⁻³–10⁻⁵ M), taurine (10⁻³–10⁻⁵ M), histamine (2 x 10⁻³–2 x 10⁻⁷ M), GM₁ (10⁻⁵–10⁻⁶ M), sucrase octasulfate (10⁻²–10⁻⁴ M), nitecapone (10⁻⁵–10⁻⁷ M), pinaverium (10⁻³–10⁻⁶ M), and KC-10667 (10⁻³–10⁻⁶ M).

After incubations the cells and the supernatant containing ethanol were separated by careful centrifugation (500 g, 8 min). The cells were resuspended in another 2.0 ml of buffer and incubated for 10 min in a shaking water bath. The ethanol-free supernatant was used for measurement of LDH leakage. The cells were dispersed in 1.5 ml of buffer (10⁷ cells/ml) and immediately distributed for simultaneous biochemical assays.
Table 1. Effects of carbachol and carbachol + atropine on pepsinogen secretion of isolated rat gastric mucosal cells

<table>
<thead>
<tr>
<th>Group</th>
<th>Carbachol, M</th>
<th>Pepsinogen Secretion, μg·min⁻¹·5×10⁶ cells⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>0</td>
<td>3.15 ± 0.56</td>
</tr>
<tr>
<td>B</td>
<td>10⁻⁴</td>
<td>5.11 ± 0.68*</td>
</tr>
<tr>
<td>C</td>
<td>10⁻³</td>
<td>5.51 ± 0.41†</td>
</tr>
<tr>
<td>D</td>
<td>10⁻²</td>
<td>6.21 ± 0.74‡</td>
</tr>
<tr>
<td>E</td>
<td>10⁻¹</td>
<td>7.50 ± 0.75§</td>
</tr>
<tr>
<td>F</td>
<td>10⁻¹</td>
<td>3.95 ± 0.49$</td>
</tr>
</tbody>
</table>

+ atropine (10⁻⁶)

Values are means ± SE. Mixed rat gastric mucosal cells (5×10⁶) containing 42% chief cells were used in each tube for 15-min incubations with pharmacological agents at 37°C. Supernatants were assayed for peptic activity at pH 2.0 using hemoglobin as substrate, and digestive products were measured by Folin reagent. *P < 0.05; †P < 0.01 as compared with group A; $P < 0.001 between groups E and F.

RESULTS

Yield, Composition, and Viability Of Isolated Rat GMC

A large number of mixed GMC (1.2–1.6×10⁸) can be isolated from a single rat glandular stomach. The ratio of parietal, chief, and mucous epithelial cells using supravital staining with 0.05% BCB was 23.0 ± 2.1, 42.1 ± 4.9, and 34.1 ± 3.7%, respectively. The average initial CV of 28 cell isolations was 90.5 ± 3.1% measured by TB dye exclusion. High CV (85–95%) was maintained for 7 h after cell isolation using our cell harvesting method and physiological solution at 37°C.

SECRETORY STUDIES

Pharmacological investigations with chief cells in mixed rat GMC revealed that the muscarinic receptor agonist carbachol significantly stimulated pepsinogen secretion from 3.15 ± 0.56 to 7.50 ± 0.75 μg·min⁻¹·5×10⁶ GMC⁻¹ (P < 0.01). Atropine given in combination with carbachol almost totally inhibited carbachol-induced increase in pepsinogen secretion (P < 0.001) (Table 1). In addition, dmPGE₂ also increased pepsinogen secretion from isolated chief cells from 8.75 ± 0.35 to 10.10 ± 0.30 μg·min⁻¹·5×10⁶ GMC⁻¹ (P < 0.01; Fig. 1).

Uptake of [¹⁴C]AP in enriched (65%) rat parietal cells was stimulated by either histamine or carbachol...
The histamine-induced increase in HCl production in parietal cells was considerably potentiated by the phosphodiesterase inhibitor IBMX (Fig. 2).

Ethanol-Induced Cellular Injury: An Assay for Cytoprotection

The average values obtained by all methods used to measure indicators of damage before and after 5-min incubation with ethanol at 37°C are shown in Table 2. Ethanol (15% vol/vol) significantly decreased CV by TB dye exclusion from 90.3 ± 3.1 to 20.9 ± 1.4 (P < 0.001) and increased LDH leakage from cytosol into media via damaged cell membrane (P < 0.001). It induced severe mitochondrial and nuclear lesions as detected by marked decrease in SDH activity from 5.7 ± 0.3 to 1.5 ± 0.1 nmol formazan·min⁻¹·mg protein⁻¹ and elevation of EB-DNA fluorescence intensity from 4.2 ± 0.5 to 17.3 ± 1.1 fluorescence units/10⁷ GMC.

