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Differential effects of selective and non-selective inhibition of nitric oxide synthase on the expression and activity of cyclooxygenase-2 during gastric ulcer healing

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Abstract

Nitric oxide synthases (NOS) and cyclooxygenase-2 (COX-2) are important enzymes involved in ulcer healing but interactions between them have not been clearly defined. The aim of this study was to investigate the effects of selective or non-selective inhibition of NOS on the expression and activity of COX-2 during healing of acetic acid-induced gastric ulcers in rats. *N*-[3-(aminomethyl)benzyl] acetamidine (1400W), a potent selective inhibitor of inducible nitric oxide synthase (iNOS), at a dose of 0.1 mg/kg/day, was found to reduce the ulcer sizes at day 3 and 7 post-ulcer induction. On the other hand, 15 mg/kg/day of N^G-nitro-L-arginine methyl ester (L-NAME), a non-selective NOS inhibitor that suppresses both iNOS and endothelial nitric oxide synthase (eNOS), enlarged the ulcer sizes over the same time periods. The expression of COX-2 and COX activity, together with NF-κB activation in the ulcer tissues were down-regulated by L-NAME but not 1400W. It is concluded that iNOS may contribute to ulcer formation while COX-2 and eNOS promote ulcer healing. eNOS enhances COX-2 expression possibly through the activation of NF-κB.

Keywords: iNOS (inducible nitric oxide synthase); eNOS (endothelial nitric oxide synthase); COX-2 (cyclooxygenase-2); *N*-(3-(aminomethyl)benzyl)acetamidine (1400W); L-NAME (N^G-nitro-L-arginine methyl ester); NF-κB (nuclear transcription factor κB)

1. Introduction

Cyclooxygenase (COX) is the rate limiting enzyme which converts arachidonic acid to bioactive prostanoids. Two isoforms of COX, namely COX-1 and COX-2, have been identified. COX-1 is constitutively expressed and produces prostaglandins (PGs) that are involved in the homeostatic and physiological functions of the gastrointestinal tract. On the contrary, COX-2 which is the inducible isoform of COX, is mainly expressed in monocytes, macrophages, fibroblasts and endothelial cells. During inflammation and mitogenic stimulation, a 10- to 20-fold increase in COX-2 expression and over-production of PGs were observed, while there are only 2- to 4-fold increase in COX-1 expression (Brooks et al., 1999). PGs and other COX-2 products enhance inflammatory cells infiltration, exudation, swelling, and pain in the wound tissues. Nonsteroidal anti-inflammatory drugs (NSAIDs) inhibit COX-2 and produce analgesic, anti-inflammatory, and antipyretic responses. The gastrointestinal side effects induced by NSAIDs are mainly due to their inhibition of COX-1 in the gastrointestinal mucosa (Wallace, 1997; Warner et al., 1999), while selective inhibition of COX-2 by COX-2 selective NSAIDs reduced much of the gastrointestinal complications (Whittle, 2000; Bombardier et al., 2000; Cannon and Breedveld, 2001). However, accumulating evidences have suggested that COX-2 and its derived PGs are important in the resolution or healing of gastric ulcer besides their involvement in inflammation. High levels of COX-2 expression and COX activity could be determined in the margin and base of gastric ulcer tissues during healing (Guo et al., 2003; Tu et al., 2001). Furthermore, selective COX-2 inhibition suppressed angiogenesis, and

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delayed the healing process (Guo et al., 2002). These results confirmed that COX-2 plays an important role in the healing of gastric ulcers.

Another inducible enzyme that is actively involved in inflammation and healing process is nitric oxide synthase (NOS). NOS is the key enzyme which catabolizes L-arginine to L-citrulline and nitric oxide (NO). There are constitutive and inducible isoforms of NOS. The endothelial NOS (eNOS), one of the constitutive isoforms of NOS (cNOS), is dependent on calcium for its activation and generates NO in only nanomolar concentrations. NO produced by eNOS mainly acts on adjacent cells regulating blood pressure, organ blood flow, platelet function and polymorphonuclear granulocytes activation. eNOS also promotes ulcer healing through enhancement of angiogenesis, maintenance of gastric blood flow, stimulation of mucus and bicarbonate secretions (Ma and Wallace, 2000). In contrast, the inducible isoform of NOS (iNOS) is transcriptionally induced in inflammatory cells by proinflammatory agents and inflammatory cytokines such as endotoxin, interleukin-1β, and tumor necrosis factor-α. iNOS is calcium independent and capable of generating micromolar concentrations of NO. High concentration and sustained release of NO by iNOS during inflammation can result in the formation of highly toxic oxidizing species such as peroxynitrites and aggravates tissue damage (Cho, 2001; Radi et al., 1991; Jaiswal et al., 2001). However, there are also observations that iNOS-derived NO may benefit ulcer healing through promotion of apoptosis in inflammatory cells (Akiba et al., 1998).

