
ADAPTATION OF GASTRIC MUCOSA TO STRESS. EFFECT OF RANITIDINE

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Gastric mucosal adaptation to injury by repeated application of stress is a well known phenomenon. This study was designed to determine the effect of gastric acid inhibition by ranitidine on gastric adaptation to repeated exposures to stress. In this study stress 3.5 h of water immersion and restraint stress (WRS) was provoked once in rats with and without pretreatment of ranitidine (40 mg/kg/s.c.) and gastric adaptation was examined by repeated exposures to 3.5 h of WRS applied every other day for up to 8 days with pretreatment with vehicle (control), with pretreatment with ranitidine (40 mg/kg/s.c.) and with withdrawal of ranitidine prior to the last exposure to WRS. Luminal acidity, mean lesion number, histology and cell proliferation (PCNA-labeling index) were determined and the expression of EGF and TGFβ was assessed by immunohistochemistry. Pretreatment with ranitidine increased significantly luminal acidity and WRS applied once with ranitidine pretreatment resulted in a significant decrease of number of lesions. Gastric mucosa adapted to repeated WRS did show a reduction in the mean lesion number by about 60% as compared to that induced by WRS applied once. About 3 fold increase in the expression of EGF was observed in the group adapted to repeated WRS. Expression of TGFβ was not significantly different from that in intact rats. We conclude that gastric adaptation to stress leads to a decrease in gastric lesions and to an increase in expression of EGF. Pretreatment with ranitidine that induces achlorhydria results in additional reduction in the number of stress lesions.

Key words: stress, gastric blood flow, epidermal growth factor, ranitidine, gastric adaptation

INTRODUCTION

Major trauma, surgery, burns or sepsis predispose to the development of stress ulcerations in the stomach or duodenum (1). The gastroduodenal mucosa has the ability to adapt to various luminal irritants and stress (2,3). This adaptation can occur over a short time, as in the adaptive cytoprotection phenomenon, or over days or weeks of repeated exposure to various irritants
or stress (4). A number of factors, such as increased gastric acid secretion, decreased mucus secretion, blood flow, prostaglandin (PG) biosynthesis, and cell proliferation have been implicated in the pathogenesis of stress ulcerations (4, 5). It has been suggested that the ability of the stomach to adapt to repeated exposures of stress is mediated, at least in part, by EGF (6, 7). EGF has a gastroprotective effect (8, 9) and accelerates the healing of gastroduodenal ulcerations (10). EGF is a 53-amino-acid polypeptide (11) produced mainly in the submandibular glands, in the Brunner’s glands of the duodenum, and in the distal tubules of the kidney (12, 13). The spectrum of activity of EGF includes inhibition of acid secretion (14—16), stimulation of epithelial cell migration (17), mucosal growth (18) and gastric mucus production (19, 20). The expression of TGFα has been considered to play a role in gastric adaptation (21). TGFα is a 50-amino-acid polypeptide that is acid-stable and highly homologous to EGF (22). TGFα is produced in the mucosa throughout the gastrointestinal tract (23), with a spectrum of biological activity that is almost identical to that of EGF (22). To prevent stress ulcerations in a clinical setting, it is common practice to suppress acid secretion using H₂ blockers such as ranitidine.

This study was designed to examine the role of mucosal expression of EGF, TGFα, and mucosal cell proliferation in the mechanisms of gastric adaptation to stress alone, in association with continuous acid suppression by ranitidine, and after withdrawal of acid suppression by this H₂-blocker before the last exposure to stress.

METHOD

Production of gastric lesions and induction of gastric adaptation

Experiments were carried out on male Wistar rats weighing 200—250 g. The animals were divided into six groups. All rats were fasted for 24 h before starting the experiment and were allowed free access only to water. The animals were placed in restraint cages similar to those described in detail by Takagi et al (24). The rats were then immersed vertically to the level of the xiphoid process in a water bath (23°C) for 3.5 h.

In group I rats were subjected to WRS only once for 3.5 h.

In group II rats were treated with ranitidine 40 mg/kg s.c. 30 min prior to WRS applied once for 3.5 h.

