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Activation of genes for growth factors and cyclooxygenases in rat gastric mucosa during recovery from stress damage

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Abstract

Growth factors and prostaglandins protect the gastric mucosa against stress-induced lesions but their role in the recovery of the mucosa from these lesions has been little studied. We evaluated gastric mucosa lesions, gastric blood flow, mucosal generation of prostaglandin E_2 and mucosal gene expression of epidermal growth factor (EGF) and transforming growth factor alpha (TGFα) as well as constitutive prostaglandin cyclooxygenase-1 and inducible cyclooxygenase-2 and the effect of the inhibition of these enzymes on the recovery of mucosa from the stress-induced lesions. Rats were exposed to 3.5 h of water immersion and restraint stress and killed at 0, 2, 4, 6, 8, 12 and 24 h after stress. The number of gastric lesions was determined and gastric blood flow was measured by H_2-gas clearance. Gastric acid secretion was tested in separate gastric fistula rats. Gastric mucosa biopsies were taken for determination of immunoreactive EGF and TGFα. Expression of EGF and TGFα mRNA and cyclooxygenase-1 and cyclooxygenase-2 mRNA was also determined by reverse-transcriptase polymerase chain reaction. The number of gastric lesions induced by 3.5 h stress averaged $\sim$ 20 per rat and declined significantly at 2, 4, 6, 8 and 12 h, to disappear almost completely after 24 h. This was accompanied by a gradual rise in gastric blood flow, mucosal generation of prostaglandin E_2 and mucosal EGF and TGFα contents, while the increased gastric acid secretion returned to normal. In the intact mucosa, EGF mRNA was not detected but TGFα mRNA was found in measurable amounts. Following exposure to stress, the expression of both these factors was significantly increased. Similarly, the expression of cyclo-oxygenase-1 and cyclooxygenase-2 mRNA was detected in the oxyntic mucosa at all time intervals after exposure to stress. Indomethacin (5 mg/kg i.p.), an inhibitor of cyclooxygenase-1 and cyclooxygenase-2, and meloxicam (1 mg/kg i.p.), an inhibitor of cyclooxygenase-2, both prolonged the healing of stress lesions and reduced the gastric blood flow, while enhancing gastric acid secretion at all times tested. We conclude that healing of stress lesions results in the restoration gastric blood flow and mucosal prostaglandin generation and that these effects are accompanied by overexpression of EGF and TGFα as well as cyclooxygenase-1 and cyclooxygenase-2 mRNA and by increased biosynthesis of gastroprotective prostaglandin. © 1998 Elsevier Science B.V.

Keywords: Stress; Cyclooxygenase; Growth factor; Prostaglandin; Ulcer

1. Introduction

Gastric stress ulceration is a serious complication which may occur in patients with major burns, surgery or central nervous system trauma (Soll, 1993, Seversten and Pranulis, 1995). Several mechanisms have been implicated in the pathogenesis of stress-induced gastric lesions such as an increase in gastric acid and pepsin secretion, a decrease in gastric blood flow, suppression of endogenous generation of prostaglandins, inhibition of mucosal growth and cell proliferation and alteration of gastric motility (Konturek et al., 1992a,b; Soll, 1993; Brzozowski et al., 1993). Among various stress models used in animals, water immersion and restraint have yielded the most reproducible results, because these two factors act synergistically to produce acute gastric lesions (Senay and Levine, 1967).

Prostaglandins are generated in gastric mucosa via the activity of an enzyme, cyclooxygenase (Eberhart and Dubois, 1995), which exists as two genetically different isoforms, constitutive cyclooxygenase-1 and inducible cyclooxygenase-2 (Kujubu et al., 1991; O'Banion et al., 1991; Xie et al., 1991; Feng et al., 1995; Hla and Neilson, 1992). Cyclooxygenase-1 has been shown to exhibit cytoprotective effects on gastric mucosa, whereas cyclooxy-

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genase-2 has been implicated in the inflammatory reactions and tissue damage involving various cytokines, endotoxins and growth factors (Kujubu et al., 1991; O'Banion et al., 1991; Xie et al., 1991).

Prostaglandins were shown to reduce the damage induced by stress (Victor et al., 1989; Yoshimura et al., 1989; Konturek et al., 1991; Brzozowski et al., 1993) but their role in the healing of stress-induced gastric lesions and gene expression of cyclooxygenase-1 and cyclooxygenase-2 during recovery from stress has not been extensively investigated. It is not known which of these two cyclooxygenase isoenzymes is involved in the mucosal recovery after stress and what could be the mechanism of the enzyme activation.

Healing of the gastric mucosa damage after exposure to stress is a complex process involving different mechanisms, the most important of which appear to be the growth factors, especially epidermal growth factor (EGF) and transforming growth factor alpha (TGFα) (Konturek, 1990; Konturek et al., 1992b, 1996; Podolsky, 1994). EGF is a 53-amino acid peptide that originates mainly from the salivary glands and no EGF mRNA has been detected in the intact gastric mucosa of rodents and humans (Polk et al., 1992; Calabro et al., 1995). Increased expression of EGF and EGF receptors was, however, reported in the gastric mucosa surrounding areas of chronic ulcerations (Wright et al., 1990) and during repair of the gastric mucosa after exposure to stress (Konturek et al., 1996). The TGFα mRNA has been detected in the intact mucosa and found to increase in damaged mucosa (Beauchamp et al., 1989; Polk et al., 1992; Thomas et al., 1992; Romano et al., 1996). It is unknown whether the genes for both EGF and TGFα, are activated during healing of stress lesions.

The present study was designed (1) to evaluate both the healing of gastric lesions induced by stress and the accompanying changes in gastric blood flow, plus the generation of endogenous prosta-glandin E_2 and mucosal contents of immunoreactive of EGF and TGFα, (2) to determine the mucosal expression of EGF and TGFα mRNA as well as cyclooxygenase-1 and cyclooxygenase-2 mRNA during mucosal recovery from the lesions induced by stress and (3) to determine the effects of blockers of cyclooxygenase-1 (indomethacin) and cyclooxygenase-2 (meloxicam) on the healing rate of stress-induced gastric ulcerations and accompanying changes in mucosal generation of prostaglandin E_2, gastric blood flow and gastric acid secretion.

2. Material and methods

The following studies were carried out under the protocol approved by the Ethical Committee of the University School of Medicine, Cracow, Poland.

