Protective role of heme oxygenase-1 on trinitrobenzene sulfonic acid-induced colitis in rats

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IN HEME METABOLISM, heme oxygenase (HO) is an initial and rate-limiting enzyme. It oxidatively degrades heme into carbon monoxide (CO), iron, and biliverdin, which is subsequently converted to bilirubin by biliverdin reductase (40). Two distinct isofoms of HO have been identified (17, 19, 41). These isofoms are different gene products and vary in their tissue expression as well as in their molecular properties (17, 19, 41). The HO-2 isofom is constitutive and physiologically expressed. In contrast, the HO-1 isofom is ubiquitously distributed in mammalian tissues and is strongly and rapidly induced by a variety of stimuli and agents that cause oxidative stress and pathological conditions (17). HO-1 induction is important in the response of tissues to oxidative stress and inflammation (12, 45). Investigations have demonstrated (11a) that heme degradation produces a variety of biological effects. Bilirubin, an antioxidant, provides cellular protection against free radical-mediated injury (12, 36). CO regulates the generation of pro- and anti-inflammatory cytokines (27). In addition, CO leads to vasodilation and inhibition of platelet aggregation (20, 24).

Reactive oxygen species (ROS), such as superoxide anion and hydrogen peroxide, as well as excess NO are involved in human inflammatory bowel disease (IBD) (6, 35). The IBD induced in rats by 2,4,6-trinitrobenzenesulfonic acid (TNBS) produced acute and chronic inflammation of the colon (15, 25, 28). This was accompanied by large quantities of ROS and NO products as well as inflammatory infiltration, which pathologically resembled human IBD (15, 25, 28). To investigate whether the expression of HO-1 is an endogenous mechanism responsible for host defense against inflammatory injury in colonic tissue, we used the TNBS-induced IBD model in this study. We utilized this model to elucidate the effect of HO-1 expression and activity on the severity of colonic lesion and inflammation and to study whether HO-1 induction could protect colonic tissue from oxidative and inflammatory damage through reduction of ROS and NO products.

MATERIALS AND METHODS

Animals. The present study was approved by the University of Hong Kong Committee on the Use of Live Animals. Male Sprague-Dawley rats (180–200 g) were fed a standard diet for 7 days before being placed on a normal rat chow diet. A total of 18 rats were divided into three groups: control, TNBS-treated, and TNBS-treated with SnMP. The TNBS-treated rats were given an enema containing TNBS (50 mg) per kg of body weight according to a previously described method (28). Control rats were given an enema containing saline instead of TNBS. The rats were killed by cervical dislocation on day 7 after the TNBS enema, and the colon was removed, rinsed, and weighed. The colon was then cut open longitudinally, and the lesion area was measured. Myeloperoxidase activity was determined in the colonic tissue from oxidative and inflammatory damage.

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laboratory diet (Ralston Purina, Chicago, IL) and given tap water. The rats were kept in a room with controlled temperature (22 ± 1°C), humidity (65–70%), and a 12:12-h light-dark cycle.

**Drugs and solutions.** Tin mesoporphyrin IX chloride (SnMP) and ferriprotoporphyrin IX chloride (hemin) (Por- phyrin Products, Logan, UT) were dissolved in 0.1 ml of 0.5 M NaOH; normal saline was added until the drug preparation equaled 0.5 ml. The solution was finally adjusted to pH 7.4. Both SnMP and hemin solutions were kept in the dark to prevent photodegradation. TNBS (Sigma, St. Louis, MO) was dissolved in 50% (vol/vol) ethanol (Merck, Darmstadt, Germany). L-Arginine (Sigma) was dissolved in normal saline.

**Experimental IBD in rats.** IBD was induced in rats according to the model described by Morris et al. (25). Rats were lightly anesthetized by diethyl ether (BDH, Poole, UK). Using a polyethylene catheter fitted onto a 1-ml syringe, we slowly injected 0.3 ml of 50% (vol/vol) ethanol containing 30 mg TNBS into the lumen of the colon (8 cm proximal to the anus through the rectum). Animals in the normal control group received 0.3 ml of 50% ethanol similarly, but normal saline was rectally administered instead. Rats were killed at specific time points after colitis induction. The distal colon was removed, opened, and thoroughly rinsed in ice-cold normal saline. The lesion size was delineated and measured with a 1-mm square grid by an observer who was unaware of the treatment. The distal colon (length, 8 cm) was weighed, and the ratio of colon weight to body wt was obtained. This ratio was used as a parameter to assess the degree of colon edema, which reflected the severity of colonic inflammation. One piece of colonic tissue (3 × 10 mm) dissected from the most ulcerative region was excised and fixed in 10% formalin buffer for subsequent immunohistochemical studies. The rest of the colonic tissue was collected, immediately frozen in liquid nitrogen, and stored at −70°C for further biochemical investigations.