In group III, IV and V animals were exposed to WRS for 3.5 h every second day for 8 days. Rats in group III received vehicle (saline) pretreatment and served as controls, group IV received ranitidine (40 mg/kg s.c.) 30 min prior to each exposure to WRS. Animals in group V received ranitidine prior to each WRS up to day 6, but ranitidine was withdrawn before the last exposure to WRS and instead, vehicle saline was administered. Group VI received no treatment at all (intact group). Each group consisted of 5—8 rats.
Determination of luminal acidity

For the determination of gastric acidity (pH), the gastric content was collected from the stomach with pylorus ligated immediately after the termination of experiment when the animals were anesthetized with ether.

Determination of gastric mucosal lesions

The stomach was then removed, opened along the greater curvature and examined with a 2x binocular magnifier for the presence of erosions by someone unaware of the treatment given using a computerized planimetry (Morphomat 10, Carl Zeiss, Berlin, Germany). The erosions in the form of round or linear erosions were counted and the average number per stomach was calculated for each group.

Mucosal histology and expression of EGF and TGFα

For histological assessment, the mucosal samples were excised, fixed in 10% formalin, embedded in paraffin and stained with hematoxilin and eosin (H&E). For immunohistochemistry, serial sections obtained from these paraffin blocks were dewaxed, rehydrated, pre-treated in citrate buffer (pH 6) in a microwave oven (3 x 5 min), and incubated with specific monoclonal antibodies (12 h. 4°C) against PCNA (1/40; PC 10, Oncogene Science, NY, USA), EGF (1/40; GF 01, Oncogene Science, NY, USA), TGF-α (1/20; GF10, Oncogene Science, NY, USA), (25). The cytoplasmic staining reactions were graded in accordance with the intensity obtained for TGF-α and EGF by examining 100 consecutive cells in the three regions of the gastric mucosa: surface epithelium (top), neck region (neck), and basal portions of the gastric glands (base). Coded specimens were independently assessed by two observers. The intensity of the staining was graded (26) as follows: 0 = no staining, I = weakly positive, II = moderately positive (cytoplasm positive but other cytoplasmic details also visible), or III = densely stained. The mean intensity per section and region was calculated. Negative control sections were processed immunohistochemically after replacing the primary antibody with PBS or an irrelevant monoclonal antibody. Positive control sections obtained from pancreatic carcinoma (TGF-α) and submandibular gland (EGF) showed grade III or maximal labeling with the appropriate antibody.

The number of cells positive for proliferating nuclear cell antigen (PCNA) was also evaluated for 100 consecutive cells in the proliferation zone of the gastric mucosa.

Statistical analysis

For statistical analysis the nonparametric Mann-Whitney U and Kruskal-Wallis tests for unpaired comparisons (two-tailed) were applied where appropriate. The results were considered significantly different if P < 0.05.

RESULTS

Gastric damage induced by water immersion and restraint stress. Effect of ranitidine on gastric adaptation

Application of WRS only once for 3.5 h resulted in numerous small bleeding erosions (mean lesion number 20 ± 3) (Table 1). In contrast the
injection of ranitidine (40 mg/kg s.c.) prior to a single exposure of WRS resulted in a significant decrease in the number of gastric lesions (mean lesion number about 8). Assessment of gastric acidity showed that pretreatment with ranitidine increased significantly gastric pH from 1.8 to about 5.

Table 1. Effects of single or repeated exposures to stress without or with pretreatment with ranitidine on number of gastric lesions and gastric luminal acidity (pH) in rats. Mean ± SEM of 5—8 rats. Asterisk indicates significant change as compared to the vehicle control. Cross indicates significant change as compared to values obtained with repeated stress for 8 days.

<table>
<thead>
<tr>
<th>Lesion number</th>
<th>Gastric acidity</th>
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<tbody>
<tr>
<td>Vehicle + WRS “once”</td>
<td>20 ± 3</td>
</tr>
<tr>
<td>Ranitidine + WRS “once”</td>
<td>8 ± 2 *</td>
</tr>
<tr>
<td>WRS for 8 days</td>
<td>7 ± 2 *</td>
</tr>
<tr>
<td>Ranitidine + WRS for 8 days</td>
<td>2 ± 1 **</td>
</tr>
<tr>
<td>Ranitidine withdrawn after 6 days + WRS for 8 days</td>
<td>5 ± 1 *</td>
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In rats adapted to WRS applied every other day for 8 days the number of gastric lesions was significantly decreased. Prior application of ranitidine to WRS resulted in a further significant decrease in the number of gastric lesions and a further rise in gastric acidity in rats adapted to repeated WRS (mean lesion number 2). Withdrawal of ranitidine in rats adapted to stress before the last application of WRS resulted in a significant increase in the number of gastric lesions and the decrease in gastric acidity to the value similar to that measured in rats exposed to WRS for 8 days without addition of ranitidine (Table 1).

