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• BASIC RESEARCH •

Expression and activities of three inducible enzymes in the healing of gastric ulcers in rats

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Abstract

AIM: To explore the roles of nitric oxide synthase (NOS), heme oxygenase (HO) and cyclooxygenase (COX) in gastric ulceration and to investigate the relationships of the expression and activities of these enzymes at different stages of gastric ulceration.

METHODS: Gastric ulcers (kissing ulcers) were induced by luminal application of acetic acid. Gastric tissue samples were obtained from the ulcer base, ulcer margin, and non-ulcerated area around the ulcer margin at different time intervals after ulcer induction. The mRNA expression and protein levels of inducible and constitutive isoforms of NOS, HO and COX were analyzed with RT-PCR and Western blotting methods. The activities of the total NOS, inducible NOS (iNOS), HO, and COX were also determined.

RESULTS: Differential expression of inducible iNOS, HO-1 and COX-2 and enzyme activities of NOS, HO and COX were found in the gastric ulcer base. High iNOS expression and activity were observed on day 1 to day 3 in severely inflamed ulcer tissues. Maximum expressions of HO-1 and COX-2 and enzyme activities of HO and COX lagged behind that of iNOS, and remained at high levels during the healing phase.

CONCLUSION: The expression and activities of inducible NOS, HO-1 and COX-2 are found to be correlated to different stages of gastric ulceration. Inducible NOS may contribute to ulcer formation while HO-1 and COX-2 may promote ulcer healing.

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INTRODUCTION

Nitric oxide synthase (NOS), heme oxygenase (HO) and cyclooxygenase (COX) are three important enzymes with constitutive and inducible isoforms. NOS catabolizes L-arginine to L-citrulline and nitric oxide (NO)[1,2], COX converts arachidonic acid to bioactive prostanoids[3,4], while HO metabolizes heme to biliverdin, carbon monoxide, and iron[5,6]. All these products play important roles in physiological and pathological conditions. The constitutive forms, namely eNOS, HO-2 and COX-1, are normally expressed in cells and tissues. Their expressions and activities are unaffected or only marginally modified during the process of inflammation. On the contrary, the inducible isoforms, namely iNOS, HO-1 and COX-2, are highly inducible in acute and chronic inflammation[7-9]. These induced enzymes may directly mediate the inflammatory reaction or contribute to the resolution of inflammation. Although the inducible property of these enzymes in inflammation has been proven and widely studied, their expression and activities at different stages of gastric ulceration have not been well defined. In this study, the temporal changes in the expressions and activities of these enzymes in rat stomaches during inflammation and ulcer healing were examined.

MATERIALS AND METHODS

Animals

The protocol of the study was approved by the Committee on the Use of Live Animals for Teaching and Research of University of Hong Kong. Male SD rats (weighing between 150-170 g) were fed on a standard laboratory diet (Ralston Purina Co., Chicago, IL) and kept inside a room with well-regulated temperature (22±1 ℃), humidity (65-70 %), and day/night cycle (12 h/12 h). The rats were starved for 24 h and water withdrawn 1 hour before the operation of the induction of gastric ulcer.

Preparation of gastric kissing ulcers

Gastric kissing ulcers were induced by luminal application of an acetic acid solution as previously described[10,11]. Briefly, the abdomen was opened under ether anesthesia, and the stomach was exposed. The anterior and posterior walls of the stomach were clamped together by a clip with metal rings of 11 mm internal diameter attached to both ends. Acetic acid solution (60 %, v/v) of 0.12 mL was injected with a syringe through the forestomach into the gastric lumen between the two rings. The acid solution was withdrawn 45 s later into the same syringe, and the operating site was disinfected with 70 % ethanol. Thereafter the animals were allowed to feed on standard diet and tap water *ad libitum* until collection of gastric tissue samples.

