Surface Epithelial Cell Damage Induced by Restraint and Water-Immersion Stress in Rats 
Effects of 16,16-Dimethyl Prostaglandin E2 on Stress-Induced Gastric Lesions 

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Abstract—The time-course of gastric mucosal surface epithelial cell damage and macroscopically visible lesions in response to restraint and water-immersion stress (22°C) in rats was examined, and the effects on it of 16,16-dimethyl prostaglandin 
E_2 (dmPGE_2) were compared with those of papaverine, timoprazole and atropine. The stress produced surface epithelial cell damage prior to visible lesion, the former increasing in severity with time and reaching a plateau 60 min later, by which time exfoliation of surface epithelial cells was observable along the mucosal folds. In contrast, macroscopically visible lesions appeared 2 hr after stress, and severity continued to increase with time. Pretreatment injections (s.c.) of dmPGE_2 (3, 30 μg/kg), papaverine (100 mg/kg) and atropine (1 mg/kg) protected the surface cells against stress (1 hr)-induced damage, and inhibited visible lesion formation after 4 hr stress. Timoprazole (30 mg/kg, s.c.) did not protect the surface cells, but did markedly inhibit visible lesion formation. dmPGE_2, papaverine and atropine, but not timoprazole, inhibited stress-induced increases in gastric contractions. dmPGE_2, timoprazole and atropine, but not papaverine, inhibited acid secretion in stress-conditions. These results indicated that stress induced damage to the gastric mucosa within 1 hr due to increased gastric contractions, and the surface epithelial cell damage developed into macroscopically visible lesions in the presence of acid, and that dmPGE_2 protected the surface epithelium against stress-induced damage probably by inhibiting gastric contractions.

The primary barrier and protective layer of the stomach is the layer of surface epithelial cells and its associated gastric mucus. We have studied the protective effects of 16,16-dimethyl prostaglandin E_2 (dmPGE_2) on gastric surface epithelial cell damage induced by various conditions (1-3); and we have found that dmPGE_2 protected surface epithelial cells against vagal nerve stimulation-induced damage, probably by inhibiting gastric contractions (3).

The rat stomach rapidly develops mucosal lesions when the animals are restrained in a cold environment (4), and vagal overactivity has been implicated in the etiology of stress-induced gastric ulcers as shown by the protective effects of pretreatment with anticholinergics, and by vagotomy. However, the relationship between surface epithelial cell damage and macroscopically visible lesions induced by stress is unknown. In the present study, we examined A) the time course of surface epithelial cell damage and macroscopically visible lesions of the rat stomach in response to stress and B) whether dmPGE_2 prevented the initial cell damage by the same
mechanism as seen in vagal nerve stimulation. To elucidate the mechanism of dmPGE2 protection, papaverine (a smooth muscle relaxant), timoprazole (a proton pump inhibitor) and atropine (an anticholinergic) were used as reference drugs.

Materials and Methods

Male Sprague-Dawley rats (240–300 g) were deprived of food but allowed free access to water for 24 hr prior to the experiments.

Determination of stress-induced gastric lesions: In the first study, the time-course of gastric mucosal damage in response to restraint and water-immersion stress was examined. The animals were placed in restraint cages and immersed vertically to the level of the xyphoid process in a water bath (22°C) for 15, 30, 60, 120 or 240 min (4). The stomach was then removed under ether anesthesia and the gastric mucosal damage determined microscopically by scanning electron microscopy, and it was macroscopically observed under a dissecting stereoscope. The scanning electron microscopic study was carried out as previously described (1). Briefly, the removed stomach was opened along the greater curvature, extended on a glass plate, washed with saline at 4°C and put into phosphate-buffered 1.2% paraformaldehyde – 2.5% glutaraldehyde – 0.03% trinitrophenol for 3 hr at 4°C, then dehydrated through a graded series of ethanol solutions, and finally placed in isoamyl acetate. The tissues were then critical point dried with CO2 (Hitachi, HPC-2), mounted on aluminum stubs and vacuum coated by a palladium-platinum ion sputter (Eiho, iB-3). The samples were examined for damage under a scanning electron microscope (Hitachi, S-510). Four parts of the corpus were individually scanned and the severity of damage in the surface epithelial cells arbitrarily scored as follows: damage index 0, almost no visible damage to the cells; damage index 1, several shallow apical erosions among the cells; damage index 2, many shallow and several deep apical erosions among the cells; damage index 3, many deep apical erosions among the cells, but without exposure of lamina propria; damage index 4, focal exfoliation of the cells thus exposing the lamina propria; damage index 5, widespread exfoliation of the surface epithelial and pit cells. The mean values obtained for the four parts of the corpus served as a microscopical damage index per stomach. The person measuring the lesions had no knowledge of which treatment each animal had received. In the macroscopic study, the removed stomach was treated with 2% formalin solution to fix both the inner and outer layers of gastric wall for 15 min, and then it was cut open along the greater curvature and examined for visible mucosal lesions under a dissecting stereoscope (x10). The sum total area (mm2) of each stomach’s lesions served as a macroscopical mucosal lesion index.