2.1. Measurement of gastric acid secretion

Ten Wistar male rats, weighing 200–250 g, were equipped surgically with a metal cannula (5 mm outside diameter) placed in the distal part of the stomach to form a gastric fistula for gastric secretory studies. The installation of this cannula allowed for the collection of the gastric juice in the conscious rats as demonstrated previously (Konturek et al., 1992a,b). The alterations in gastric acid secretion during recovery from 3.5 h of stress and the influence of indomethacin and meloxicam (kindly supplied by Dr. M. Trybulec of Boehringer Ingelheim, Warsaw, Poland) on this secretion were examined. On the day before the experiment, the rats were housed in individual cages to avoid coprophagy and fasted 24 h with ad libitum access to drinking water. Then, the cannula of the gastric fistula was opened and the stomach was cleared from debris by washing with 5 ml of tap water. Then, the gastric contents were collected as 30-min aliquots, the volume of each collection was measured and the concentration and output of acid were determined and expressed as 1-h outputs as described before (Konturek, 1991, Brzozowski et al., 1993). In tests with inhibitors of cyclooxygenase-1 and cyclooxygenase-2, respectively, indomethacin (5 mg/kg i.p.) and meloxicam (1 mg/kg i.p.) were injected 30 min before the start of the secretory studies and collection of gastric content was continued for 1 h at 0, 4, 8, 12 and 24 h after the termination of the stress. After each hour of gastric collection, the gastric fistula was closed and then reopened at the next time interval tested.

2.2. Production of gastric lesions and measurement of gastric blood flow and luminal content of prostaglandin E_2

Stress-induced gastric lesions were provoked by placing the animals in individual cages, causing immobilization and immersing them in water at 23°C up to the xyphoid process as described previously (Konturek et al., 1991; Brzozowski et al., 1993). This protocol was repeated after the administration of indomethacin (5 mg/kg i.p.) or meloxicam (1 mg/kg i.p.) just before the start of the stress. The animals were then lightly anesthetized with ether immediately (time 0 h) or after 2, 4, 6, 8, 12 and 24 h after termination of 3.5 h stress, for each time period 8–10 animals being used. The abdomen was opened, the stomach was exposed and gastric blood flow was measured by the H_2-gas clearance technique as described previously (Konturek, 1991). Briefly, double needle electrodes were inserted into the mucosa through the serosa with the tips located in the mucosa, one electrode being used for local generation of H_2-gas and the other for the measurement of tissue H_2. With this method the H_2 generated by water hydrolysis is carried away by the blood and a polarographic current detector shows the decreasing tissue H_2 as a clearance curve which is used to calculate absolute flow

rate (ml/min 100 g). Gastric blood flow was measured in three areas of the oxyntic portion of the stomach and the mean value of three recordings was calculated and expressed as the percentage of the flow recorded in the intact mucosa of non-stressed rats.

Gastric contents were then collected after washing out of the stomach with 1 ml of saline injected into the stomach with the pylorus sphincter ligated to prevent escape of the saline into the duodenum in animals lightly anesthetized with ether. Washings were immediately neutralized with NaOH to pH 7.0, centrifuged and then frozen with liquid nitrogen to be stored at −80°C until the radioimmunoassay of prostaglandin E_2, EGF and TGFα as described below.

Finally, the stomach was removed and the number of gastric lesions was counted using a computerized planimeter (Morphomat 10, Carl Zeiss, Berlin) by a person blinded to the origin of coded specimens. The stress lesion was defined as a round or linear mucosal black or red defect of at least 0.1 mm in diameter Fig. 1. The number of gastric lesions in the rat stomach from each study group was counted, summed and divided by the number of rats in each experimental group to give the means (±S.E.M.). Biopsy samples (50–100 mg) of the oxyntic mucosa were then taken and placed either in phosphate-buffered 10% formalin for histology or weighed and used for the assessment of mucosal generation of prostaglandin E_2 or frozen in liquid nitrogen to be stored at −80°C for radioimmunoassay of EGF and TGFα as well as for the detection of mRNA for EGF, TGFα, cyclooxygenase-1 and cyclooxygenase-2 as described below. All these determinations were done in the same animals that were anesthetized with ether and used either before (time 0) or at various time intervals after the stress.

2.3. Radioimmunoassay of EGF and TGFα

Specific radioimmunoassays were used for determination of the concentration of EGF and TGFα in luminal washings and in gastric mucosal biopsies. The mucosal biopsy samples were homogenized in 1.0 ml of 50 mM Tris–HCl adjusted to pH 7.4 using a Potter S homogenizer (Unipam, Warsaw Poland) at 1500 rpm for 20 s. The homogenates were centrifuged at 16 000 × g for 20 min at 4°C and the supernatants were used for radioimmunoassay of EGF as described previously (Konturek et al., 1991). EGF was determined using an EGF antiserum (Amersham, UK) raised in rabbits against rat EGF (purified from rat salivary glands) and used in a final dilution of 1:210 000. The antiserum showed full cross-reactivity with synthetic and natural rat EGF. Iodinated (3-^{125}Iodotyrosyl) peptide and rat EGF were used as the calibration standards (Amersham, UK). The detection limit of the assay was 0.1 ng/ml. The intra-assay and the inter-assay variability was about 15 and 10%, respectively. For the radioimmunoassay of TGFα, a TGFα antiserum (Gesellschaft fur Immunchemie und Biologie MBH, Hamburg, Germany) was used, that showed no cross-reactivity with human EGF and other closely related peptides. A final dilution of 1:20 000 was used. Recombinant (natural sequence) TGFα was used as standard and as ^{125}I-labeled tracer. The intra-assay and the inter-assay variability was about 12 and 15%, respectively. The sensitivity of the assay was 0.1 ng/ml.

2.4. Determination of mucosal generation of prostaglandin E_2

The generation of prostaglandin E_2 in mucosal biopsies was determined according to the method described in details elsewhere (Konturek et al., 1991). The mucosal samples were placed in preweighed Eppendorf vials and 1 ml of Tris buffer (50 mM, pH 3.5) was added to each vial. The samples were finely minced (for about 15 s) with scissors, washed and centrifuged for 10 s and the pellet was resuspended in 1 ml of Tris. Each sample was then stirred on a vortex mixer for 1 min and centrifuged for 15 s. The pellet was weighed and the supernatant was transferred to a second Eppendorf vial containing indomethacin (10 mM) and was kept at −20°C until radioimmunoassay. Prostaglandin E_2 generated by the biopsy samples and the prostaglandin E_2 content were measured using radioimmunoassay kits (New England Nuclear, Munich). The capacity of the mucosa to generate prostaglandin E_2 was expressed in ng/g wet tissue weight.