Sample collection

The rats were killed by ether anesthetization at 2 h, 6 h, 12 h, 1 d, 2 d, 3 d, 5 d, 8 d, and 15 d after ulcer induction and their stomachs were excised. The stomach was opened along the greater curvature and rinsed with cold normal saline, then blotted dry. The ulcer area (mm^2) was traced onto a transparency and then copied to a grid paper with 1 mm^2 square. The ulcer area was determined by counting the numbers of square it covered. Gastric tissues from the ulcer base, ulcer margin (1-2 mm adjacent to the ulcer base) and intact tissues around the ulcer margin were obtained and immediately frozen

in liquid nitrogen before storage at -70 ℃ until used for reverse transcription polymerase chain reaction (RT-PCR), Western blot and enzyme activities analysis. Gastric samples obtained from each time point were also fixed in 10 % buffered formalin for histological examinations. Gastric tissues from the rats without kissing ulcers were used as the control.

Histology

Paraffin embedded sections were prepared and hematoxylin and erosin staining (H&E) was used for morphological examinations of histological changes during tissue inflammation and ulcer healing.

RT-PCR analysis of mRNA expression

Total RNA was isolated from gastric tissues using Trizol reagent (Gibico BRL, Gathersburg, MD, USA). First strand complementary DNA (cDNA) was synthesized from 5 μg RNA by using oligo $(dT)_{20}$ primer with the thermoscript RT-PCR system (Gibico BRL). PCR cycles were performed for amplification of iNOS, eNOS, COX-1, COX-2, HO-1, HO-2 and β-actin cDNA using a PCR thermal cycler (Gene Amp PCR System 9700; Perkin-Elmer Corp., Norwalk, CT, USA) and oligonucleotides (Gibico BRL) of sequences are listed in Table 1. The number of PCR cycles was adjusted carefully to avoid saturation of the amplification system. PCR products were visualized by UV illumination (Bio-Rad, Hercules, CA, USA) after electrophoresis through 1 % agarose gel containing 0.5 μg/mL ethidium bromide. The gel photographs were scanned with a computerized densitometer (Multi-Analyst, Bio-Rad, Hercules, CA).

Table 1 Primer sequences of iNOS, eNOS, COX-1, COX-2, HO-1, HO-2 and β-actin

Primer	Primer sequences	Amplicon-length (bp)
HO-1	Sense: 5'-CAGTCGCCTCCAGAGTTTCC-3'	
	Antisense: 5'-TACAAGGAGGCCATCACCAGC-3'	284
HO-2	Sense: 5'-AGAAGTATGTGGATCGGA-3'	
	Antisense: 5'-TACTCAGGTCCAAGGCA-3'	242
COX-1	Sense: 5'-TGCTGCTGAGAAGGGAGTTCATTC-3'	
	Antisense: 5'-CAAGTCACACACACGGTTATGCTC-3'	403
COX-2	Sense: 5'-ACACTCTATCACTGGCATCC-3'	
	Antisense: 5'-GAAGGGACACCCTTTCACAT-3'	584
iNOS	Sense: 5' -TGGCTTGCCCTTGGAAGTTTCTC-3'	
	Antisense: 5'-TCCAGGCCATCTTGGTGGCAAGA-3'	574
eNOS	Sense: 5'-TACGGAGCAGCAAATCCAC-3'	
	Antisense: 5'-CAGGCTGCAGTCCTTTGATC-3'	812
β-actin	Sense:5'-GTGGGGCGCCCCAGGCACCA-3'	
	Antisense: 5'-CTCCTTAATGTCACGCACGATTTC-3'	540