Microscopical damage to the surface epithelial cells became evident 30 min after induction of stress, and a plateau was reached after 60 min, while macroscopical hemorrhagic lesions of the gastric mucosa appeared after 2 hr, and continued to increase in severity with time. Thus, in the second series of experiments, a pharmacological study was performed to examine the correlation between microscopical surface epithelial cell damage and macroscopical mucosal hemorrhagic lesions. The drugs used were dmPGE2 (a cytoprotective agent, Funakoshi), papaverine HCl (Nakarai), timoprazole (synthesized in the Suntory laboratory) and atropine sulfate (Merck). The effects of these compounds were examined on surface epithelial cell damage induced by 1 hr-stress, and on hemorrhagic mucosal lesions induced by 4 hr-stress. dmPGE2 (3 and 30 μg/kg) was first dissolved in absolute ethanol and then diluted with saline to the appropriate concentrations immediately before use. Papaverine HCl (100 mg/kg) and timoprazole (30 mg/kg) were suspended in saline, and atropine sulfate (1 mg/kg) was dissolved in saline. Each agent was given s.c. as a bolus injection in a volume of 0.5 ml/100 g body wt. 10 min before exposure to stress. The animals were sacrificed 1 hr or 4 hr after stress initiation, and either the surface epithelial cell damage or the visible mucosal lesions were examined as described above.

Determination of gastric acid: The effects
of dmPGE₂, papaverine, timoprazole and atropine on gastric acid secretion during conditions of stress were investigated in pylorus-ligated rats. Ten min after treatment with each agent, the abdomen of each rat was incised and the pylorus ligated under light ether anesthesia. The wound was sutured and covered with collodion (Wako) to prevent water infiltration during water immersion. After recovery from anesthesia (about 5 min), each animal was put into a stress cage and immersed in water as described above. Control animals were kept in stress cages at room temperature. One or 4 hr after stress loading, the animals were sacrificed, and the gastric contents collected and analyzed for volume (ml/rat), acidity (µEq/ml) and acid output (µEq/rat). Acidity was determined by automatic titration of gastric juices against 0.1 N NaOH to pH 7.0 (Metrohm Herisau, Combi-titrator 3D).

Measurement of gastric motility: Gastric motility was measured in conscious rats using a miniature balloon according to the method described by Takeuchi and Nobuhara (5). Briefly, under ether anesthesia, a water-filled balloon attached to a support catheter was inserted into the stomach through a cautery hole made in the forestomach. The catheter was tied in place so that the balloon lay in the glandular part of the stomach. Pressure in the balloon was measured with a pressure transducer (Nihon Kohden, LPU-0.1-350-0-11) and recorded continuously with a polygraph (Nihon Kohden, RM-8200). The incision was then closed with a ligature, covered with collodion, and each rat was put into an individual stress cage. Gastric motility was quantitated both by measuring the maximum amplitude of intragastric pressure (cmH₂O) and by counting the number of pressure spikes with an amplitude of 20 cmH₂O or greater. Approximately 1 hr after basal gastric motor activity had stabilized, each of the test agents was given subcutaneously, and 10 min later, the animals were immersed in water; the intragastric pressure was recorded for 4 hr thereafter.

In addition, the left femoral artery was cannulated with a polyethylene tube (Clay Adams, PE 50) in order to monitor blood pressure with the aid of a pressure transducer (Nihon Kohden, MPU-0.5-290-0-III). Heart rate was measured using a cardiotachometer (Nihon Kohden, AT-600) triggered by blood pressure pulse.

Statistics: Data were presented as means±S.E.M. of 4–8 animals per group. Damage indices from the scanning electron microscopic study were analyzed by the x² test, and the other parameters were compared by the two-tailed Dunnett multiple comparison test (analysis of variance) (6). A 5% level of significance was used throughout.