2.5. Reverse-transcriptase polymerase chain reaction for detection of mRNA for EGF, TGFα, cyclooxygenase-1 and cyclooxygenase-2

Total RNA was isolated from the mucosal specimens and stored at −80°C. A rapid quanidinium isothiocyanate/phenol chloroform single step extraction kit from Stratagene® was used. Following precipitation, the RNA was resuspended in RNase-free TE buffer and the concentration was estimated by absorbance at 260 nm wavelength. The quality of each RNA preparation was determined by agarose-formaldehyde gel electrophoresis and ethidium bromide staining. RNA samples were stored frozen at −80°C until analysis. First-strand cDNA was synthesized from total cellular RNA (5 μg) using a 200 U StrataScript™ reverse transcriptase kit (Stratagene, La Jolla, USA) and oligo (dT) primers (Stratagene, La Jolla, USA) according to standard techniques. Briefly, 5 μg of total RNA was used as the template to synthesize complementary DNA with 2.5 U of Moloney murine leukemia virus reverse transcriptase in 5 μg of buffer containing 10 mM/l Tris–HCl, pH 8.3; 50 mM/l KCl; 5 mM/l $MgCl_2$; 1 mM/l each deoxyribonucleoside triphosphates; 2.5 mM/l of oligo (dt) primers and 1.4 U/μg of RNAse block. Reverse transcription was performed at room temperature for 20 min, then at 37°C for 15 min, at 90°C for 5 min and at 5°C for 5 min. The resulting complementary

DNA was used as a template for the subsequent polymerase chain reaction.

The reaction mixture for the polymerase chain reaction contained cDNA template (2 μl), 50 pmol of each primer and 2.5 U of Taq DNA polymerase (Promega) in 10 mM Tris–HCl (pH 8.8), 50 mM KCl, 1.5 mM $MgCl_2$ and 0.5 mM dNTPs in a volume of 50 μl. Reverse transcriptase blanks (no RNA included) and polymerase chain reaction blanks (no cDNA products included) were included in each analysis. Primers were synthesized by Biometra® (Göttingen, Germany). The nucleotide sequences of the rat EGF primers were based on the published cDNA sequences encoding rat preproEGF and preproTGFα (Fan et al., 1995, Saggi et al., 1992). Rat EGF sense primer was 5′-GACAACTCCCCTAAGGCTTA-3′ (nucleotides 2804–2823); the EGF antisense primer was 5′-CATGCA-CACGCCACCATTGAGGCAGTACCCATCGTACGA-3′ (nucleotides 3332–3370) (Saggi et al., 1992; Fan et al., 1995). The rat TGFα sense primer was 5′-ATGGTCCC-CGCGGCCGGACA-3′ and the rat TGFα antisense primer was 5′-GACCACTGTCTCAGAGTGGCAGCAGG-CAGTCCTTCCTTT-3′ as described elsewhere (Saggi et al., 1992).

The sequence of oligonucleotide primers used for cyclooxygenase-1 upstream primer was AGC CCC TCA TTC ACC CAT TT and that for cyclooxygenase-1 downstream primer was CAG GGA CGC CTG TTC TAC GG, respectively (Gustafson-Svard et al., 1996). The rat cyclooxygenase-2 upstream primer was TGGTGC CGG GTCTGA TGA TG and the cyclooxygenase-2 downstream primer was GCA ATG CGG TTC TGA TAC TG (Gustafson-Svard et al., 1996). Concomitantly, amplification of control rat β-actin (ClonTech, Palo Alto, CA) was performed on the same samples to asses RNA integrity. To maximize amplifi-cation specificity, a hot start polymerase chain reaction was performed in a Perkin Elmer Cetus DNA thermal cycler for 33 cycles (94°C for 1 min, 60°C for 45 s, 72°C for 2 min) using AmpliWax® PCR Gen 50 beads. Briefly, after adding primers, buffer and dNTPs, an AmpliWax polymerase chain reaction Gen was added and heated to 80°C for 10 min. Then the DNA Taq polymerase, cDNA sample and buffer were pipetted into the PCR mixture. 8-μl aliquots of amplified polymerase chain reaction product were electrophoresed on a 1.5% agarose gel stained with ethidium bromide and then visualized under UV light. The location of predicted polymerase chain reaction products (base pairs) was confirmed by using DNA digest PhiX174/*Hae*III as a standard size marker. The gel was

Table 1
Mean number of gastric lesions, prostaglandin E_2 generation and gastric blood flow in rats pretreated with vehicle, indomethacin (5 mg/kg i.p.) or meloxicam (1 mg/kg i.p.) given alone or combined with stress and recorded at 0, 2, 4, 6, 8, 12 and 24 h after stress

	Lesion number	Prostaglandin E_2 generation (ng/g)	Gastric blood flow (% control)
Vehicle-control	0	224 ± 12	100%
Indomethacin (alone)	0	28 ± 2[a]	89 ± 4[a]
Meloxicam (alone)	0	85 ± 8[a]	86 ± 5[a]
After stress (time)			
0 h	18 ± 4	101 ± 6	48 ± 4
2 h	17 ± 2	125 ± 8	78 ± 6[a]
4 h	12 ± 2[a]	130 ± 10[a]	82 ± 8[a]
6 h	9 ± 1[a]	155 ± 12[a]	85 ± 8[a]
8 h	8 ± 0.5[a]	192 ± 14[a]	92 ± 10[a]
12 h	5 ± 0.6[a]	211 ± 15[a]	95 ± 7[a]
24 h	2 ± 0.3[a]	240 ± 30[a]	98 ± 12[a]
Indomethacin + stress (time)			
0 h	31 ± 6[b]	24 ± 2[b]	35 ± 3[b]
2 h	27 ± 5[b]	30 ± 3[b]	38 ± 4[b]
4 h	24 ± 3[b]	28 ± 3[b]	45 ± 5[b]
6 h	23 ± 3[b]	32 ± 4[b]	49 ± 5[b]
8 h	18 ± 4[b]	45 ± 5[b]	68 ± 7[b]
12 h	12 ± 2[b]	42 ± 4[b]	75 ± 6[b]
24 h	8 ± 1[b]	52 ± 5[b]	86 ± 7[b]
Meloxicam + stress (time)			
0 h	29 ± 6[b]	95 ± 10[b]	32 ± 3[b]
2 h	28 ± 5[b]	89 ± 9[b]	35 ± 3[b]
4 h	26 ± 4[b]	112 ± 8[b]	48 ± 5[b]
6 h	25 ± 4[b]	128 ± 8[b]	52 ± 4[b]
8 h	23 ± 5[b]	140 ± 7[b]	71 ± 5[b]
12 h	19 ± 3[b]	122 ± 9[b]	78 ± 6[b]
24 h	11 ± 3[b]	109 ± 8[b]	81 ± 7[b]

Results are means ± S.E.M. for 8–10 rats.
[a]Significant ($P < 0.05$) change as compared to the value recorded in non-stressed vehicle-controls or in rats exposed to stress at time 0.
[b]Significant ($P < 0.05$) change as compared to the value recorded at time 0 in rats after exposure to stress alone without indomethacin or meloxicam.