Western blot analysis of NOS, HO and COX proteins

Gastric tissues for the analysis of protein expressions of iNOS, eNOS, HO-1, HO-2, COX-1 and COX-2 were homogenized in a proteinase inhibitor buffer (50 mmol/L Tris HCl, pH 7.5, 150 mmol/L NaCl, 0.5 % α-cholate sodium, 0.1 % SDS, 2 mmol/L EDTA, 1 % Triton X-100, 10 % glycerol, 1 mmol/L PMSF and aprotinin) and then centrifuged at 10 000 rpm for 15 min at 4 ℃. The supernatant was collected and the protein content was determined with dye-binding (Bio-Rad) method. 30 μg of total protein was loaded onto SDS-polyacrylamide gel and blotted onto hybrid C membranes (Amersham Life Science, Little Chalfont, Buckinghamshire, England) by electrophoresis. Pre-stained rainbrow recombinant protein molecular weight markers (Amersham International plc, Little Chalfont, Buckinghamshire, England) were used for molecular weight determinations. Membranes were blocked with blocking buffer containing 5 % fat free milk powder, 10 mmol/L Tris-HCl (pH 7.5), 100 mmol/L NaCl and 0.1 % Tween 20 for 1 h at room temperature. The blots were incubated overnight at 4 ℃ with 1:500 dilution of polycolonal antibodies against HO-1 and HO-2 (Stress-Gen, Victoria, Canada), monoclonal antibodies against iNOS and eNOS (Transduction Lab, Lexington, Kentucky, USA), polyclonal antibodies against COX-1 and COX-2 (Santa Cruz Biotechnology INC, Santa Cruz, California, USA). After washed in washing buffer for 30 min, the membranes were treated with HRP conjugated secondary antibody (1:5 000) (Bio-Rad) for 1 h at room temperature followed by another 30 min of washing. The ECL Western blotting system (Amersham Life Sciences) was used in accordance to the manufacturer's instructions for chemiluminescence of proteins, and the blots were then exposed to photographic films (Fuji Photo Film Co., Tokyo, Japan).

Determination of NOS activity

NOS activity in the gastric tissue was measured as the ability of tissue homogenates to convert L-[^{3}H]-arginine to L-[^{3}H]-citrulline[12]. Gastric samples were homogenized at 4 ℃ in a buffer containing 10 mmol/L HEPES (pH 7.2), 320 mmol/L surcose, 0.1 mmol/L EDTA, 1 mmol/L dithiothreitol, 10 μg/mL leupeptin, 2 μg/mL aprotinin (Sigma, St. Louis, MO, USA) and 1 mmol/L PMSF (Sigma), then centrifuged at 12 000 rpm for 30 min at 4 ℃. The supernatant was collected and the protein contents were measured. 100 μL of supernatant was then mixed with a buffered solution consisting of 0.7 mmol/L NADPH, 150 μmol/L $CaCl_2$, 7 mmol/L L-valine, 10 mmol/L HEPES (pH 7.2) and 1μ Ci [^{3}H]-L-arginine (Gibico BRL) and incubated at 37 ℃ for 30 min to determine the total NOS activity. For determination of the iNOS activity, 1 mmol/L EGTA was used to inhibit the activity of calcium-dependent constitutive eNOS. The reaction was stopped by adding 50 μL 20 % perchloric acid, 160 mL 1 mmol/L NaOH and 540 mL dilution solution containing 1 mmol/L each of L-arginine and DL-citrulline. The newly formed L-[^{3}H]-citrulline was separated from L-[^{3}H]-arginine by passing the reaction mixture over 1 mL AG50W-X8 resin columns (Bio-Rad), and the eluted labelled material was measured using a Beckman scintillation counter (LS-6500, Beckman Instrument, USA). The final result was expressed as pmol of L-[^{3}H]-citrulline formed per milligram of protein per 30 min.

Determination of HO activity

Heme oxygenase activity was measured as the ability of tissue homogenates to metabolize heme to bilirubin[13]. In brief, gastric tissues were homogenized in 0.1 mol/L potassium phosphate-buffered saline (pH 7.4) and centrifuged at 12 000 rpm for 30 min. Then 500 μL supernatant (about 4 mg total protein) was added to an equal volume of reaction mixture (2 mmol/L $MgCl_2$, 30 μmol/L hemin, 30 mg rat liver cytosol, 0.2 U glucose-6-phosphate dehydrogenase, 2 mmol/L glucose-6-phosphate, and 0.8 mmol/L NADPH), and incubated at 37 ℃ in the dark for 1 h. The formed bilirubin was extracted with benzene and the absorbance of bilirubin at 462 nm was measured against a baseline absorbance at 530 nm. Heme oxygenase activity was expressed as μg of bilirubin formed/mg protein per hour. The protein content in the supernatant was determined by dye-binding method with BSA as a standard.