Histologic findings

Table 2 shows the results of histological assessment of surface epithelium, gastric mucosa and submucosa in rats exposed to WRS once or after repeated treatment with WRS for 8 days with or without ranitidine or vehicle. A single exposure to 3.5 h WRS (group I) resulted in multiple deep haemorrhagic erosions. The erosions were extending to the muscularis mucosae and there was extensive desquamation of the surface epithelium. The submucosa was edematous and did show vasodilatation. In contrast, the gastric mucosa of rats pretreated with ranitidine (group II) did show only superficial erosions, desquamation of surface epithelium, edema of the submucosa and vasodilation.

After 8 days of repeated exposures to stress every second day only few superficial lesions were observed (group III). Compared to group I and II there
was only occasional focal desquamation of superficial epithelial cells. The proliferation zone appeared thicker and in the mucosa healing of erosions was observed. The submucosa still showed edema and dilation of blood vessels. Histology of gastric mucosa of rats adapted to 3.5 h of WRS every second day and pretreated with ranitidine (group IV) did show almost no superficial erosions and very little desquamation of superficial epithelial cells (Table 2).

Withdrawal of ranitidine after 8 days of repeated exposures to stress before the last stress exposure produced deep erosions in the gastric mucosa and desquamation of surface epithelium. The submucosa did show edema with dilatation of veins. Group VI (control group) did show normal gastric mucosa without any erosions, submucosal edema or dilation of vessels in the submucosa (Table 2).

Table 2. Histology of the gastric mucosa after WRS applied once or repeatedly throughout the period of 8 days with or without ranitidine pretreatment. Group I received WRS once for 3.5 h. Group II was treated with ranitidine 30 min prior to WRS for 3.5 h. Group III, IV and V received WRS for 3.5 h for every second day for 8 days. Group III received pretreatment with vehicle 30 min prior to each exposure to WRS, group IV received ranitidine 30 min prior to each exposure to WRS. Animals in group V received ranitidine up to day 6, and then ranitidine was withdrawn before the last exposure to WRS. Group VI received no treatment at all (intact group). N =

<table>
<thead>
<tr>
<th></th>
<th>group I (3.5h WRS and vehicle)</th>
<th>group II (3.5h WRS and ranitidine)</th>
<th>group III (8 × WRS and vehicle)</th>
<th>group IV (8 × WRS and ranitidine)</th>
<th>group V (8 × WRS withdrawal of ranitidine)</th>
<th>group VI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Surface epithelium</td>
<td>desq.</td>
<td>desq.</td>
<td>Occasional desq.</td>
<td>Occasional desq.</td>
<td>desq.</td>
<td>N</td>
</tr>
<tr>
<td>Mucosa</td>
<td>deep erosions</td>
<td>superficial erosions</td>
<td>few superficial erosions</td>
<td>few superficial erosions</td>
<td>deep erosions</td>
<td>N</td>
</tr>
<tr>
<td>Submucosa</td>
<td>marked edema vasodil.</td>
<td>marked edema vasodil.</td>
<td>edema vasodil.</td>
<td>edema vasodil.</td>
<td>marked edema vasodil.</td>
<td>N</td>
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Expression of PCNA