Results

Development of stress-induced damage: Surface epithelial cell damage was detected prior to macroscopically visible hemorrhagic mucosal lesions in the stress-loaded rat stomachs. As shown in Figs. 1 and 2A, extensive damage to the apical membranes of the surface epithelial cells appeared in stomachs excised 30 min after stress initiation. The severity of the damage increased to the level of surface epithelial cell exfoliation and exposure of lamina propria along the mucosal folds. The severity of the damage 2 and 4 hr after stress initiation was much the same as that observed at 1 hr, the microscopical damage indices being 4.0±0.2, 4.2±0.2 and 4.1±0.2 at 1, 2 and 4 hr, respectively. In contrast to the surface epithelial cell damage observed under a scanning electron microscope, macroscopically visible hemorrhagic lesions in the gastric mucosa rarely appeared within 1 hr after stress initiation, but were visible after 2 hr. Their severity increased with time, as reflected by the gastric mucosal lesion indices of 0.4±0.2, 3.4±1.2 and 7.9±1.4 mm² at 1, 2 and 4 hr after stress initiation, respectively (Fig. 1 and 2B). The macroscopically visible lesions were either linear or focal in shape, and occurred along the mucosal folds. Thus, surface epithelial cell damage observed under a scanning electron microscope, macroscopically visible hemorrhagic lesions in the gastric mucosa rarely appeared within 1 hr after stress initiation, but were visible after 2 hr. Their severity increased with time, as reflected by the gastric mucosal lesion indices of 0.4±0.2, 3.4±1.2 and 7.9±1.4 mm² at 1, 2 and 4 hr after stress initiation, respectively (Fig. 1 and 2B). The macroscopically visible lesions were either linear or focal in shape, and occurred along the mucosal folds. Thus, surface epithelial cell damage reached a maximum 1 hr after stress initiation, and visible lesions were consistently formed 4 hr after stress initiation. For these reasons, in the following experiments we examined the effects of dmPGE₂, papaverine, timoprazole and atropine on initial surface epithelial cell damage and macroscopically visible mucosal lesions at 1 hr and 4 hr after stress initiation,
Fig. 1. Scanning electron micrographs (upper) and gross appearance (lower) of stress-induced gastric lesions in rats. Damage to surface epithelial cells appeared after 30 min stress, and a plateau was reached after 60 min (No stress x 500, 30 min x 500, 60 min x 100, 240 min x 100). Hemorrhagic lesions were observed after 2–4 hr stress. Note that both exfoliation of surface epithelial cells and hemorrhagic lesions occurred along the mucosal folds.

Fig. 2. Time-course study of microscopical damage to the surface epithelial cells (A) and macroscopical lesions (B) of the rat stomach induced by restraint and water-immersion stress. Surface epithelial cell damage was determined by scanning electron microscopy (SEM).

Fig. 3. Effects of dmPGE2, papaverine (Pap), timoprazole (Tim) and atropine (Atr) on stress-induced gastric mucosal damage. Surface epithelial cell damage was determined after 1 hr stress by scanning electron microscopy (SEM, A), and the macroscopically visible lesions were determined after 4 hr stress (B). Note that timoprazole did not prevent the initial damage, but markedly prevented macroscopical lesion formation.
Effects of drugs on stress-induced gastric mucosal damage: The effects of dmPGE² on the stress-induced initial surface epithelial cell damage (1 hr) and on the more extensive visible lesions (4 hr) were compared with those of papaverine, timoprazole and atropine. Pretreatment with both 3 and 30 μg/kg dmPGE² dose-dependently suppressed surface epithelial cell damage induced by 1 hr stress-loading, the protection being 37% and 61%, respectively (Fig. 3A). Representative photomicrographs are shown in Figs. 4B and 4C. The surface epithelial cells were also protected against stress-induced damage by pretreatment with papaverine (100 mg/kg, Figs. 3A and 4D) and with atropine (1 mg/kg, Figs. 3A and 4F), the protection being 45%.
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and 50%, respectively. In contrast, timoprazole (30 mg/kg) exerted no protective effect against the initial cell damage (Figs. 3A and 4E).

Exposure of the animals to stress for 4 hr consistently caused hemorrhagic lesions in the gastric mucosa, the area of mucosal lesions being 7.5±0.8 mm². dmPGE₂ (3 and 30 μg/kg), papaverine, timoprazole and atropine all significantly inhibited the formation of these lesions (Fig. 3B), the protection being 71%, 92%, 57%, 91% and 91% respectively.

Effects of drugs on acid secretion during stress: Gastric acid secretion tended to decrease in response to 4 hr stress, but not significantly (Fig. 5). Pretreatment with dmPGE₂ (30 μg/kg), timoprazole (30 mg/kg) or atropine (1 mg/kg) significantly inhibited acid output in both 1 hr- and 4 hr-stress-loaded pylorus ligated rats, although papaverine had no significant influence (Fig. 5).