Assessment of COX activity

Cyclooxygenase activity was measured as the ability of tissue homogenates to metabolize arachidonic acid to PGE_2 according to the method described by Tomlinson and Vane[7, 8]. Gastric tissues were homogenized at 4 ℃ in proteinase inhibitory

buffer containing 50 mmol/L Tris-Cl (pH 7.4), 3.15 % trisodium citrate, 1 mmol/L PMSF, 0.2 mmol/L leupeptin. The protein concentration in the homogenates was measured. Homogenates were incubated at 37 ℃ for 30 min in the presence of excess arachidonic acid (30 mmol/L). The samples were then boiled and centrifuged at 12 000 rpm for 30 min. The concentration of PGE_2 in the supernatant was measured by immunoassay, using R&D PGE_2 kits (R&D Systems, Inc. Minneapolis, MN, USA). Results were expressed as ng PGE_2 produced per mg protein in 30 min.

Statistical analysis

All the data were expressed as mean ± S.E.M. Statistical analysis was performed using Student' s *t*-test. Values of $P<0.05$ were considered statistically significant.

RESULTS

Morphology and histology

Two symmetrical ulcers were induced in the anterior and posterior walls of the stomach by acetic acid injection. The ulcer base was denuded of mucosal layers because of the necrotic changes and it reepithelized gradually during ulcer healing. Histological accumulation of neutrophils was found at 6 h and prominent inflammatory infiltration was observed at the ulcer base on day 1 to day 3 after ulcer induction. This period was defined as the inflammatory stage of gastric ulceration. After 3 days, the ulcer healed rapidly and was characterized by a reduction in ulcer area (Figure 1). There was intensive proliferation of epithelial cells at the ulcer margin, accompanied by the development of granulation tissues with angiogenesis. The period from day 3 onwards was considered to be the healing phase of gastric ulceration. Complete reepithelization of the ulcer craters was found in some stomaches on day 15 after ulcer induction.

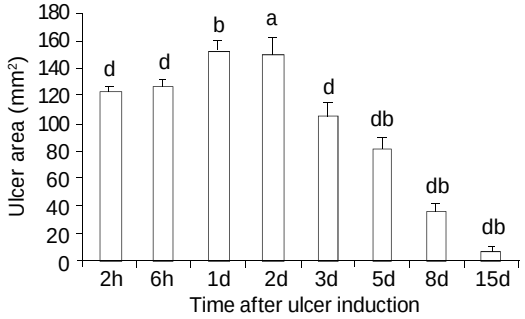

Figure 1 Sequential change of ulcer areas at different time points after ulcer induction. Significant decrease of ulcer area was found on day 3 after ulcer induction. The data were represented as mean ± S. E. M of 20 rats in each group, $^aP<0.05$ *vs* the 2 h group; $^bP<0.001$ *vs* the 2 h group; $^dP<0.001$ *vs* the day 1 group.

Expression of HO, NOS and COX mRNA

Basal levels of COX-2 and HO-1 mRNA expression were detected in normal and non-ulcerated gastric tissues around the ulcers, while expression of iNOS mRNA could only be detected in the ulcer tissue 6 h after ulcer induction. Dramatic increase in mRNA expression of HO-1, iNOS and COX-2 was found from 6 h onwards. High level of iNOS expression persisted for only 3 days then declined rapidly over the healing phase. The expression of HO-1 and COX-2 mRNA remained at high levels during the healing stage from day 3 to day 8. Unlike their inducible isoforms, mRNA expression of HO-2, eNOS and COX-1 was relatively stable. The HO-2 mRNA appeared to be slightly increased in the ulcer base at 12 h after ulcer induction (Figure 2).