Effects of Thiol Compounds on Ethanol-Induced Cell Injury

NAC, taurine, or cysteamine alone did not induce any injury in mixed GMC (data not presented). The effects of 60-min incubation of isolated rat GMC with NAC against ethanol-induced cellular injury are shown in Fig. 3. Cell membrane damage quantified by TB dye exclusion and LDH leakage, mitochondrial SDH activity, and EB-DNA fluorescence revealed a mild (12–21%) protection against ethanol-induced damage (P < 0.05).

As shown in Fig. 4, a similar (10–20%) increase in cell resistance was detected after 60-min preincubation

Table 2. Ethanol-induced injury in isolated gastric mucosal cells after 5-min incubation

<table>
<thead>
<tr>
<th>Method</th>
<th>Subcellular Targets</th>
<th>Before ethanol</th>
<th>After ethanol</th>
</tr>
</thead>
<tbody>
<tr>
<td>TB exclusion</td>
<td>Cell membrane</td>
<td>90.3 ± 3.1</td>
<td>20.9 ± 4.4*</td>
</tr>
<tr>
<td>LDH-media</td>
<td>Cell membrane</td>
<td>17.0 ± 9</td>
<td>104.9 ± 12.7*</td>
</tr>
<tr>
<td>LDH-cells</td>
<td>Cell membrane</td>
<td>19.0 ± 1.5</td>
<td>7.8 ± 1.1*</td>
</tr>
<tr>
<td>SDH</td>
<td>Mitochondria</td>
<td>5.7 ± 0.3</td>
<td>1.5 ± 0.1*</td>
</tr>
<tr>
<td>EB-DNA</td>
<td>Nucleus</td>
<td>4.2 ± 0.5</td>
<td>17.3 ± 1.1*</td>
</tr>
</tbody>
</table>

Data are expressed as means ± SE of 28 cell isolations and treatments. TB, trypan blue (% of unstained cells); LDH, lactic dehydrogenase (mU·min⁻¹·mg protein⁻¹); SDH, succinic dehydrogenase (nmol formazan·min⁻¹·mg protein⁻¹); EB, ethidium bromide (fluorescence/10⁷ gastric mucosal cells). *P < 0.001.
with taurine (2-aminoethanesulfonic acid) against ethanol injury, and the difference was statistically significant with LDH leakage and EB-DNA fluorescence ($P < 0.05$). The protection of mitochondria and nuclei reached the level of significance ($P < 0.05$).

In contrast, cysteamine moderately aggravated ($P < 0.05$) the ethanol-induced cell membrane and mitochondrial damage measured by TB exclusion and SDH, respectively. However, some concentration-dependent nuclear protection ($P < 0.01$) was measured by EB-DNA fluorescence. No major changes were found in LDH leakage from cysteamine-treated GMC after incubation with 15% ethanol (Fig. 5).

**Effects of dmPGE$_2$ and Ethanol**

Concentration-dependent protection (15–21%) against ethanol-induced injury was found after 60-min incubation with dmPGE$_2$ as measured by TB exclusion ($P < 0.05$), LDH release ($P < 0.05$), and changes in activity of SDH from 1.95 ± 0.11 to 3.31 ± 0.21 ($P < 0.05$). Only a slight increase ($P > 0.05$) in nuclear resistance was observed after incubation with dmPGE$_2$ in ethanol-treated GMC (Fig. 6).

**Effects of SOS and Ethanol**

Incubation of GMC for 60 min with SOS dose-dependently decreased the ethanol-induced LDH release and increased CV measured by TB dye exclusion; the differences reached statistical significance ($P < 0.05$) at $10^{-3}$ M of SOS (Fig. 7). The effect of SOS on mitochondrial and nuclear membranes detected by changes in the activity of SDH and EB-DNA binding was not statistically significant.