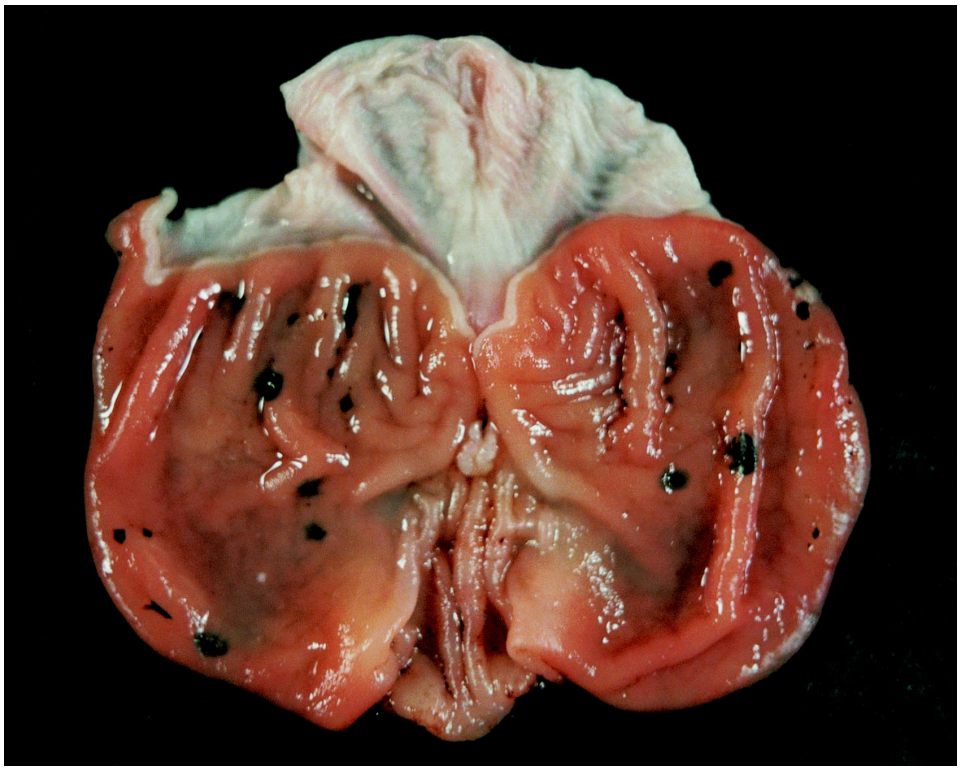

Fig. 1. Gross appearance of typical gastric lesions induced by 3.5 h exposure of rats to water immersion and restraint stress. Note that the lesions are round, bleeding erosions located predominantly in the oxyntic mucosa.

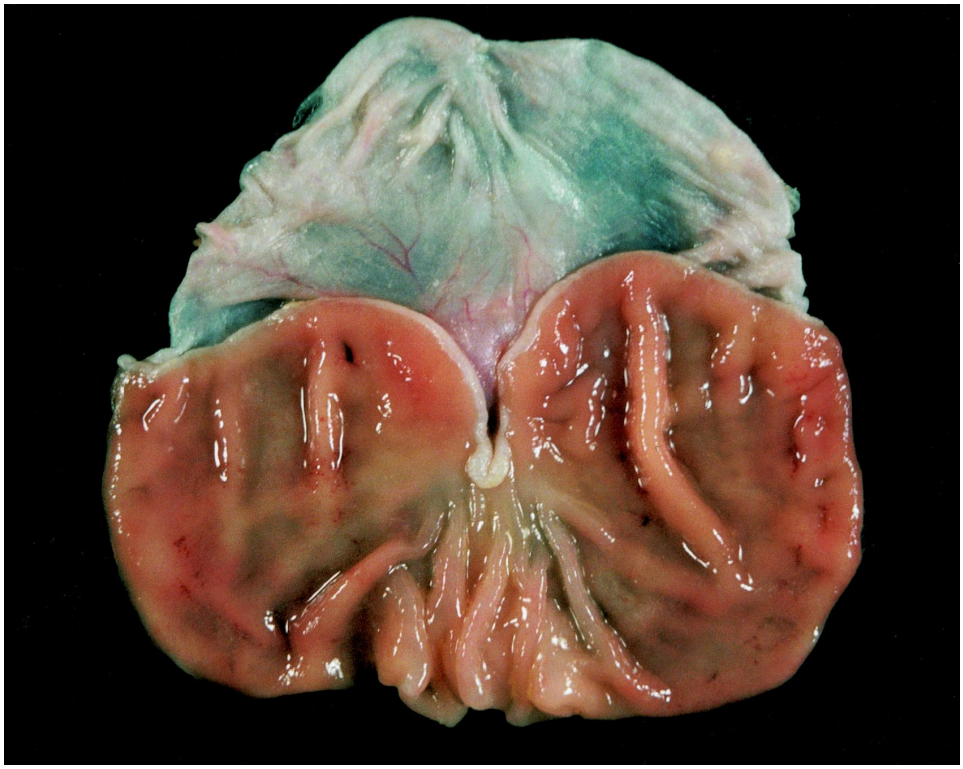

Fig. 2. Gross appearance of the stomach 24 h after the exposure to water immersion and restraint stress. Note the presence of only single healing erosions.