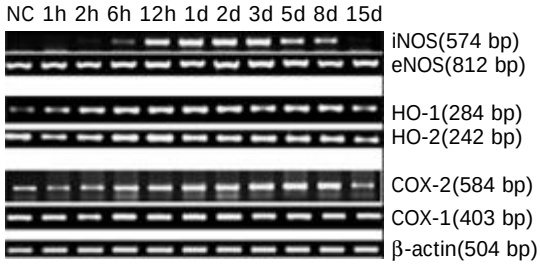

Figure 2 Time course of mRNA expression of inducible and constitutive isoforms of NOS, HO and COX in the ulcer bases. NC=normal control group.

Western blot analysis of HO, NOS and COX

A basal level of HO-1 protein was found in normal gastric tissues, while the iNOS and COX-2 proteins were undetectable. Acetic acid-induced ulceration resulted in a transient loss of HO-1 protein at 2 h, but re-appeared at 6 h and then increased persistently until its peak expression on day 3 to day 5. Its level decreased but still remained higher than normal during day 5 to day 15, at which the ulcer decreased in size and reepithelized.

Expression of COX-2 protein was found to be induced 6 h after ulcer induction and peaked on day 5. Afterwards it remained at a high level during the healing stage. Unlike HO-1 and COX-2, high level of iNOS protein was only detected at the inflammatory stage from day 1 to day 3. The protein expressions of HO-2, eNOS and COX-1 were relatively stable. Transient losses of eNOS and COX-1 proteins were observed at 2 h after acetic acid injection, and then gradually returned to normal levels during the healing process. The level of HO-2 protein was slightly increased after ulcer induction and peaked on day 1, then returned to normal level during ulcer healing (Figure 3).

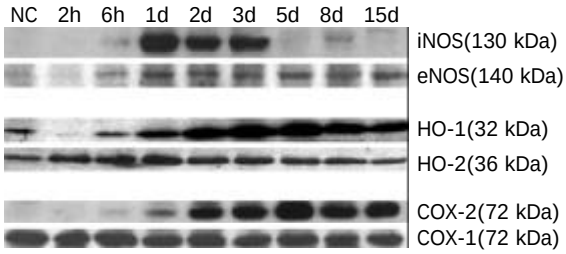

Figure 3 Western blot analysis of the protein levels of inducible and constitutive isoforms of NOS, HO and COX in the ulcer bases at different time after ulcer induction. NC= normal control group.

Expressions of iNOS and COX-2 in the ulcer margin were lower than those in ulcer base during the inflammatory phase. HO-1 expression in the ulcer margin was higher than that in non-ulcerated tissue but lower than that in the ulcer base both in the inflammatory and healing stages.

Enzyme activities of HO, NOS and COX

Marked increase of HO, NOS and COX activities at the ulcer base was found 6 h after ulcer induction. High level of iNOS activity was detected in ulcer base on day 1 to day 3, which was in consistent with the Western blot findings of iNOS protein expression. Similar trend was found in the margins but to a lesser extent (Figure 4). The activity of HO increased in 6 h and peaked on day 2, then remained at an elevated level on day 3 to day 8 (Figure 5). COX activity was persistently increased during the healing stage and was markedly elevated on day 15. Similar trend was found in the margin tissues but to a lesser extent (Figure 6). The trends for HO, NOS and COX activities in the ulcer margin were similar to those found in the ulcer base but with a lower value.