**Drug administration.** Rats were administered either SnMP (20 μmol/kg) subcutaneously into posterior neck tissue 3 h before and 21 h after colitis induction or hemin (50 μmol/kg) intravenously 1 h before the TNBS enema application. L-Arginine (50 mg/kg) was administered subcutaneously once daily over 4 days before the TNBS enema. Rats were administered the TNBS enema as described above.

**Determination of HO activity in colonic tissue.** HO activity in colonic tissue was measured as previously described (18). Briefly, the colonic tissue was homogenized in 10 mM PBS (pH 7.2) containing 1 mM phenylmethylsulfonyl fluoride, 1.5 mM pepstatin, and 0.2 mM leupeptin. The homogenate was centrifuged at 10,000 g for 15 min at 4°C. The supernatant fraction (10 mg of protein/ml) from rat colonic tissue was measured as previously described (18). Both SnMP and hemin solutions were kept in the dark to prevent photodegradation. TNBS (Sigma, St. Louis, MO) was dissolved in 50% (vol/vol) ethanol (Merck, Darmstadt, Germany). L-Arginine (Sigma) was dissolved in normal saline.

**Myeloperoxidase activity in colonic tissue.** Myeloperoxidase (MPO) activity was determined by a modified method as described previously by Suzuki et al. (39). In brief, colonic tissue was minced with scissors and homogenized in an ice-cold 50 mmol/l PBS (pH 6.0) solution containing 0.5% hexadecyltrimethylammonium bromide. The homogenate was freeze-thawed three times, followed by repeated sonication for 30 s each. The MPO activity assay has been described previously in detail by Guo et al. (8). The final value of MPO activity was represented as units per gram of tissue.

**HO-1 immunohistochemistry in colonic tissue.** The formalin-fixed colonic tissue was embedded in paraffin, and sections were dewaxed. Endogenous peroxidase activity was blocked with 3% H2O2 in methanol for 30 min. After blocking in 0.05 mol/l Tris-buffered saline (TBS) containing 3% normal horse serum and 0.3% Triton X-100 for 30 min, sections were rinsed with TBS buffer and incubated with mouse monoclonal HO-1 antibodies (Stressgen, Victoria, BC, Canada) diluted to 1:200 overnight at 4°C. The tissue was stained for antigen-antibody complexes using a peroxidase detection system (LSAB kit, DAKO).

**Total RNA isolation and RT-PCR.** Total RNA was isolated from rat colonic tissue with TRizol reagent (GIBCO BRL, Gaithersburg, MD). cDNA was amplified from RNA with the use of Thermoscript RT-PCR system reagent (GIBCO BRL). Primers were designed according to previously published sequences (2, 5). Sense (CAG TCT CCT CCA GAG TTT CC) and antisense primers (GTA CAA GGA GGC CAT CAG C) for HO-1 (284-bp fragment), sense (CTT ACC AAC GAG CAG TCA TCT T) and antisense primers (ATG TCT TTG AAG CTC TGG GGA AAA TTC) for HO-2 (251-bp fragment), sense (ATG CTT TGG CCT TGG AAG CTC) and antisense primers (TCC AGG CCA TCT TGG TGG CAA GA T) for iNOS (251-bp fragment), and sense (GTA GGC CGC CCC AGC CAG CA) and antisense primers (CTC CTT AAT GTC AGC GAT TTC) for β-actin (540-bp fragment) were used. β-actin was employed as an internal control. For HO-1 and HO-2 amplification, the processes involved 30 cycles of PCR (1 min at 95°C for denaturing, 1 min at 58°C for annealing, and 2 and 7 min (final cycle) at 72°C for elongation). For iNOS and β-actin, after denaturation at 95°C for 5 min, 30 cycles of PCR were performed with 30 s at 95°C for denaturing, 45 s at 60°C for annealing, and 1 and 7 min (final cycle) at 72°C for elongation.

**Western blot analysis.** Fifty micrograms of microsomal protein and the supernatants of tissue homogenate described above were respectively fractionated in a 10% or 7.5% SDS-polyacrylamide gel, then transferred to nitrocellulose membranes. After incubating in blocking buffer containing 5% skim milk dissolved in 10 mmol/l Tris-HCl, pH 7.5, 100 mmol/l NaCl, and 0.1% Tween 20, blots were probed with mouse monoclonal antibodies for HO-1 (1:400), iNOS (1:1,000), and rabbit polyclonal antibody for HO-2 (1:400) in blocking buffer for 1 h at room temperature. Antigen-antibody complexes were visualized by using horseradish peroxidase-conjugated goat anti-mouse or anti-rabbit antibodies.