It has been demonstrated that NO interacts with COX pathways in several in vivo and in vitro experimental systems. NO has been shown to amplify COX-2 expression in rat mesangial cells and hepatic macrophages under stimulation of interleukin-1β or endotoxin (Tetsuka et al., 1996; Ahmad et al., 2002). In animal model of myocardial ischemic pre-conditioning, NO has been found to trigger the activation of transcription factor nuclear factor-κB (NF-κB) and induce COX-2, which contributed to its protective action against subsequent myocardial ischemia insult (Bolli et al., 2003; Shinmura et al., 2002). However, contradictory results have been reported with respect to whether NO enhances, inhibits, or has minimum effect on COX activity (Goodwin et al., 1999; and references therein). The inconsistency may be due to the use of different cell types, enzyme isoforms, inhibitors, timing in sampling and concentrations of mediators in these studies.

Little is known as to the in vivo cross-talk between NOS/NO and COX-2/PGs systems during gastric ulcer healing, and the underling mechanisms of regulation. It has been shown that NF-κB responsive element is present in the promoter region of COX-2 and the expression of which is regulated by NF-κB in many cell types (D'Acquisto et al., 1997; Kojima et al., 2000; Schmedtje et al., 1997). Persistent inhibition of NF-κB activation would result in a decrease of COX-2 expression and delay ulcer healing (Takahashi et al., 2001). On the other hand, NO has been found to have a modulating role in NF-κB activation in previous studies (Spiecker et al., 1997; Chen et al., 1995). However, whether there is any involvement of NF-κB activation in the putative modulating effect of NO on COX-2 expression and activity during gastric ulcer healing is unknown. The aims of this study were to investigate the effects of selective or non-selective inhibition of NOS, and the role of NF-κB, in the expression and activity of COX-2 during gastric ulcer healing.

2. Materials and methods

2.1. Animals

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

2.2. Ulcer induction and drugs administration

Gastric kissing ulcers were induced in male Sprague–Dawley rats by luminal application of acetic acid solution as previously described (Guo et al., 2002). Thereafter the animals were allowed to feed on standard diet and tap water ad libitum until collection of gastric tissue samples. A potent selective iNOS inhibitor, *N*-[3-(aminomethyl) benzyl] acetamidine (1400W, 0.1 mg/kg/day, Tocris Cookson Limited, Bristol BS11 8TA, UK), or a non-selective NOS inhibitor, N^G-nitro-L-arginine methyl ester (L-NAME, 15 mg/kg/day, Sigma Chemical, St. Louis, MO, USA), was intraperitoneally administered to the rats in the 1400W group and L-NAME group, respectively. The dosages of 1400W and L-NAME were selected with reference to the results obtained from a small pilot study and previous published findings (Guo et al., 2004; Ma and Wallace, 2000). While the ulcer control (UC) group received same volume of normal saline as the vehicle. All injections were given at 1 h before ulcer induction and once daily throughout the whole experimental period.

2.3. Sample collection

Rats in each group were killed by ether anesthetization at day 1, 3, and 7 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 squares. The ulcer area was determined by counting the numbers of square it covered. Gastric tissues from the ulcer base were immediately frozen in liquid nitrogen and stored at −70 °C.

2.4. Determination of NOS activity

NOS activity in the gastric tissue was determined as the ability of tissue homogenates to convert L-[^{3}H]-arginine to L-

[^{3}H]-citrulline (Guo et al., 2003; Ma and Wallace, 2000). Gastric samples were homogenized at 4 °C in a buffer containing 10 mM HEPES (pH 7.2), 320 mM sucrose, 0.1 mM EDTA, 1 mM dithiothreitol, 10 μg/ml leupeptin, 2 μg/ml aprotinin (Sigma, St. Louis, MO, USA) and 1 mM PMSF (Sigma) then centrifuged at 12,000 ×*g* for 30 min at 4 °C. The supernatant was collected and the protein contents were measured using dye-binding method (Bradford, 1976). One hundred microlitres of supernatant were then mixed with a buffered solution consisting of 0.7 mM NADPH, 150 μM $CaCl_2$, 7 mM L-valine, 10 mM HEPES (pH 7.2) and 1 μCi [^{3}H]-L-arginine (Gibco BRL, Gaitherburg, MD, USA) and incubated at 37 °C for 30 min to determine the total NOS activity. For determination of the iNOS activity, 1 mM 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 M NaOH and 540 μl dilution solution containing 1 mM 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 through 1 ml AG50W-X8 resin columns (Bio-Rad), and the activity of the eluted labeled material was recorded with a Beckman scintillation counter (LS-6500, Beckman Instrument, USA). The final result was expressed as picomoles of L-[^{3}H]-citrulline formed per milligram of protein per 30 min. The calcium-dependent eNOS activity was calculated by subtracting calcium-independent activity from total activity.