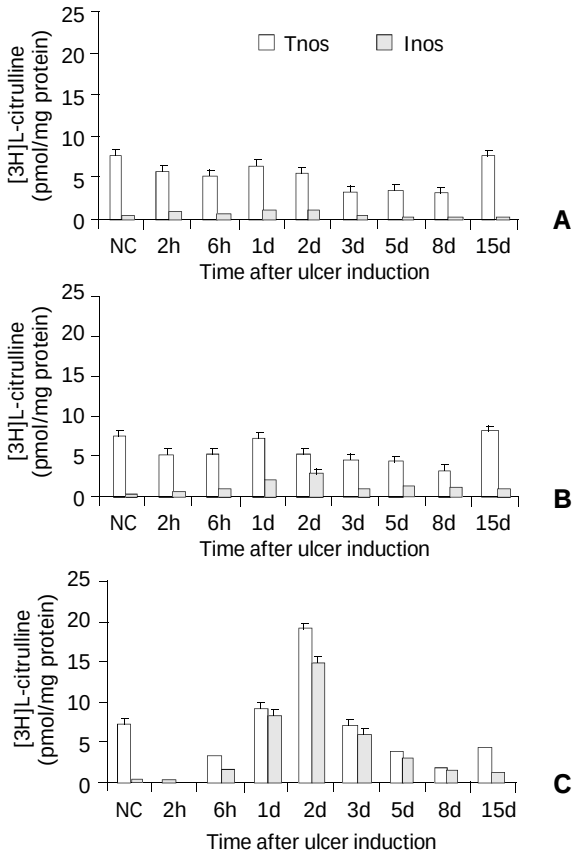

Figure 4 Total NOS (tnos) and iNOS (inos) activities at the non-ulcerated tissues (A), margins (B), and ulcer base (C) of acetic acid induced gastric ulcer. Each bar represents the mean ± S.E.M of 8 rats in each group. NC=normal control group.

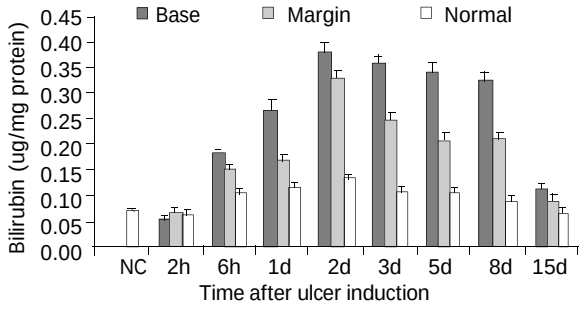

Figure 5 HO activities in the gastric tissues of normal control rats (NC) and the non-ulcerated tissues, ulcer margins and ulcer bases of acetic acid induced gastric ulcer. Each bar represents the mean ± S. E. M of 8 rats in each group.

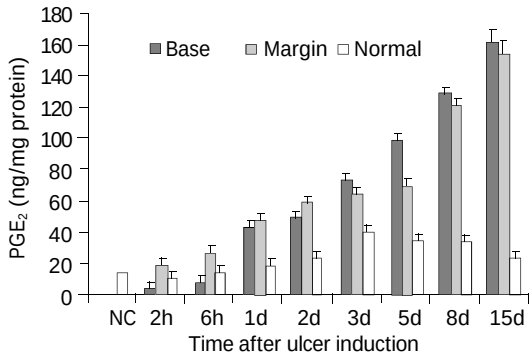

Figure 6 COX activities in the gastric tissues of normal control rats (NC) and the non-ulcerated tissues, ulcer margins and ulcer bases of acetic acid induced gastric ulcer. Each bar represents the mean ± S.E.M of 8 rats in each group.

DISCUSSION

In this study, the expression and activity patterns of NOS, HO and COX were investigated in rats with acetic acid-induced kissing ulcers. The results showed that the inducible isoforms of HO-1, iNOS and COX-2 were all up-regulated during the inflammatory phase. High expression and activity of iNOS were found to coincide with severe inflammation in the ulcer tissue, suggesting that it could be contributed by inflammatory cells that were involved in the inflammatory process. Maximum expressions and activities of HO-1 and COX-2 were found during the ulcer healing phase and thus they might play a role in ulcer healing.