In rats exposed to WRS once the immunostaining was observed in the gastric mucosal neck area (Fig. 1A). In rats treated with WRS for 8 days expansion of the mucosal proliferative zone was observed and the number of PCNA labeled cells was significantly increased (Fig. 1B). The strong PCNA immunoreactivity was observed in the area of healing of gastric erosions in rats exposed to repeated WRS with ranitidine pretreatment (Fig. 1C).
Fig. 1 A-C. Photomicrographs of gastric mucosa immunostained with antibody against PCNA. A) In rats treated with WRS once labeled cells are distributed in the gastric mucosal neck area (proliferative zone). B) In rat gastric mucosa adapted to WRS the proliferative zone is increased and there is an increased number of PCNA labeled cells. C) Gastric mucosa adapted to WRS. Increased expression of PCNA in a healing erosion. (Magnification × 180)
Fig. 2. Proliferating cell nuclear antigen (PCNA) expression in gastric mucosa after single exposure to WRS (group I), ranitidine plus WRS (group II) and after 8 repeated exposures to WRS alone (group III) or with the combination with ranitidine (group IV) or with ranitidine withdrawn before last exposure to WRS (group V). Last column represents the PCNA-labeling index in the intact gastric mucosa. (Mean values ± standard deviation).

The labeling index for PCNA increased significantly (p < 0.05) in the group of animals adapted to WRS and in those treated with ranitidine prior to each: WRS exposure (group IV and V) compared to the group treated with WRS once for 3.5h without ranitidine pretreatment (group I and II) (Fig. 2).

Expression of EGF and TGFα

Fig. 3A-C shows the immunohistochemical expression of EGF in the gastric mucosa of rat exposed to WRS once and in that exposed to WRS every other day throughout the period of 8 days.

In the intact gastric mucosa, a weak staining for EGF was localized in the cells of the neck region but staining for EGF in the lumen of the gastric gland or in surface epithelial cells was observed and these results were omitted for the sake of clarity. In rats exposed to 3.5 h of WRS once, the expression of EGF
Fig. 3 A—C. Photomicrographs of gastric mucosa immunostained with antibody against EGF. A) Gastric mucosa treated with WRS once. B) Gastric mucosa adapted to WRS. Increased immunostaining of EGF-positive material in the lumen of the gastric glands and in the apical part of cells at the base and neck region (arrows). C) Control section with irrelevant primary antibody (S = surface epithelium; B = base of gastric glands). (Magnification × 180)
was localized predominantly in the neck region of gastric glands (Fig. 3A). The marked rise in EGF immunoreactivity was observed in the base and neck region of gastric glands of the regenerative mucosa in rats exposed to repeated WRS treatment (Fig. 3B) In contrast, control section with irrelevant antibody failed to show immunoreactive staining for EGF (Fig. 3C).

In gastric mucosa adapted to WRS without or with the combination with ranitidine the expression of EGF was increased up to 3 fold predominantly in the neck region (Fig. 3B and 4).

![Figure 4](image)

*Fig. 4. Epidermal growth factor (EGF) expression in gastric mucosa after single exposure to WRS and after 8 repeated exposures to WRS (group I), ranitidine plus WRS (group II) alone (group III) or with the combination with ranitidine (group IV) or with ranitidine withdrawn before last exposure to WRS (group V). Last column represents the EGF staining intensity in the intact gastric mucosa (Mean values ± standard deviation). Values in group III, IV and V are significantly different (p<0.05) from those of the intact group.*

The immunochemical staining for TGFα in rats exposed to single or repeated WRS in presented in Fig. 5 A-C. The normal gastric mucosa showed TGFα immunoreactivity throughout the whole gastric gland with predominant staining of superficial epithelial cells and no significant changes of TGFα staining intensity was observed in rats with single or repeated exposure to WRS without or with ranitidine (Figs. 5 A-C and 6).
Fig. 5 A—C. Immunohistochemical localization of transforming growth factor alpha (TGFα). A) Gastric mucosa exposed to WRS once. B) Treatment with ranitidine prior to exposure of WRS once. C) Gastric mucosa adapted to WRS. Treatment with ranitidine prior to each exposure to WRS. (Magnification × 180)
Fig. 6. Transforming growth factor α (TGFα) expression in gastric mucosa after single exposure to WRS (group I), ranitidine plus WRS (group II) and after 8 repeated exposures to WRS alone (group III) or with the combination with ranitidine (group IV) or with ranitidine withdrawn before last exposure to WRS (group V). Last column represents the TGFα staining intensity in the intact gastric mucosa. (Mean values ± standard deviation).

As expected the immunohistochemical controls were negative or did show only weak background staining while positive controls (submandibular gland for EGF and pancreatic carcinoma for TGFα) were strongly stained.