2.5. RT-PCR for COX-2 mRNA expression

Total RNA was isolated from gastric tissue with Trizol reagent (Gibco BRL). First-strand complementary DNA was synthesized from 5 μg RNA using oligo dT_{20} primer and Thermoscript RT-PCR system (Gibco BRL). PCR was performed for COX-2 and β-actin with the same complementary DNA sample in a PCR Thermal Cycler (Gen Amp PCR System 9700, The Perkin-Elmer Corporation, Norwalk, CT, USA). The sequences of the oligonucleotide primers were designed according to previously published sequences. For COX-2, the primers were sense 5′-ACACTCTATCACTGGCATCC-3′ and antisense 5′-GAAGGGACACCCTTTCACAT-3′ (Genebank accession no. S67722, nt 1229–1249 and 1794–1813, with 585 bp amplicon); while for the β-actin, the sense and antisense primers were respectively 5′-GTGGGGCGCCCCAGG-CACCA-3′ and 5′-CTCCTTAATGTCACGCACGATTTC-3′ (Genebank accession no. BC063166, nt 184–203 and nt 700–723, with 539 bp amplicon). β-actin was employed as an internal control. After denaturation for 5 min at 94 °C, 28 cycles of amplification were carried out followed by final extension of 10 min at 72 °C. RT-PCR products were electrophoresed in a 1% agarose (Gibco BRL) gel containing 0.5 μg/ml ethidium bromide. Locations of the predicted products were confirmed using ϕX 174 RF DNA/Hae III fragments (Gibco BRL) as standard size markers. The intensity of bands was quantified using a computerized densitometer. The amplification signals of COX-2 cDNA fragment were standardized against the β-actin signal for each sample and the results were expressed as COX-2/β-actin mRNA ratio.

2.6. Assessment of COX activity

COX activity was measured as the ability of tissue homogenates to metabolize arachidonic acid to PGE_2 according to the method previously described (Vane et al., 1994). Gastric tissues were homogenized at 4 °C in proteinase inhibitory buffer containing 50 mM Tris–HCl (pH 7.4), 3.15% trisodium citrate, 1 mM PMSF, 0.2 mM leupeptin. The protein concentration in the homogenates was measured. Homogenates were incubated at 37 °C for 30 min in the presence of excess arachidonic acid (30 μM). The samples were then boiled and centrifuged at 12,000 ×*g* for 30 min at 4 °C. The concentration of PGE_2 present 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.

2.7. Isolation of nuclear proteins

Gastric tissues were homogenized in ice-cold buffer A containing 10 mM HEPES (pH 7.9), 1.5 mM $MgCl_2$, 10 mM KCl, 1 mM DTT, 1 mM PMSF, and 2 μg/ml each of aprotinin, chymostatin, pepstatin, and leupeptin protease inhibitor mixtures. The lysate was centrifuged at 800 ×*g* for 10 min at 4 °C after a 10 min incubation on ice. The cytoplasmic supernatant was removed, and the cell pellet was reconstituted in ice-cold buffer B (1% Triton X-100 in buffer A), then incubated on ice for 10 min, and centrifuged as above. The crude nuclear pellet was rinsed once with buffer A and resuspended in 100 μl buffer C containing 20 mM HEPES (pH 7.9), 25% glycerol (v/v), 0.4 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, and protease inhibitor mixtures and incubated on ice for 30 min. Nuclear proteins were recovered after centrifugation at 14,000 ×*g* for 15 min at 4 °C. The supernatant containing nuclear protein was aliquoted and stored at −80 °C. The protein concentrations were quantified using dye-binding method.