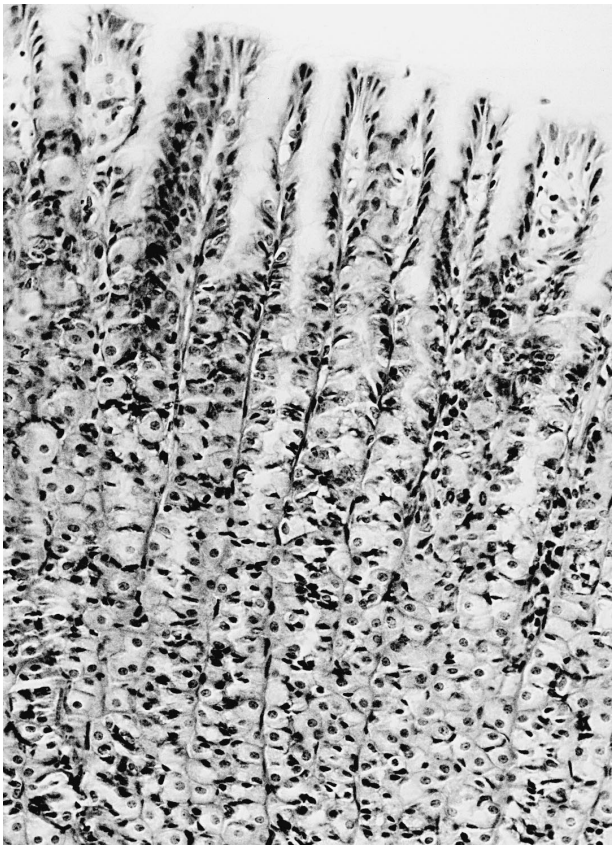

Fig. 3. Histological appearance of intact gastric mucosa. Hematoxylin and eosin stain, magnification ×260.

then photographed under UV transillumination. Oligonucleotides primer sequences are specific, as ascertained by computer-assisted search of the updated version of GeneBank. In addition to size analysis by agarose gel electrophoresis, specificity of the primer pair for prepro-EGF, TGFα and for cyclooxygenase-1 and cyclooxygenase-2 was assessed by sequencing of polymerase chain reaction products.

2.6. Statistical analysis

The results are expressed as means ± S.E.M. Statistical comparisons were made with the non-parametric Mann–Whitney *U*-test and the Kruskal–Wallis test for unpaired comparisons (two-tailed) was applied, where appropriate, with a P-value < 0.05 considered significant.

3. Results

3.1. Gastric lesions, gastric acid secretion, gastric blood flow and mucosal generation and luminal release of prostaglandin E_2 after exposure to stress

Table 1 shows the time-course of gastric lesions at 0, 2, 4, 6, 8, 12 and 24 h after stopping the 3.5 h stress with the accompanying changes in gastric blood flow and prostaglandin E_2 generation in gastric mucosa. In vehicle-control, non-stressed animals, no gastric lesions were observed in the intact oxyntic mucosa. The exposure to 3.5 h stress resulted in the appearance of multiple bleeding erosions in the oxyntic mucosa but without any evidence of gross damage in the forestomach (Fig. 1). The number of lesions was significantly reduced at 4 h and these lesions almost completely had disappeared at 24 h after the stress (Fig. 2).

Histological examination of the oxyntic mucosa after 3.5 h of stress revealed damage to the surface epithelium with many cells sloughed off into the gastric lumen and deep necrosis occupying about 40% of the mucosal strip length (Figs. 3 and 4). Histology showed healed stress erosions with depressed gastric mucosa covered with regenerated gastric surface epithelium and elongated neck and foveolar glandular areas (Fig. 5).

The healing of stress lesions was accompanied by a significant rise in gastric blood flow and mucosal generation of prostaglandin E_2 (Table 1). For 6, 8, 12 and 24 h after stress, the number of lesions was progressively reduced, by 50, 56, 72 and 89%, respectively, accompanied by a gradual rise in gastric blood flow and prostaglandin E_2 generation in the gastric mucosa.

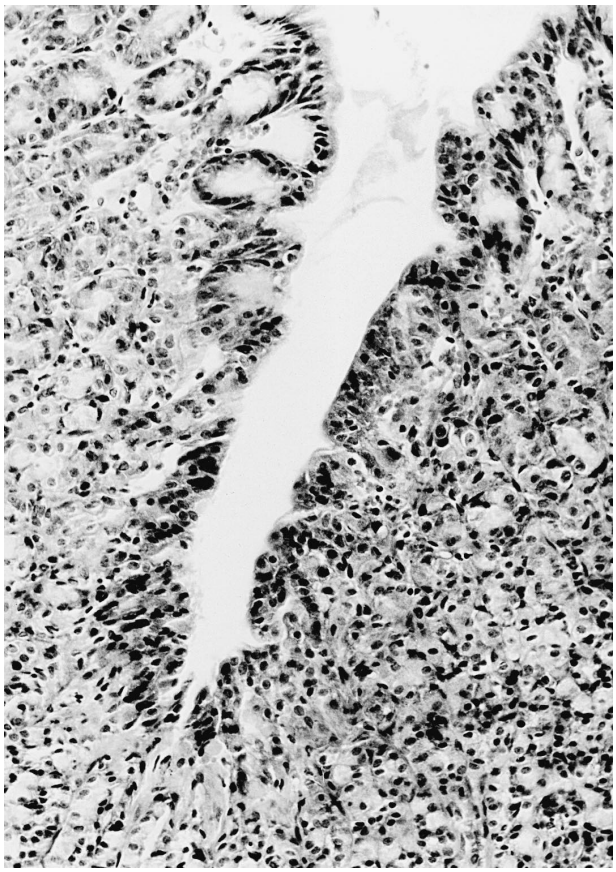

Fig. 4. Histological appearance of typical erosion in the oxyntic mucosa after 3.5 h of exposure to water immersion and restraint stress. Hematoxylin and eosin stain, magnification ×260.