DISCUSSION

The present study shows that repeated exposure to stress leads to a reduction in the number of mucosal lesions in the stomach. The ability of the gastric mucosa to adapt to repeated stress (6, 7), repeated applications of various irritants (27) including aspirin (21, 27) and alcohol (28), has been demonstrated previously. The mechanism of this protection is not clear but, as shown in this study it may be due to increased expression of growth factors, especially that of EGF and augmented mucosal cell proliferation and mucosal repair as reflected by the increase in PCNA immunoreactivity.
In the present study, we investigated the effect of gastric acid suppression on the adaptive ability of the gastric mucosa to repeated stress. We found that the administration of H$_2$-blockers such as ranitidine and the resulting significant increase in the pH leads to an additional reduction in the number of stress lesions in the stomach. In animals that had been adapted to stress over a period of six days and prior to each stress exposure had received ranitidine, the cessation of administration of this H$_2$-blocker prior to the last exposure to stress, resulted the reappearance of stress lesions again. However, this increase in the number of stress lesions was not significantly greater than that seen in rats exposed to repeated stress but without acid suppression by ranitidine. These results indicate that even in a stomach adapted to stress, the suppression of gastric acid has an additional protective effect over that obtained with repeated stress alone. The increase in the number of stress lesions in adapted gastric mucosa following discontinuation of ranitidine provides a further evidence for the protective role of this H$_2$-blocker against stress ulcerogenesis and supports the notion that gastric secretion plays an important role in the gastric adaptation to stress.

To date, the factors involved in the adaptation of the gastric mucosa to stress and the adaptive action of other irritants, have not yet been fully identified (4, 5). However, evidence is accumulating to suggest that prostaglandins may be involved in stress adaptation (6).

The role of other factors as mucosal blood flow, increased cell regeneration and proliferation has been implicated in a number of different adaptation processes to injurious actions of various ulcerogens such as alcohol, aspirin or ammonia in experimental animals and humans (4).

The central role played by the growth factors EGF and TGF$_x$ is still controversial. Polk et al. was able to show that exposure of gastric mucosa to bile salts triggered an increase in mRNA for TGF$_x$ but not for EGF (29). In our previous study the exposure to stress was followed by a dynamic change in the expression of TGF$_x$, but the peak in the mucosal expression of TGF$_x$ occurred after 12 hours (30). Romano et al. (21) reported an increase in TGF$_x$ immunoreactivity in gastric mucosa adapted to aspirin (ASA) at 4 to 12 h after termination of ASA treatment. In contrast, our studies assessing the contents of mucosal growth factors were made immediately after the end of the treatment pointing to a role of EGF in the adaptation of the gastric mucosa because the mucosal adaptation to ASA was accompanied by a significant elevation of luminal EGF increments and the rise in mucosal EGF expression (27).

The salivary glands appear to contribute to gastric adaptation to stress (31). Earlier studies showed that exposure of the gastric mucosa to a wide range of irritants was followed by an increase in the EGF concentration in the salivary glands (32). Our results confirm that adaptation of the gastric mucosa to stress situations increases the EGF immunoreactivity, while the expression of TGF$_x$
remains without significant alterations. This is in keeping with our previous finding that when the major source of EGF such as salivary glands are removed, the adaptation of the gastric mucosa to stress no longer occurs (7). Since damaged gastric mucosa is capable of producing EGF via the formation of new cell lines (33), it is likely that EGF is produced locally in damaged mucosa by the stress.

To date, the Northern blot method has failed to detect any mRNA for EGF in the gastric mucosa. However, using the much more sensitive method of RT-PCR, we have recently been able to demonstrate the presence of EGF mRNA in gastric mucosa adapted to ammonia (34,35). This is a further evidence supporting that there is a local production of EGF in gastric mucosa adapting to topical irritant such as ammonia.

In summary, we have shown that the increase in cell proliferation detected by PCNA labeling index and the increased expression of EGF are important mechanisms involved in the adaptation of the gastric mucosa to stress. Furthermore, it can be seen that, even in the case of gastric adaptation to stress, the suppression of acid secretion provides an additional gastric protective effect that may be of clinical significance.

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