2.8. Western blotting of COX-2 and NF-κB protein levels

The protein levels of COX-2 and the activated NF-κB in ulcer tissues were analyzed with Western blotting. Gastric ulcer tissues were homogenized in a proteinase inhibitor buffer (50 mM Tris–HCl; pH 7.5, 150 mM NaCl, 0.5% α-cholate sodium, 0.1% SDS, 2 mM EDTA, 1% Triton X-100, 10% glycerol, 1 mM PMSF and 2 μg/ml aprotinin) and then centrifuged at 10,000 ×*g* for 15 min at 4 °C. The supernatant was collected and the protein content was determined with dye-binding method (Bio-Rad). Thirty micrograms of total protein were loaded onto SDS-polyacrylamide gel and blotted onto hybond 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% non-fat milk powder, 10 mM Tris–HCl (pH 7.5), 100 mM 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 polycolonal antibodies against COX-2 and β-actin (Santa Cruz Biotechnology INC, Santa Cruz, CA). After washing in washing buffer for 30 min the membranes were treated with HRP-conjugated secondary antibody (1:5000) (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 exposed to photographic films (Fuji Photo Film Co., Tokyo, Japan). For the determination of activated nuclear transcription factor κB (NF-κB) level, 30 μg of the nuclear protein extracts were subjected to SDS-PAGE. An anti-p65 subunit monoclonal antibody (MAB3026, CHEMICON International Inc, Temecula, CA) which recognizes an epitope overlapping the nuclear location signal of the p65 subunit of the NF-κB heterodimer and thus selectively binds to the activated form of NF-κB, was used as the primary antibody.

2.9. Statistical methods

All the data are expressed as the means±S.E.M. Statistical analyses were performed using one-way analysis of variance (ANOVA) followed by the Dunnet's multiple comparison tests (SPSS statistical package, version 11.0, SPSS Inc., Chicago, IL). Values of $P<0.05$ were considered statistically significant.

3. Results

3.1. Effects of NOS inhibitors on gastric ulcer healing

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 which re-epithelialized gradually during ulcer healing. The ulcer areas were found to be largest at day 1 and reduced at day 3 and 7 after ulcer induction (Fig. 1). 1400W administration significantly reduced the ulcer sizes at day 3 and 7 when compared with the ulcer control rats (Fig. 1). On the contrary, rats treated with L-NAME showed larger ulcer areas (Fig. 1). The results indicated that 1400W accelerated, while L-NAME delayed ulcer healing.

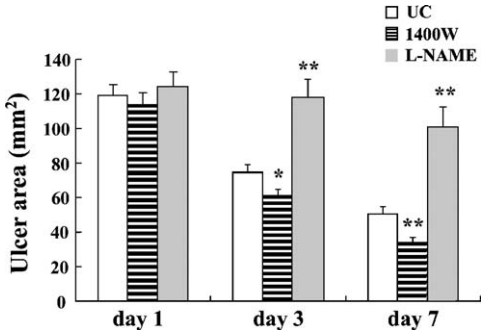

Fig. 1. Effects of nitric oxide synthase (NOS) inhibitors on gastric ulcer healing. 1400W (0.1 mg/kg/day), or L-NAME (15 mg/kg/day), or the same volume of normal saline vehicle was administered intraperitoneally to rats 1 h before ulcer induction and once daily for 7 days. The ulcer area was determined at day 1, 3, and 7 after ulcer induction. UC: the normal saline treated ulcer control group; 1400W: 1400W treated group; L-NAME: L-NAME treated group. Each column represents the mean±S.E.M. of 8 rats in each group. *$P<0.05$; **$P<0.01$ when compared with the UC group.

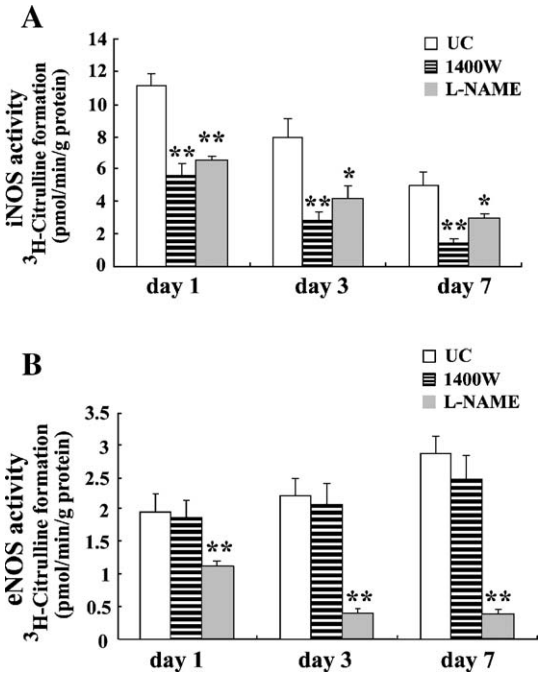

Fig. 2. Effects of NOS inhibitors on NOS activity. 1400W (0.1 mg/kg/day), or L-NAME (15 mg/kg/day), or the same volume of normal saline vehicle was administered intraperitoneally to rats 1 h before ulcer induction and once daily for 7 days. Gastric ulcer tissues were collected at day 1, 3, and 7 after ulcer induction. The iNOS (A) and eNOS (B) activities in the gastric ulcer tissues were measured as the ability of tissue homogenates to convert L-[^{3}H]-arginine to L-[^{3}H]-citrulline. UC: the normal saline treated ulcer control group; 1400W: 1400W treated group; L-NAME: L-NAME treated group. Each column represents the mean±S.E.M. of 8 rats in each group. *$P<0.05$; **$P<0.01$ when compared with the UC group.