**Effects of Various Gastroprotective Compounds on Ethanol Toxicity**

The effects of different agents that in vivo exert gastroprotection in isolated GMC with or without incubation with 15% ethanol are summarized in Table 3. Only the statistically significant changes in TB dye exclusion and biochemical assays are listed, indicating cell injury alone or aggravation of ethanol-induced...
Table 3. Effects of various gastroprotective compounds alone and in presence of 15% ethanol on isolated rat gastric mucosal cells

<table>
<thead>
<tr>
<th>Compounds, M</th>
<th>Treatments</th>
<th>Plasma membrane</th>
<th>Mitochondria</th>
<th>Nuclei</th>
</tr>
</thead>
<tbody>
<tr>
<td>Histamine</td>
<td>(2 x 10^{-3} – 2 x 10^{-7})</td>
<td>+ Ethanol</td>
<td>Biphasic</td>
<td>Biphasic</td>
</tr>
<tr>
<td>GM1</td>
<td>(10^{-3} – 10^{-6})</td>
<td>+ Ethanol</td>
<td>A</td>
<td>A</td>
</tr>
<tr>
<td>Nitecapone</td>
<td>(10^{-5} – 10^{-7})</td>
<td>+ Ethanol</td>
<td>A</td>
<td>A</td>
</tr>
<tr>
<td>Pinaverium</td>
<td>(10^{-5} – 10^{-7})</td>
<td>+ Ethanol</td>
<td>A</td>
<td>A</td>
</tr>
<tr>
<td>KC-10667</td>
<td>(10^{-5} – 10^{-6})</td>
<td>+ Ethanol</td>
<td>A</td>
<td>A</td>
</tr>
<tr>
<td>t-Arginine</td>
<td>(10^{-5} – 10^{-6})</td>
<td>+ Ethanol</td>
<td>A</td>
<td>A</td>
</tr>
<tr>
<td>d-Arginine</td>
<td>(10^{-5} – 10^{-6})</td>
<td>+ Ethanol</td>
<td>A</td>
<td>A</td>
</tr>
<tr>
<td>L-Alanine</td>
<td>(10^{-3} – 10^{-6})</td>
<td>+ Ethanol</td>
<td>A</td>
<td>A</td>
</tr>
<tr>
<td>Glycine</td>
<td>(10^{-3} – 10^{-6})</td>
<td>+ Ethanol</td>
<td>A</td>
<td>A</td>
</tr>
</tbody>
</table>

Data from 3–6 cell isolations and experiments are pooled and calculated. Plasma membrane, mitochondrial and nuclear membrane integrities were assessed by TB exclusion and LDH leakage, SDH activity, or EB-DNA fluorescence, respectively. Only the statistically significant changes (P < 0.05) are listed. I, cell injury; A, aggravation of ethanol-induced cell injury; biphasic, concentration-dependent protection or aggravation; no, no effect.

DISCUSSION

Our study demonstrates that preincubation of isolated rat gastric mucosal cells with dmPGE_2, SOS, or the SH compounds NAC, taurine, or cysteamine alone did not cause any measurable cell injury, and these agents slightly increased resistance (up to 21%) against ethanol-induced cellular damage. Similar protection was found with a low concentration of histamine, but at a higher concentration it aggravated ethanol toxicity. Partial or general aggravation of ethanol injury was detected on GMC membranes after preincubation with wide concentration ranges of amino acids such as L-arginine, D-arginine, glycine, L-alanine, and D-alanine or in vivo gastroprotective compounds such as nitecapone, ganglioside GM_1, the spasmodytic pinaverium, and KC-10667. Some of these agents alone (i.e., without ethanol) exerted some direct cellular toxicity. Our results also revealed that the isolated chief cells (pepsinogen secretion) or enriched parietal cells (HCl production) have an excellent membrane receptor-mediated responsiveness to pharmacological stimuli such as cholinergic and histaminergic stimulators or PG. These results are in accordance with other studies in cultured and isolated parietal cells (3, 33, 34) or peptic cells (2, 8, 18, 19).

Acute gastric mucosal injury is a complex process because of the heterogeneous structure and multiple functions of the stomach wall. In the pathways of direct and indirect chemical injury, vascular damage, inflammatory processes, free radicals, and proteases are involved (41, 45). The list of gastroprotective agents has been growing since the introduction of the concept of gastric cytoprotection, without a proportionate increase in our understanding of the mechanisms of gastric mucosal injury and protection. We nevertheless know that most of the mucosal protection is relative and indirect. Namely, despite the initial destruction of superficial epithelial cells in rats given ethanol intragastrically after pretreatment with gastroprotective agents, the histological integrity of the gastric mucosa is restored by rapid epithelial restitution and the organ structure and function are maintained.