Effects of drugs on gastric motility during stress: Gastric motility in normal rats usually consisted of 6-13 contractions per 10 min (9.6±1.2 contractions/10 min), with an amplitude of 25-40 cmH₂O (30.4±2.2 cmH₂O), and it did not change with time (4 hr). The initiation of restraint and water-immersion stress was associated with an initial stimulation of gastric contractions in saline-treated rats, as shown in Fig. 6. The amplitude of the contractions increased with time, the maximal level being reached after 90 min. Thus, the basal level increased from 35.4±1.6 cmH₂O before stress to 47.8±6.2, 57.6±4.3, 56.0±4.9 and 57.0±2.8 cmH₂O at 60, 90, 120 and 240 min after stress initiation, respectively (Fig. 7). The frequency of contractions also increased with time, the plateau being reached also after 90 min (10.8±0.9/10 min before stress vs. 15.4±1.6/10 min at 90 min after stress initiation).

Pretreatment with dmPGE₂ (3 and 30 μg/kg) 10 min before stress initiation caused a

![Graphs showing effects of dmPGE₂, papaverine (Pap), timoprazole (Tim) and atropine (Atr) on gastric acid secretion in stress-loaded pylorus-ligated rats (1 hr or 4 hr). Note that dmPGE₂, timoprazole and atropine, but not papaverine, inhibited acid secretion.](image-url)
Fig. 6. Representative figures showing the effects of water-immersion stress on gastric motility, blood pressure and heart rate in rats. The initiation of water-immersion stress was associated with an initial stimulation of gastric contractions and bradycardia.

Fig. 7. Effects of dmPGE₂, papaverine, timoprazole and atropine on rat gastric motility enhanced by restraint and water-immersion stress. Drugs were given s.c. after stabilization of spontaneous gastric contractions. The amplitude and frequency of gastric contractions before and after stress were reduced by dmPGE₂, papaverine and atropine.
Table 1. Effects of dmPGE₂, papaverine, timoprazole and atropine on changes of blood pressure and heart rate induced by restraint and water-immersion stress in rats

| Drugs    | Dose mg/kg s.c. | Before stress | Stress loading |
|----------|-----------------|---------------|---------------|
|          | Control         | 10 min after dosing | 30 min | 1 hr | 2 hr | 3 hr | 4 hr |
| Saline   | BP 103±2        | 104±3         | 101±4        | 97±5 | 98±4 | 98±5 | 96±4 |
|          | HR 493±16       | 492±15        | 285±15       | 235±13 | 217±12 | 208±9 | 199±11 |
| dmPGE₂   | 0.003 BP 105±4  | 105±3         | 105±6        | 104±7 | 104±6 | 101±7 | 100±6 |
|          | HR 520±10       | 518±6         | 307±11       | 225±13 | 200±10 | 194±8 | 189±5 |
|          | 0.03 BP 101±4   | 96±4          | 104±5        | 106±5 | 106±5 | 100±3 | 97±2 |
|          | HR 495±14       | 498±16        | 261±16       | 255±11 | 218±3 | 200±4 | 194±4 |
| Papaverine | 100 BP 106±3    | 97±2          | 86±5         | 85±5 | 84±4 | 88±6 | 91±8 |
|          | HR 522±13       | 510±13        | 301±15       | 258±9 | 245±6 | 228±6 | 212±8 |
| Timoprazole | 30 BP 105±6    | 103±7         | 95±5         | 91±6 | 93±6 | 95±8 | 94±9 |
|          | HR 477±12       | 479±13        | 270±11       | 228±10 | 217±8 | 198±3 | 181±3 |
| Atropine | 1 BP 93±3       | 92±4          | 90±5         | 88±6 | 88±4 | 90±5 | 91±6 |
|          | HR 500±18       | 513±21        | 286±14       | 239±14 | 217±9 | 210±9 | 203±8 |

BP: mean blood pressure (mmHg). HR: heart rate (beats/min). n=4 each.
dose-dependent inhibition of both spontaneous gastric motor activity and stress-induced gastric hypermotility, both in terms of amplitude and frequency (Fig. 7). dmPGE2 at 30 µg/kg produced a slight increase in gastric tone, but clearly inhibited motor activity, causing the stress-induced high amplitude contractions to all but completely disappear. Similar inhibition was also observed at 3 µg/kg, but the duration of its effects was shorter. Pretreatment with papaverine (100 mg/kg) and atropine (1 mg/kg) also significantly suppressed the amplitude and frequency of gastric contractions, but timoprazole (30 mg/kg) had essentially no influence on stress-induced gastric motor hyperactivity (Fig. 7).