3.2. Effects of NOS inhibitors on NOS activity

As was shown in Fig. 2, a high level of iNOS activity was detected in the ulcer base at day 1 but decreased at day 3 and 7 after ulcer induction in the control group. The iNOS activity was significantly inhibited by selective or non-selective NOS inhibitor at day 1, 3, and 7 after ulcer induction (Fig. 2A). However, the activity of constitutive eNOS was inhibited only by L-NAME but not by 1400W (Fig. 2B). The result was in consistence with the selectivity of these inhibitors on different forms of NOS isozymes.

3.3. Effects of NOS inhibitors on COX-2 expression

The expressions of COX-2 mRNA and protein were detectable at day 1 after ulcer induction in the ulcer control group and increased markedly in day 3 and day 7, when the ulcers were healing. Administration of L-NAME reduced the mRNA and protein levels of COX-2 in the ulcer tissues, especially at day 3

and 7 (Fig. 3). However, there was no difference in COX-2 mRNA and protein expressions in 1400W treated group when it was compared with the ulcer control group (Fig. 3).

3.4. Effects of NOS inhibitors on COX activity

As was observed in Fig. 4, the COX activity in gastric ulcer tissues, which was determined as the ability of COX to generate PGE_2 in the tissue homogenates, was well co-related with COX-2 protein expression in all rats (Fig. 5). The COX activity and the protein level of COX-2 were highest at day 7 in the ulcer control group when compared with that at day 3 and day 1 (Figs. 4, 5). The administration of L-NAME significantly decreased COX activity and COX-2 protein level in the gastric ulcer tissues, especially at day 3 and 7 (Figs. 4, 5). There was no difference in COX activity and COX-2 protein level in the ulcer tissues of rats treated with 1400W when compared with the ulcer control rats (Figs. 4, 5).

3.5. Effects of NOS inhibitors on NF-κB activation

High level of activated NF-κB was found in the gastric ulcer tissues taken from ulcer control group at day 1 after ulcer

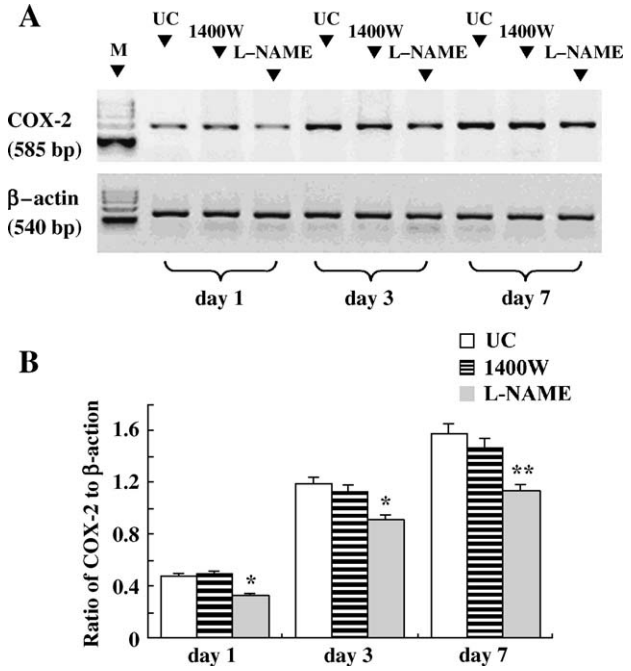

Fig. 3. Effects of NOS inhibitors on COX-2 expression. 1400W (0.1 mg/kg/day), or L-NAME (15 mg/kg/day), or the same volume of normal saline vehicle was administered intraperitoneally to rats 1 h before ulcer induction and once daily for 7 days. Gastric ulcer tissues were collected at day 1, 3, and 7 after ulcer induction. The mRNA expression of COX-2 in the gastric tissues was determined with RT-PCR. (A) Representative RT-PCR profile of COX-2 mRNA expression in gastric ulcer tissues. The PCR products were detected as 585 and 540 bp bands for COX-2 and β-actin mRNA, respectively. (B) Semi-quantitative analysis of COX-2 mRNA using densitometric scanning of amplified PCR products. Each COX-2 signal was standardized against the corresponding β-actin signal and the results are expressed as COX-2/β-actin ratios. UC: the normal saline treated ulcer control group; 1400W: 1400W treated group; L-NAME: L-NAME treated group. Each column represents the mean±S.E.M. of 8 rats in each group. $*P<0.05$; $**P<0.01$ when compared with the UC group.