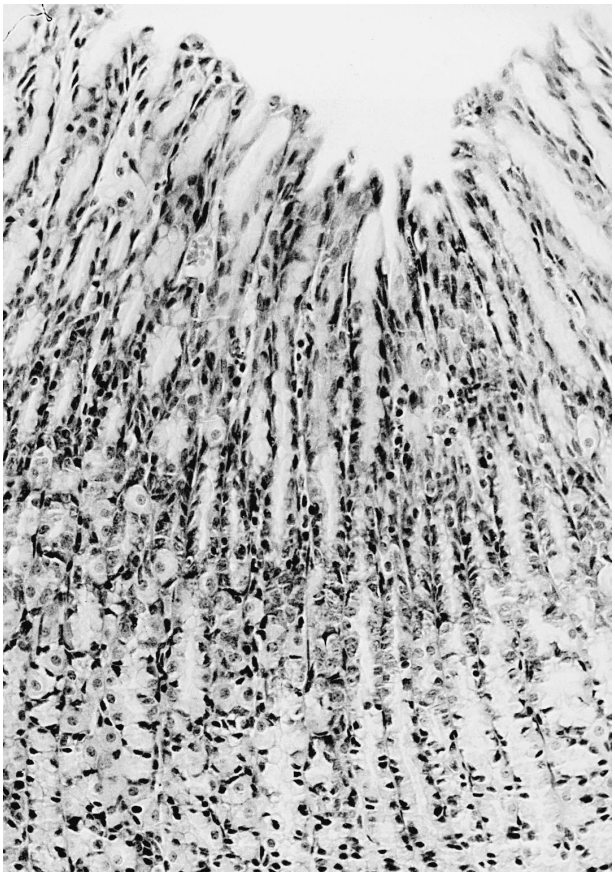

Fig. 5. Healed stress erosion. Depressed gastric mucosa is covered with regenerated gastric surface epithelium with elongated neck and faveolar glandular areas as well as enhanced proliferation in the regeneration zone of the glands. Hematoxylin and eosin method, magnification ×260.

Basal gastric acid outputs measured in separate groups of conscious animals with gastric fistulas reached 225 ± 28 μmol/h in intact non-stressed rats (Table 2). It was significantly higher (341 ± 18 μmol/h) after 3.5 h of stress but then gradually declined after the cessation of stress, to reach after 12 h, a value similar to that observed in the control non-stressed animals.

Gastric blood flow in the gastric mucosa of intact, non-stressed rats was 52 ± 8 ml/min 100 g tissue (taken as 100%) and was significantly decreased (by about 50%) when measured immediately after the 3.5 h stress (Table 1). Prostaglandin E_2 generation in vehicle-treated gastric mucosa averaged 224 ± 12 ng/g, and was also significantly reduced by (about 55%) on exposure to the 3.5 h stress (Table 1). At 12 and 24 h after the stress, both the gastric blood flow and prostaglandin E_2 generation were restored to values similar to those recorded in control animals not exposed to stress. The reduction in the number of stress lesions observed after the withdrawal from the stress was accompanied by a gradual increase in both prosta-glandin E_2 generation and gastric blood flow, reaching at 12 and 24 h after the stress, the values not significantly different from those recorded in non-stressed animals.

3.2. Effects of inhibitors of cyclooxygenase-1 and cyclooxygenase-2 on gastric mucosal lesions, gastric acid secretion, gastric blood flow and mucosal generation of prostaglandin E_2 after exposure to stress

Administration of indomethacin (5 mg/kg i.p.) or meloxicam (1 mg/kg i.p.), just before the start of stress, increased significantly the number of mucosal lesions, to reach a value of ~30 per rat immediately (time 0 h) after the stress (Table 1). The number of stress lesions gradually declined on cessation of the stress but at all time intervals tested the number was significantly higher than that in control rats not given indomethacin or meloxicam.

Gastric blood flow in tests with indomethacin and meloxicam was significantly lower at all time intervals after the stress than that recorded in vehicle-treated (control) animals after this stress. This blood flow in indomethacin- and meloxicam-treated animals, similarly to that in vehicle-treated rats, gradually increased with increasing time intervals after the termination of the stress (Table 1).

Gastric mucosal generation of prostaglandin E_2 was significantly reduced (by about 90%) during all post-stress periods after indomethacin but after meloxicam the reduc-

Table 2
Gastric acid output from the gastric fistula rats under basal conditions or after i.p. administration of indomethacin (5 mg/kg) or meloxicam (1 mg/kg) alone and after 3.5 h stress without and with pretreatment with indomethacin or meloxicam

	Acid output (μmol/h)
Basal secretion in intact rats (without stress)	225 ± 28
Indomethacin (5 mg/kg i.p.)	238 ± 16
Meloxicam (mg/kg i.p.)	226 ± 12
After 3.5 h stress-time	
0 h	341 ± 18[a]
4 h	287 ± 18[a]
8 h	263 ± 13[a]
12 h	228 ± 11
24 h	218 ± 9
After indomethacin (5 mg/kg i.p.) plus 3.5 h stress-time	
0 h	402 ± 31[b]
4 h	363 ± 25[b]
8 h	260 ± 19
12 h	218 ± 17
24 h	210 ± 12
After meloxicam (1 mg/kg i.p.) plus 3.5 h stress-time	
0 h	398 ± 28[b]
4 h	360 ± 19[b]
8 h	289 ± 15
12 h	254 ± 12
24 h	225 ± 9

Mean ± S.E.M. for 10 rats.
[a] Indicates significant ($P < 0.05$) change as compared to the value obtained for intact rats without stress.
[b] Indicates significant ($P < 0.05$) change as compared to the value obtained after exposure to stress at time 0 and 4 h.

Table 3
EGF and TGFα immunoreactivity in gastric juice and in gastric mucosa in rats immediately after 3.5 h of water immersion and restrain stress (0 h) and rats

	EGF	
	gastric juice (ng/ml)	gastric mucosa (ng/g)
Intact	5.3±0.7	0.19±0.08
After stress (time)		
0	36.6±3.2[a]	0.36±0.06[a]
2	42.3±3.1[a]	0.39±0.05[a]
4	48.1±3.8[a]	0.35±0.08[a]
6	55.3±5.1[a]	0.45±0.01[a]
8	62.2±5.1[a]	0.49±0.09[a]
12	63.8±6.8[a]	0.41±0.06[a]
24	70.1±5.2[a]	0.37±0.11[a]

[a]Indicates a significant change ($P < 0.05$) as compared to the value recorded in intact non-stressed rats.

tion was less pronounced and was observed later after the cessation of the stress.

Gastric acid outputs from the gastric fistula rats stressed for 3.5 h and treated with indomethacin or meloxicam were significantly higher immediately after withdrawal from the stress but after 8 h they decreased to the values observed after stress in rats without indomethacin or meloxicam administration (Table 2). Indomethacin or meloxicam given alone to the intact rats (without stress) did not affect significantly the basal acid output in these animals.

