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

Jin-Sheng Guo, Chi-Hin Cho, Wei-Ping Wang, Xi-Zhong Shen, Chuen-Lung Cheng, Marcel Wing Leung Koo

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 times 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

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 (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 The acid solution was withdrawn 45 s later into the same syringe, and the operating site was disinfected with 70 % ethanol.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 anesthesia 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 (mm²) was traced onto a transparency and then copied to a grid paper with 1 mm² 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 °C 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 eosin 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)12 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-Analyt, Bio-Rad, Hercules, CA).

<table>
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<tr>
<th>Primer sequences of iNOS, eNOS, COX-1, COX-2, HO-1, HO-2 and β-actin</th>
<th>Amplicon length (bp)</th>
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<tr>
<td>HO-1</td>
<td>Sense: 5’-CAGTGCCTCAGAGTTCC-3’ 284</td>
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<tr>
<td></td>
<td>Antisense: 5’-TACAAGGAGCCATACCAAGC-3’</td>
</tr>
<tr>
<td>HO-2</td>
<td>Sense: 5’-AAGAAGTATGGTGACCG-3’ 242</td>
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<tr>
<td></td>
<td>Antisense: 5’-TACTCAGGTCCAAGGCT-3’</td>
</tr>
<tr>
<td>COX-1</td>
<td>Sense: 5’-TGCTGTGAAAGGAGATGTTCT-3’ 403</td>
</tr>
<tr>
<td></td>
<td>Antisense: 5’-CAAGTCACACACATGCTC-3’</td>
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<td>COX-2</td>
<td>Sense: 5’-ACACCTTCACTTCGACCT-3’ 584</td>
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<td></td>
<td>Antisense: 5’-GAAGGGACACCCCTCCTAC-3’</td>
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<tr>
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<tr>
<td></td>
<td>Antisense: 5’-TCCACCATCCTGACAGGTC-3’</td>
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<tr>
<td>eNOS</td>
<td>Sense: 5’-TAGGAGACAGGAAATACTAC-3’ 812</td>
</tr>
<tr>
<td></td>
<td>Antisense: 5’-CGGGTGAATGTCCGCTG-3’</td>
</tr>
<tr>
<td>β-actin</td>
<td>Sense: 5’-GTGGGCGGCCTGACAGGAC-3’ 540</td>
</tr>
<tr>
<td></td>
<td>Antisense: 5’-CCTCCTTAATGTCACGCAG-3’</td>
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**Western blot analysis of NOS, HO and COX proteins**

Gastric tissues for the analysis for protein expression 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 °C. 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 rainbow 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 °C with 1:500 dilution of polyclonal 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 (Amer sham 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 Film Photo Co., Tokyo, Japan).

**Determination of NOS activity**

NOS activity in the gastric tissue was measured as the ability of tissue homogenates to convert L-[3H]-arginine to L-[3H]-citrulline.[12] Gastric samples were homogenized at 4 °C in a buffer containing 10 mMol/L HEPES (pH 7.2), 320 mMol/L sucrose, 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 °C. 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 CaCl2, 7 mMol/L L-valine, 10 mMol/L HEPES (pH 7.2) and 1 µg Cl [3H]-L-arginine (Gibico BRL) and incubated at 37 °C 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 µL 1 MOL/L NaOH and 540 mMol dilution solution containing 1 mMol/L each of L-arginine and DL-citrulline. The newly formed L-[3H]-citrulline was separated from L-[3H]-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-[3H]-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.[31] 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 MgCl2, 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 °C 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 PGE2, according to the method described by Tomlinson and Vane.[7,8] Gastric tissues were homogenized at 4 °C in proteinase inhibitory
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 ulcer induction (Figure 2).

**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 reepithelialized 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 stomachs on day 15 after ulcer induction.

![Figure 1](image1.png)

**Expression of HO, NOS and COX mRNA**

Basal levels of COX-2 and HO-1 mRNA expression were found 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](image2.png)

**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 reappeared 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](image3.png)

**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.

**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.
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 HO 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 after ulcer induction was accompanied by enlargement of ulcer crater. The expression of iNOS protein and activity were observed to be decreased 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].

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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 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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