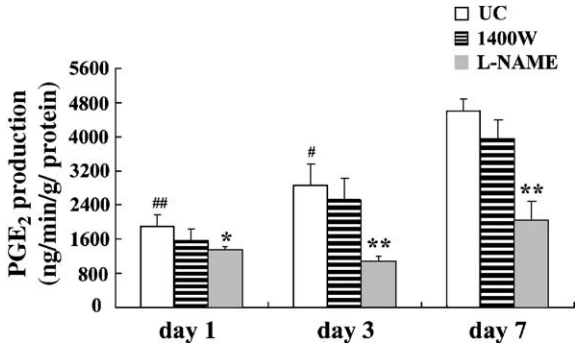

Fig. 4. Effects of NOS inhibitors on COX activity. 1400W (0.1 mg/kg/day), or L-NAME (15 mg/kg/day), or the same volume of normal saline vehicle was administered intraperitoneally to rats 1 h before ulcer induction and once daily for 7 days. Gastric ulcer tissues were collected at day 1, 3, and 7 after ulcer induction. COX activity was assayed as the ability of COX in the tissue homogenate to produce PGE_2. UC: the normal saline-treated ulcer control group; 1400W: 1400W treated group; L-NAME: L-NAME treated group. Each column represents the mean±S.E.M. of 8 rats in each group. $*P<0.05$; $**P<0.01$ when compared with the UC group. $^{\#}P<0.05$; $^{\#\#}P<0.01$ when compared with the UC group at day 7 post-ulcer induction.

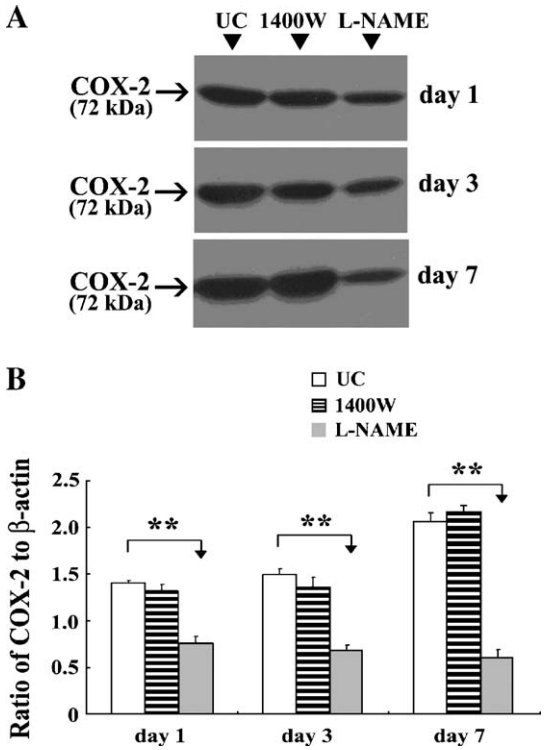

Fig. 5. Effect of NOS inhibitors on COX-2 protein expression. 1400W (0.1 mg/kg/day), or L-NAME (15 mg/kg/day), or the same volume of normal saline vehicle was administered intraperitoneally to rats 1 h before ulcer induction and once daily for 7 days. Gastric ulcer tissues were collected at day 1, 3, and 7 after ulcer induction. The protein expression of COX-2 in the gastric ulcer tissues was determined with Western blot method. (A) Representative Western blot profile of COX-2 protein in gastric ulcer tissues of each group at day 1, 3, and 7 post-ulcer induction. (B) Semi-quantitative analysis of COX-2 protein signals using densitometric scanning. Each COX-2 signal was standardized against the corresponding β-actin signal and the results are expressed as ratio of COX-2 to β-actin. Data are expressed as mean±S.E.M. of 8 rats in each group. UC: the normal saline treated ulcer control group; 1400W: 1400W treated group; L-NAME: L-NAME treated group. $**P<0.01$ when compared with the UC group.