It has been found that NO released from endothelial cells and neuronal cells by the constitutive isoform of NO synthase has regulatory roles in blood flow, motility and secretion. It also protects the gastrointestinal tract against injurious substances. NO produced from inflammatory cells by the inducible isoform of NOS has antimicrobial, antitumor and cytotoxic effects, but excessive amount may lead to peroxynitrite formation, protein tyrosine nitration, hydroxyl radical production and tissue damage[14, 15]. The present study also demonstrated that overexpression of iNOS on day 1 and day 2 after ulcer induction was accompanied by enlargement of ulcer crater. The expression of iNOS protein and activity were observed to be declined when the ulcer began to heal. NO generated from iNOS may also play a beneficial role during ulcer healing by inducing apoptosis in inflammatory cells[16].

The roles of HO-1 and COX-2 in gastric ulcer healing have not been clearly defined. HO-1 has been shown to possess cyto-protective and anti-inflammatory actions. Its expression is associated with the resolution of non-immune as well as immune-driven inflammation[9]. The role of HO-1 as an inflammatory defensive factor may be due to its conversion of oxidant heme to antioxidants biliverdin and bilirubin. It also elevates intracellular free iron levels to facilitate ferritin up-regulation, and regulation of vascular tension through CO generation. Moreover, a recent study has found that CO is able to inhibit the expression of lipopolysaccharide-induced pro-inflammatory cytokines and increase the anti-inflammatory cytokine through a pathway involving the mitogen-activated protein kinases (MAPK)[17]. Besides a role in resolution of inflammation, evidence has also been found that HO-1 may participate in the regulation of endothelial cells activation, proliferation and angiogenesis[18,19], which is essential for wound healing. In this study, the expression and activity of HO-1 were significantly elevated during early ulcer healing and this could be due to the contributory actions of HO-1 in inflammatory resolution and angiogenesis in tissue regeneration.

High expression of COX-2 protein and enzymatic activity of COX at the late ulcer healing stage were observed in this study. The expression of COX-1 protein was relatively stable, thus the change of COX activity might mainly be due to the change of protein level and activity of COX-2. Since tissue remodeling which includes reepithelization of gastric mucosa, maturation of granulation tissue, and reconstruction of extracellular matrix (ECM) mainly occurs at the late ulcer healing stage, the results of this study suggest that COX-2 may play an essential role in these remodeling processes. This is supported by the fact that non-selective as well as selective COX-2 inhibitors delay ulcer healing and prevent regeneration of the mucosa, maturation and angiogenesis in the ulcer base[20-26].

There may be interactions among NOS, HO and COX. HO may modulate NOS and COX systems since they are all heme-containing enzymes. NO and NO donors have been found to stimulate HO-1 expression in different cell lines[27-31] and NO was found to be able to stabilize HO-1 mRNA[32]. On the other hand, HO-1 may have a negative feedback regulation on NO production. Induction of HO-1 by cadmium, bismuth salts,

heme, and nitric oxide (NO) donors have been shown to inhibit the expression of iNOS[33, 34], while inhibition of HO-1 by its inhibitor enhanced iNOS expression. In the present study, a dramatic decrease of iNOS expression and activity was accompanied by an increase in expression and activity of HO-1 on day 5 after ulcer induction. This inverse relationship between iNOS and HO-1 expression and activities supports the existence of close interaction among these enzymes.

In summary, differential expression and activity patterns of inducible enzymes of iNOS, HO-1 and COX-2 during gastric ulceration and healing were found in the present study. The results indicate that iNOS may contribute to tissue inflammation during ulcer formation, while HO-1 and COX-2 may promote ulcer healing, since their expression and activities correlate with the resolving of inflammation and remodeling of ulcer tissues. Close interaction between iNOS and HO-1 may exist because the decrease of iNOS expression and activity coincide with the increase in expression and activity of HO-1. However, further experiments that enroll the use of selective blockers of these inducible enzymes should be conducted to substantiate these conclusions.

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