**Chemiluminescence assay in colonic tissue.** Immediately after the colonic lesion was recorded, two full-thickness biopsy specimens (50–100 mg) were taken from the most ul-
cerative lesion and inflammatory area. The samples were
initially placed in preoxygenated (95% O₂-5% CO₂ for 20 min)
PBS solution with concentrations of calcium (1.13 mmol/l)
and glucose (5 mmol/l) at ambient temperature and then
transferred to 300 μmol/l luminol immediately before assess-
ment of the chemiluminescence response. The chemilumines-
cence was measured in a liquid scintillation counter (2000
CA, Packard) with an analyzer operated in “the out-of-co-
cidence” mode for 2 min. The final result of each colonic
determination was the value after subtraction of background
counts. The value was expressed as the number of photons
per minute per milligram of wet tissue (34).

Determination of iNOS activity in colonic tissue. iNOS
activity in colonic tissue was measured as previously de-
scribed (3) by the conversion of [3H]arginine to citrulline. To
determine iNOS activity, EGTA (91 mmol/l) was used to
inhibit the activity of calcium-dependent (constitutive) NOS.
The final result was expressed as picomoles of [3H]citrulline
per minute per milligram of protein.

Statistical analysis. Results were expressed as means ±
SE. Differences between two groups were examined using
one-way ANOVA followed by unpaired Student’s t-test unless
specified. The correlation between the two parameters was
tested using the Pearson correlation test. P < 0.05 was
considered statistically significant.

RESULTS

Effects of HO activity on colonic lesion and MPO
activity in TNBS-induced IBD in rats. Intracolonic
administration of TNBS-ethanol resulted in noticeable
hyperemia and exfoliation of epithelial lining at 2 and
6 h, and maximal mucosal lesion and inflammation in
the distal colon (e.g., extensive ulceration, bowel wall
thickening, and hyperemia) were grossly and also his-
tologically evident at 24 and 48 h. The weight of the distal
colon was also markedly increased because of
tissue edema.

Figure 1 shows that the TNBS enema markedly
increased HO activity in the colonic tissue at 6 and
24 h, with maximal activity level reached at 72 h.
Administration of 20 μmol SnMP 3 h before and 21 h
after TNBS induction remarkably inhibited TNBS-in-
duced HO activity. The colonic lesion area in animals
receiving SnMP was significantly potentiated (Fig. 2).
MPO activity was dramatically increased at 6, 24, and
72 h after TNBS administration. SnMP administration
enhanced MPO activity stimulated by the TNBS en-
ema. However, SnMP alone did not change MPO activ-
ity compared with the normal control group (Fig. 3).

HO-1 immunohistochemistry in TNBS-induced colitis.
HO-1 immunoreactivity was absent in normal colon
tissue (Fig. 4A). HO-1 immunoreactivity was detected
in the vascular endothelial, epithelial, and smooth
muscle cells of colonic tissues 2 h after application of
the TNBS-ethanol enema (Fig. 4, B and C). This was
accompanied by some positive-stained infiltrated inflam-
matory cells. The HO-1 staining was evident and
predominantly localized in the submucosa underneath
the mucosal lesion area in the tissue 24 h after colitis
induction (Fig. 4D). The cells contributing to an in-
crease in HO-1 expression were infiltrated neutrophils,
macrophages, and other inflammatory cells (Fig. 4D,
inset).

Expression profiles of HO mRNA and proteins in
TNBS-induced colitis. Total RNA and protein were
isolated at various times after colitis induction. As
shown in Fig. 5A, RT-PCR yielded a 284-bp product for
HO-1 and a 242-bp product for HO-2 when HO-1 and
HO-2-specific primers, respectively, were used. The
level of HO-1 mRNA was low in normal colonic tissue.
After TNBS administration, HO-1 mRNA transcripts
rapidly increased starting at 6 h and peaked at 3 days
with an ~25-fold increase over the normal level (Fig.
5A). On the other hand, there was no significant
change for HO-2 mRNA expression during the inflam-
lation period (Fig. 5B). The pattern of HO-1 protein
accumulation showed a trend similar to that of mRNA
expression. HO-1 protein level was elevated starting at
6 h and reached its peak at day 3 (Fig. 6A). Again,
the protein level of HO-2 was not significantly changed
during the inflammation period (Fig. 6B). However,
It was expressed more during this period of time com-
pared with normal tissue.