Effects of drugs on stress-induced changes in the cardiovascular system: Blood pressure in stress-loaded rats did not change during the experimental period, but the heart rate gradually decreased with time (Fig. 6). Pretreatments with dmPGE2, papaverine, timoprazole and atropine had no influence on this bradycardia (Table 1).

Discussion

We found that restraint and water-immersion stress produced in the rat stomach microscopic damage to the surface epithelial cells prior to formation of macroscopically visible hemorrhagic lesions. Pretreatments with dmPGE2, papaverine and atropine suppressed both the initial cell damage and the more extensive visible lesions. Timoprazole on the other hand, did not inhibit the initial cell damage, but potently inhibited the formation of visible lesions.

Water-immersion stress is widely used as an experimental model to induce acute stress ulcers in rats because of its reliable reproducibility. Changes in gastric secretion (7, 8), abnormal gastric motility (9, 10), and disturbance of gastric mucosal microcirculation (11, 12) have been implicated as underlying pathogenetic mechanisms.

It was reported following a scanning electron microscopic study of rat stomachs (13) that surface epithelial cells were damaged following acute stimulation of gastric acid secretion by either pentagastrin administration or intragastric instillation of 150 mM HCl. Acid hypersecretion has been noted by some investigators to be associated with lesion formation in water-immersion stressed rats (7, 14). However, others have reported either no effects or even suppression of acid secretion (8). In our experiments with pylorus-ligated rats, gastric acid hypersecretion was not found to be a physiological response to restraint and water-immersion stress, indicating that gastric acid secretion was not involved in the pathogenetic mechanisms of the stress-induced surface epithelial cell damage. This correlates well with the observations that timoprazole all but completely inhibited gastric acid secretion but exerted no cytoprotective effect though it did prevent the later outgrowth of visible lesions after 4 hr stress. These results indicated that acid secretion played an important role in aggravating the gastric lesions, but did not contribute to the initial cell damage. Furthermore, the protective effect of dmPGE2 against the stress-induced initial cell damage does not seem to be attributable to its antisecretory activity.

Pretreatments with dmPGE2, papaverine and atropine protected the surface epithelial cells from stress-induced damage, and they significantly inhibited the gastric hypercontractions with the same doses. Water-immersion stress-induced increases in amplitude and frequency of gastric contractions have been reported to occur along with the formation of gastric lesions (9, 15, 16); and furthermore, the authors showed that vagal overactivity was involved in both stress-induced gastric hypermotility and mucosal lesion formation, inasmuch as both parameters were decreased or blocked by atropine or vagotomy (15, 16). Yano et al. (9) and Garrick et al. (10) underlined the role of muscular elements in the pathogenesis of gastric lesions induced by water-immersion stress; they found that either a continuous infusion of papaverine during (9) or a single injection prior to stress initiation (10) suppressed lesion formation without inhibiting acid secretion. In the present study, we have shown that high-amplitude gastric contractions induced by stress triggered the initial damage to the surface epithelial cells, and the vulnerable mucosa, once so-induced,
was closely linked to subsequent induction of macroscopically visible lesions. Mersereau and Hinchey (17, 18) recently reported that a deficiency in prostaglandins could render the mucosal folds more vulnerable to compression injury at stress-concentrating sites. However, the detailed mechanisms by which gastric motor alterations induce the initial surface epithelial damage remain unknown.

Mersereau and Hinchey (15) reported that the gastric lesions induced by stress were formed at specific sites along the gastric mucosal folds as a result of mucosal compression due to vagal overactivity. We observed that the exfoliation of surface epithelial cells after stress loading for 1 hr were also localized along the crests of the mucosal folds. Small amounts of acid may then be corrosive to the vulnerable regions, resulting in macroscopically visible lesions either linear in shape or linearly distributed along the mucosal folds.

Decreased gastric mucosal blood flow has been reported as one important factor related to lesion formation induced by restraint and water-immersion stress (11, 12). In the present study, we cannot discuss the correlation between the initial surface epithelial cell damage and decreased gastric mucosal blood flow in stressed rats. However, it is possible that dmPGE₂, papaverine and atropine improved the regional mucosal blood flow as a result of dissolution of mucosal folds initially formed as a result of the stress-induced high-amplitude gastric contractions which may have caused a temporarily restricted blood flow to the crests of the mucosal folds, thus resulting in hypoxia.

We concluded that A) restraint and water-immersion stress within 1 hr induced gastric mucosal surface epithelial cell damage due to increased gastric contractions. The initial cell damage developed into macroscopically visible lesions in the presence of acid; and B) dmPGE₂ protected the surface epithelial cells from stress-induced damage, probably by inhibiting gastric contractions.

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