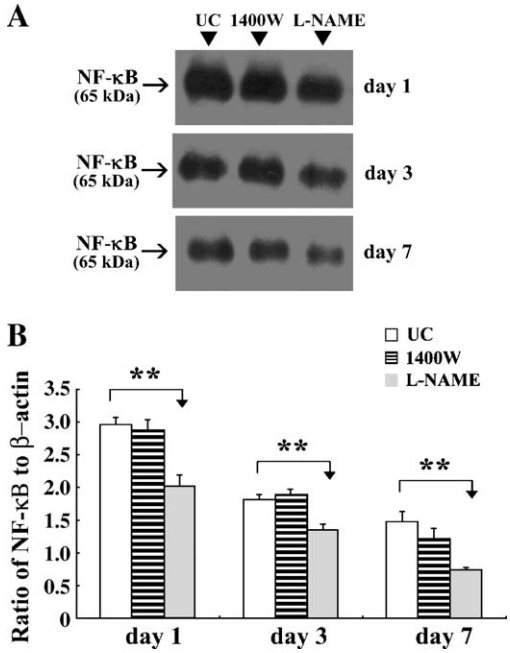

Fig. 6. Effects of NOS inhibitors on NF-κB activation. 1400W (0.1 mg/kg/day), or L-NAME (15 mg/kg/day), or the same volume of normal saline vehicle was administered intraperitoneally to rats 1 h before ulcer induction and once daily for 7 days. Gastric ulcer tissues were collected at day 1, 3, and 7 after ulcer induction. The nuclear protein of the gastric ulcer tissues was isolated and the activated NF-κB in the nuclear protein extract was analyzed with Western blotting. (A) Representative Western blot profile of the activated NF-κB in the nuclear protein extracts of each group at day 1, 3, and 7 post-ulcer induction. (B) Semi-quantitative analysis of the activated NF-κB protein signals using densitometric scanning. Each NF-κB signal was standardized against the corresponding β-actin signal and the results are expressed as ratio of NF-κB to β-actin. Data are expressed as mean±S.E.M of 8 rats in each group. UC: the normal saline treated ulcer control group; 1400W: 1400W treated group; L-NAME: L-NAME treated group. $^{**}P<0.01$ when compared with the UC group.

induction, but it was decreased at day 3 and 7. The nuclear level of activated NF-κB was down-regulated with the administration of L-NAME (Fig. 6). However, it was not affected by 1400W administration.

4. Discussion

In the present study, selective iNOS and non-selective NOS inhibitor promoted or delayed healing of gastric ulcers, respectively. The former also demonstrated no effect while the latter decreased the COX-2 expression and COX activity, accompanied with an inhibition of NF-κB activation in the gastric ulcer tissues.

1400W is a relatively non-toxic and irreversible inhibitor of iNOS (Babu and Griffith, 1998). Its selectivity on iNOS over eNOS is at least 5000-fold in vitro (Garvey et al., 1997). Results obtained in this study demonstrated that 1400W inhibited iNOS activity therefore may reduce the over-production of NO and highly toxic oxidizing species. These toxic reactive free radicals such as peroxynitrites can oxidize tissue sulfhydryls, degrade proteins and DNA, and thus aggravate tissue damage. Although there are reports on the limiting action of iNOS-derived NO in inflammation or reducing the production of inflammatory cytokines (Kolb and Kolb-Bachofen, 1998; Akiba et al., 1998), results obtained from the present study supported a beneficial role of selective iNOS inhibitor on gastric ulcer healing. This is further substantiated by recent studies in which selective inhibition of iNOS ameliorated different types of experimentally produced inflammatory diseases such as colitis (Menchén et al., 2001), collagen-induced arthritis (Cuzzocrea et al., 2002), allergic airway inflammation (Koarai et al., 2000), and autoimmune myocarditis (Shin et al., 1998). A small but effective dose of 1400W on iNOS inhibition was used in the present study. It was found to reduce the ulcer sizes at day 3 and 7 post-ulcer induction, at which time the ulcers were in the healing stage. Whether there is a dose-dependent anti-inflammatory and wound healing effect of 1400W warrants further investigation.