3.3. EGF and TGFα concentrations in gastric mucosa and gastric content

Tables 3 and 4 show the EGF and TGFα concentrations in gastric contents and in the gastric mucosa of rats with or without exposure to stress. EGF was present in relatively large concentrations in gastric juice but was found in only minute amounts in the gastric mucosa of vehicle-treated non-stressed rats. In rats exposed to stress, EGF was increased several-fold in the gastric lumen compared with the value measured in intact non-stressed animals. This increase remained significantly higher at all time intervals following the stress. The EGF content in gastric mucosa was also significantly increased after exposure to stress compared to that in non-stressed rats and this increase was observed at all periods after the stress. The contents of TGFα both in the gastric juice and gastric mucosa were several-fold higher than those of EGF in these animals. Following exposure to the stress, the contents of TGFα both in the gastric lumen and in gastric mucosa were significantly higher at all time intervals when compared to the values recorded in the intact rats (Table 4).

Table 4
TGFα immunoreactivity in gastric juice and in gastric mucosa in rats immediately after 3.5 h of water immersion and restrain stress (0 h) and at 2, 4 rats

	TGFα	
	gastric juice (ng/ml)	gastric mucosa (ng/g)
Intact	24.4±2.3	5.4±1.3
After stress (time)		
0 h	36.6±1.2[a]	9.4±2.1[a]
2 h	41.1±5.3[a]	12.2±3.2[a]
4 h	51.3±6.3[a]	10.1±2.6[a]
6 h	68.3±4.7[a]	9.5±1.8[a]
8 h	56.5±6.8[a]	11.3±2.4[a]
12 h	43.1±2.8[a]	7.2±1.8[a]
24 h	39.2±3.6[a]	9.2±1.6[a]

[a]Indicates a significant ($P < 0.05$) change as compared to the value recorded in intact non-steroid rats.

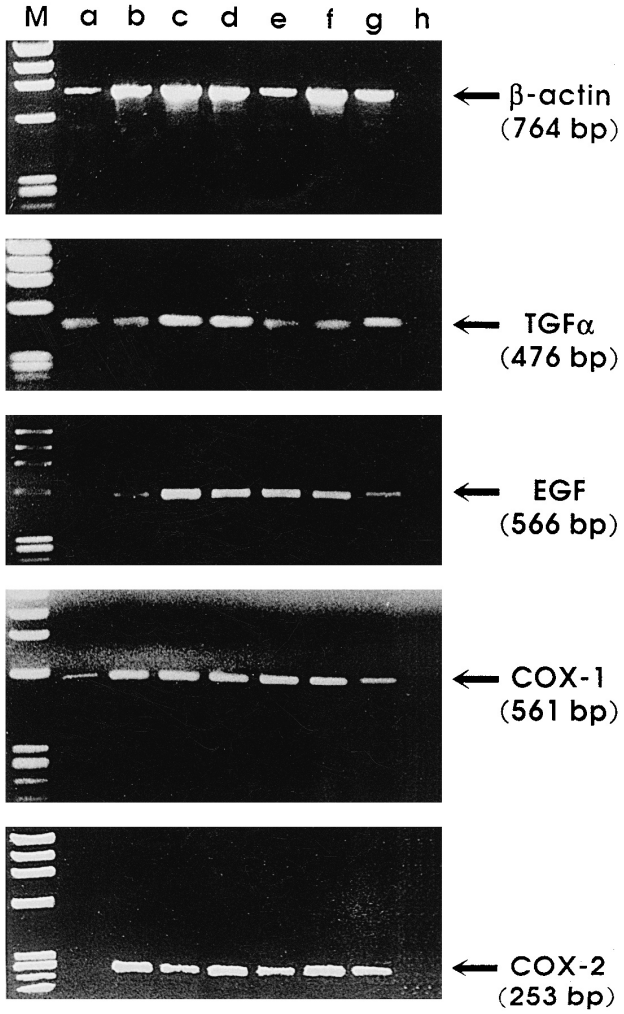

Fig. 6. Messenger RNA expression for rat β-actin, TGFα, EGF, cyclooxygenase-1 and cyclooxygenase-2 with reverse transcriptase polymerase chain reaction in intact gastric mucosa (lane a) and at 0, 2, 4, 6, 8 and 12 h after exposure to stress (lanes b–g). Lane h: negative control (water). M: size marker ϕX 174 *Hae*III digest. Arrow-expected polymerase chain reaction product (bp). Note that the gene expression of TGFα and cyclooxygenase-1 was detected in intact gastric mucosa, while the signal for mRNA EGF and cyclooxygenase-2 was not detected in the intact gastric mucosa.

3.4. EGF, TGFα and cyclooxygenase-1 and cyclooxygenase-2 mRNA expression during recovery from stress.

The β-actin mRNA was expressed in the mucosa of intact, non-stressed rats and in those exposed to stress, at all time periods tested (from 0 to 12 h) after withdrawal from stress (Fig. 6).

The expression of TGFα mRNA was detected using reverse transcriptase polymerase chain reaction in intact gastric mucosa and at all time intervals tested (from 0 to 12 h) after the stress (Fig. 6). In contrast, the expression of EGF mRNA was undetectable in the intact gastric mucosa but appeared at 0, 2, 4, 6, 8 and 12 h after stress (Fig. 6). The expression of cyclooxygenase-1 mRNA was detected in intact mucosa and at all time periods tested (from 0 to 12 h) after the stress (Fig. 6). Cyclooxygenase-2 mRNA was not expressed in the intact mucosa but appeared during recovery at all time periods tested (from 0 to 12 h) after the stress (Fig. 6).

4. Discussion

The present results confirmed and extend previous findings that the exposure to stress produces gastric mucosal damage and that following the termination of the stress, a progressive decrease in the number of mucosal lesions is observed (Victor et al., 1989; Brzozowski et al., 1993; Konturek et al., 1996). Recovery in the gastric mucosa following withdrawal from the stress, was accompanied by a rise in gastric blood flow and an increase in luminal and mucosal concentrations of EGF and TGFα. Exposure to stress reduced by ~50% the mucosal generation of prostaglandin E_2 but generation of this prostaglandin was restored time-dependently during the recovery from the stress. It is of interest that the expression of cyclooxygenase-1, as identified by reverse transcriptase polymerase chain reaction, was detected in the mucosa of intact animals as well as after the stress. In contrast, cyclooxygenase-2 was not expressed in the intact mucosa but was expressed immediately after the exposure to stress when multiple gastric ulcerations occurred.