One of the new possibilities for assessing the phenomenon of direct gastric cytoprotection is to investigate in vitro the possible protection of isolated GMC by naturally occurring or exogenous compounds against chemically induced and measurable cell damage. We have recently developed and optimized morphological and biochemical methods for harvesting mixed populations of rat GMC to investigate reversible and irreversible cell damage and protection at the level of cell membranes in organelles (13, 25). Selective and parallel assessment of chemical injury and protection of plasma membrane, mitochondria, and nuclei can be investigated with sensitive measurements such as TB exclusion, LDH leakage, SDH activity, or EB-DNA binding. These targets were selected because of their critical roles in reversible and irreversible cell injury. That is, the extent of plasma membrane and mitochondrial damage is currently accepted as the rate-limiting step between reversible and irreversible cell damage, whereas nuclear damage is an indicator of cell death by either necrosis or apoptosis (7, 38). This short incubation (5 min) of rat GMC with a low concentration of ethanol induced reversible and/or irreversible cell membrane, mitochondrial, or nuclear injury that can be measured by morphological and biochemical assays (25). The short exposure is meant to imitate the in vivo conditions when fluids like ethanol are rapidly emptied from the stomach and mucosal lesions develop within minutes (10, 16, 20, 40). Our previous experiments revealed only minimal or no protection of isolated GMC against ethanol injury after preincubation with PG for 30 min, whereas the present results indicate a moderate increase in cellular resistance of GMC after 1-h incubation with dmPGE_2.

The SH compounds constitute one of the groups of endogenous mediators of acute gastroprotection (10, 20, 30, 39, 42, 44). Only a moderate but statistically significant direct gastroprotection was observed in rat
GM1 against ethanol-induced cytotoxicity by preincubation with NAC (in all organelles examined) or taurine (in mitochondria and nuclei). Taurine is a metabolite of L-cysteine. In vivo, it protects several organs against chemical injury, and we recently found gastrointestinal protection against ethanol in rats (unpublished data). Cysteamine, another SH-containing agent, has also been shown to exert in vivo gastroprotection (42). Romano et al. (31) demonstrated that cysteamine has a direct protective effect in vitro against damage induced by taurocholate or indomethacin in gastric epithelial monolayers derived from a human cell line. In our study with freshly isolated rat GMC, cysteamine did not induce significant enhancement of cell resistance.

SOS, a derivative of sucralfate, had a moderate protective effect against ethanol injury in isolated rat GMC. This direct cellular protection might be one of the mechanisms of acute gastroprotection by sucralfate (36).

GM1, a ganglioside and sialic acid-containing glycosphingolipid, exerts acute gastric mucosal protection against ethanol (37, 48). Gangliosides play a role in the regulation of transmembrane signaling, cellular differentiation and proliferation, membrane fluidity, and ion transport (35). In the present study, exogenous GM1 was not able to protect against ethanol-induced damage in isolated GMC.

Amino acids such as D- or L-arginine, glycine, and D- or L-alanine are either modulators of neural transmissions or sources of vasoactive nitric oxide. They are also gastroprotective against ethanol injury in rats (unpublished data), and they reduce tubular cell damage in the kidney (1). In our study they did not have any direct cytoprotective effect in rat GMC against ethanol.

Our findings show the following. 1) A large number of mixed GMC with high and long viability and preserved membrane receptor sensitivity can be isolated from rat stomach. 2) Selective examination of ethanol injury and protection of plasma, mitochondrial, and/or nuclear membranes may be demonstrated biochemically and morphologically. 3) Only a slight or moderate concentration-dependent protection was detected by thiolic compounds, dmPGE2, or SOS. Of the few chemicals that exerted direct cytoprotection in vitro (e.g., NAC, taurine, dmPGE2, SOS), all decreased plasma membrane damage as revealed by TB exclusion and/or LDH release, whereas only taurine and NAC diminished nuclear damage, i.e., cell death. This implies that it is much easier to offer protection against the reversible than against the irreversible stage of cell injury. 4) In contrast, a large number of naturally occurring or synthetic in vivo gastroprotective compounds have no direct cytoprotective effects against ethanol cytotoxicity in a mixed population of GMC. 5) Consequently, minimal or no correlation could be demonstrated between in vitro cytoprotection and in vivo gastroprotection by most compounds investigated. These findings indicate that a major part of the beneficial effect of gastroprotective agents seems to be mediated via complex mechanisms at the tissue and organ levels.

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REFERENCES