Non-selective NOS inhibitor L-NAME was found to postpone gastric ulcer healing in the present study, which was in accordance with the findings in previous reports (Akiba et al., 1998; Ma and Wallace, 2000). Due to the highly selective nature of 1400W on iNOS, it is reasonable to consider that the difference between L-NAME and 1400W on gastric ulcer healing is mainly due to the inhibition of eNOS activity. In acetic acid-induced gastric ulcer model, the expression and activity of eNOS in the ulcer tissues were found to increase gradually during ulcer healing after a transient loss at the time of ulcer induction when the epithelial cells were denuded (Guo et al., 2003), since eNOS protein is mainly found in vascular endothelium of granulation tissues and the mucosal epithelium (Ma and Wallace, 2000). Studies performed in eNOS-deficient mice or with NOS inhibitors and NO donors (Lee et al., 1999; Ma and Wallace, 2000; Sanchez-Fidalgo et al., 2005), showed that eNOS derived NO contributed significantly to wound healing through promotion of angiogenesis in wounded tissues. The mechanisms through which NO modulates angiogenesis may involve the suppression of protein kinase C-δ and the modification of adhesion molecule expression such as αvβ3-integrin on endothelial cells. Moreover, as it was shown in the present study, L-NAME but not 1400W down-regulated COX-2 expression and COX activity during the healing of ulcers. Previous studies have demonstrated that COX-2 but not COX-1 is highly induced in the gastric ulcer tissues leading to a higher level of PGE_2 production (Guo et al., 2003; Takahashi et al., 1998; Poonam et al., 2005). Highly selective inhibitors of COX-2 significantly reduced the PGE_2 production in the gastric ulcer tissues, impaired angiogenesis of the healing ulcers and delayed ulcer healing (Guo et al., 2002; Shigeta et al., 1998). These results provide evidences that COX-2 rather than COX-1 is the major source of the total COX activity in healing gastric ulcer tissues and it plays an important role in promoting angiogenesis and ulcer healing. The inhibition of COX-2 expression and PGE_2 production by L-NAME but not 1400W, as it was shown in the present study, further suggested that eNOS derived NO may promote angiogenesis via the induction of COX-2.

The present study also revealed that NF-κB may be involved in the regulation of COX-2 expression by NO and NOS in the gastric ulcer tissues. Again, this regulation may be mediated by

eNOS derived NO, as the activation of NF-κB was only inhibited by L-NAME but not 1400W. NF-κB is a pleiotropic transcription activator that exists in the majority of cell types. It consists of homo- or heterodimers of structurally related proteins including p65 (Rel A), C-Rel, and p50. In resting cells, NF-κB is coupled with inhibitor-κB (iκB) and resides in the cytosol as an inactive form. In response to inflammatory stimuli and mitogens, iκB is phosphorylated and degradated, thus releasing free NF-κB and allowing it to translocate into the nucleus to activate transcription. Among the inducible genes that can be activated by NF-κB are those encoding for inflammatory cytokines, growth factors and cell adhesion molecules. COX-2 gene also contains NF-κB binding site in its promoter region and its expression is regulated by NF-κB in many cell types, including gastric fibroblasts. In gastric ulcer tissues, NF-κB was found to be activated particularly in fibroblasts, macrophage, and neutrophils in rat. Since NF-κB is involved in the gene expressions of iNOS, interleukin-1β, cytokine-induced neutrophil chemoattractant-1 and COX-2, therefore it plays an important role in the ulcer healing processes, such as neutrophil infiltration, epithelial regeneration, and angiogenesis. Inhibition of NF-κB activation by NF-κB decoy oligonucleotide and pyrrolidine dithiocarbamate (PDTC, an inhibitor of NF-κB activation) resulted in the down-regulation of healing factors, including COX-2, and impairment in wound healing (Takahashi et al., 2001). In the present study, non-selective NOS inhibitor L-NAME but not 1400W induced a similar down-regulating effect as NF-κB decoy oligonucleotide and PDTC on NF-κB activation and COX-2 expression. These results suggest that eNOS derived NO may activate NF-κB activation and increase COX-2 expression.

The present findings could not exclude the possibility that pathways other than NF-κB are also involved in the regulation of COX activity by NO/eNOS. The activation of NF-κB was found to be at its peak in the inflammatory stage at day 1 but decreased at day 3 and 7 during ulcer healing. However, the expressions of COX-2 and other healing factors were still elevated. The sustained expression of these healing factors might partly be attributed to the activation of other signal pathways or transcriptional factors, such as protein kinase C/mitogen activated protein kinase-dependent pathway and transcription factors STATs (signal transducers and activators of transcription) that have been shown to control COX-2 expression in other biological systems (Barry et al., 1999; Bolli et al., 2003). Further studies should be performed to delineate whether these alternative pathways regulate COX-2 expression during gastric ulcer healing.

The present study showed that non-selective inhibition of nitric oxide synthase by L-NAME delayed gastric ulcer healing, decreased COX-2 expression and COX activity, and inhibited NF-κB activation in the ulcer tissues. The potent and selective iNOS inhibitor 1400W, on the contrary, accelerated the ulcer healing. It did not inhibit COX-2 expression and COX activity, as well as NF-κB activation in the healing ulcers. It is concluded that iNOS may contribute to gastric ulceration while COX-2 and eNOS promote ulcer healing.

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