The mechanism for enhancement of cyclooxygenase-2 mRNA expression by the stress is not explained but the concomitant expression of EGF-mRNA and TGFα-mRNA in the gastric mucosa of the same animals exposed to stress suggest that these growth factors might contribute to activation of the genes encoding cyclooxygenase-2. It is also likely that, under stress conditions, the mucosal expression of cyclooxygenase-2 can be rapidly induced by proinflammatory mediators including cytokines and proteases (O'Banion et al., 1991; Xie et al., 1991; Feng et al., 1995). Also these mediators may contribute to the formation of mucosal lesions and the fall in gastric blood flow. We thought, therefore, that a specific cyclooxygenase-2 inhibitor, such as meloxicam could be useful to accelerate healing of stress-induced gastric lesions. However, the inhibition of cyclooxygenase-2, gave results opposite to those expected, because pretreatment with meloxicam increased the number of stress-induced lesions and caused a marked delay in healing of these lesions, suggesting that the predominant factors generated by cyclooxygenase-2 are cytoprotective prostaglandins. These substances biosynthesized by cyclooxygenase-1 and cyclooxygenase-2 in the gastric mucosa appear to limit the extent of mucosal damage by stress and the suppression of their release by indomethacin or meloxicam greatly augmented the stress-induced ulcerogenesis and prolonged the healing rate of these ulcers. Thus, meloxicam, which is a rather weak inhibitor of prostaglandin formation and was expected to provide a more favorable risk:benefit ratio in the stomach (Engelhardt et al., 1996) was now found to augment rather than to reduce the stress-induced lesions and to delay ulcer healing in the gastric mucosa. Further studies with more potent and specific inhibitors of cyclooxygenase-2 are needed to assess whether the products of this enzymes has a protective or a noxious influence on the gastric mucosa.

It is of interest that the growth factors, especially EGF, whose mRNA was not expressed in the intact mucosa (but which was found in gastric lumen possibly originating from salivary secretion), was expressed in the mucosa of stressed animals. TGFα, that is normally present in the gastric mucosa, was also increased by the stress. As both EGF and TGFα are effective to protect gastric mucosa from the stress-induced lesions (Konturek et al., 1992b), it is reasonable to assume that these factors limit the extent of mucosal damage caused by stress and probably contribute to the early recovery of the mucosa from the stress-induced lesions. Thus, the upregulated expression of EGF and TGFα is likely to be involved in the recovery of gastric mucosa from stress damage, because both EGF and TGFα were shown to enhance mucosal cell migration, cell proliferation and DNA synthesis as well as mucus secretion and gastric blood flow (Konturek, 1990; Konturek et al., 1991, 1992b; Hui et al., 1993).

The results of our present study emphasize the importance of endogenous prostaglandins in mucosal repair after stress by showing a progressive rise in mucosal generation of prostaglandin E_2 during the first 24 h of recovery from the stress damage. Exposure to stress produced an initially significant decline in the prostaglandin E_2 generation but the fall in prostaglandin generation was reversed during the recovery from this stress. To determine if the prostaglandin production correlates with increased cyclooxygenase-1 or cyclooxygenase-2 expression, we used the reverse transcriptase polymerase chain reaction technique with specific primers to detect the signals for increased gene expression of both these isoforms. The expression of β-actin mRNA was used to verify the integrity of mRNA. Indeed, it was found that the increase in generation of prostaglandin E_2 after stress was associated with the upregulated expression of genes for cyclooxygenase-1 at

each time intervals after stress exposure. The expression of cyclooxygenase-2 mRNA in this study was not detected in the intact mucosa but showed a strong signal immediately after the stress and at 2, 4, 6, 8 and 12 h after the exposure to stress. Thus, it is reasonable to assume that recovery from the stress results from the upregulated expression of both cyclooxygenase-1 and cyclooxygenase-2 transcripts, which could explain the gradual rise in the rate of prostaglandin generation observed during mucosal repair after stress.

The inhibition of activity of cyclooxygenase-1 by indomethacin or of cyclooxygenase-2 by meloxicam in this study made the mucosa more vulnerable to stress damage. This exacerbated response to stress when protective prostaglandins were eliminated by these agents probably originates from the reduced mucosal blood flow and impaired synthesis of the elements of gastric mucous barrier. Thus, prostaglandins, like growth factors, seem to play an important role in the repair of the gastric mucosa after acute damage caused by stress, through their antisecretory, mucoprotective and hyperemic activities (Konturek, 1990; Brzozowski et al., 1993; Podolsky, 1994).

We found that the exposure to stress produced a marked fall in gastric blood flow but that following recovery from the stress, the gastric microcirculation was gradually restored, reaching at 12 h after stress values similar to those observed in the control rats. EGF and TGFα could be the mediators of this rise in gastric microcirculation, which would be consistent with the vasoactive influence of growth factors recently implicated in the mechanism preserving mucosal integrity (Konturek et al., 1992b; Hui et al., 1993). Our previous studies revealed that both EGF and TGFα attenuated the reduction in mucosal blood flow caused by ethanol or stress and that indomethacin abolished these effects. This indicates that mucosal prostaglandins are necessary for the maintenance of gastric blood flow by growth factors (Konturek et al., 1992a). Since prostaglandins, which are known to protect the gastroduodenal mucosa from damage caused by various ulcerogens, including stress (Konturek, 1990; Konturek et al., 1992a), are important vasodilators, an alternate hypothesis might be that the rise in gastric blood flow observed during the healing of stress-induced damage results from an enhanced generation of prostaglandins as a result of increased cyclooxygenase-1 and cyclooxygenase-2 activity observed in this study. This does not exclude the growth factors from the mediation of the recovery of the mucosa from the stress lesions because these factors have been shown to stimulate both prostaglandin-cyclo-oxygenase activity and the mucosal generation of prostaglandins (Mori et al., 1987; Nakano et al., 1995). Thus, it is possible that these growth factors are responsible for the induction of cyclooxygenase-2 in the mucosa under the stress conditions. This possibility is supported by our finding that the strong signal of expression of cyclooxygenase-2 mRNA appeared during recovery from stress damage when the mucosal contents of both EGF and TGFα were greatly increased. Furthermore, the involvement of the growth factors in the activation of cyclooxygenase-2 during recovery after the stress is supported by the fact that upregulated expression of mRNA for these factors was detected during healing of stress lesions, concomitantly with the expression of mRNA for cyclooxygenase-1 and cyclooxygenase-2 in the mucosa.

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