Elevation of luminol-amplified chemiluminescence
by SnMP treatment in TNBS-induced colitis. Luminol
reacts with oxidants such as ROS, to form 3-aminoph-
thalate. Electrons in the luminol are raised to higher
energy levels during the reaction with oxidants. On
reverting to the basal state, the energy is released in
the form of photons (chemiluminescence), which are
detected by the photomultiplier tubes of a scintillation

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counter (6). The changes in chemiluminescence can reflect ROS production in tissues. Luminol-amplified chemiluminescence in colonic tissue was markedly increased at 6 h and even more at 24 h after the TNBS enema. Pretreatment with SnMP produced significantly more luminol-amplified chemiluminescence in rats with TNBS-induced colitis and did not increase chemiluminescence in normal rats (Fig. 7).

**Inhibition of iNOS activity and expression with HO-1 induction.** The TNBS enema led to a significant increase in iNOS activity in colonic tissue at 6, 24, and 72 h. SnMP administration further elevated iNOS activity compared with the respective TNBS control groups. Pretreatment with SnMP alone did not cause the alteration of iNOS activity in normal rats (Fig. 8). RT-PCR showed that iNOS mRNA expression was increased by SnMP administration compared with the TNBS control at 24 h (Fig. 9A, lanes 3 and 4 vs. lanes 1 and 2). The protein level of iNOS in Western blots coincided with its mRNA expression at 24 h (Fig. 9B, lanes 3 and 4 vs. lanes 1 and 2). To confirm further the effect of HO activity on iNOS, the expression of iNOS mRNA and protein was examined in the TNBS-treated colonic tissue pretreated with hemin, a stimulator for HO. Administration of hemin increased the HO activity by about twofold (from 5.34 to 10.43 nmol bilirubin·h⁻¹·mg protein⁻¹) 24 h after TNBS administration and reduced iNOS mRNA expression and protein production in colonic tissue (Fig. 9A and B, lanes 5 and 6 vs. lanes 1 and 2).

**Aggravated colonic damage by L-arginine pretreatment in TNBS-induced colitis.** There was a strong correlation between iNOS activity and colonic lesion area (Pearson correlation coefficient = 0.816, \( P < 0.01 \); data not shown). Supplemented with the substrate of iNOS for the production of NO, L-arginine further exacerbated the severity of the mucosal lesion induced by the TNBS enema and also the injury action of SnMP at 24 and 72 h after TNBS administration (Fig. 10).

**DISCUSSION**

Recent studies (14, 27, 29, 42, 45) with HO-1-knockout mice have shown that induction of HO-1 helps to ameliorate tissue injury or inflammation changes in a variety of experimental models. However, the exact role of HO-1 in IBD is unclear. In this study, HO-1 was induced in colonic tissue damaged by the TNBS-ethanol enema. Prior administration with the HO activity inhibitor SnMP potentiated the colonic damage. The protective effect of HO could result from radical scavenging and inhibition of iNOS expression.

TNBS significantly increased HO activity in the colonic tissue. Administration of SnMP 3 h before the TNBS enema resulted in an apparent decrease of HO activity (Fig. 1). The changes in HO activity were shown to be associated with the severity of mucosal macroscopic lesion (Fig. 2). MPO activity was used as an index of quantitative inflammation and neutrophil infiltration in tissues (30). SnMP administration was able to aggravate neutrophil infiltration and inflammation (Fig. 3). These results suggest that generation of endogenous HO-1 may possibly protect colonic tissue against inflammation. RT-PCR showed a trace of HO-1 expression in normal rats, whereas SnMP administration significantly increased HO-1 expression (Fig. 8B, lanes 3 and 4 vs. lanes 1 and 2). The protein level of HO was further confirmed by Western blots (Fig. 9B, lanes 3 and 4 vs. lanes 1 and 2).
mRNA in normal colon, which was below the detection limit of Western immunoblotting. The TNBS-ethanol enema increased HO-1 mRNA transcription and protein level starting at 6 h and peaked at 72 h (Figs. 5A and 6A). On the other hand, the expression of HO-2 mRNA and protein was slightly elevated in TNBS-induced IBD; however, there was no significant change at different time points (Figs. 5B and 6B). These results indicate that the altered activity and probable protective effect of HO are mainly due to HO-1 induction in IBD. HO-1 is a stress-response protein and can be induced by a variety of oxidant stimuli that facil-
tate the generation of ROS. TNBS can be rapidly metabolized to generate ROS such as superoxide anion in rat colonocytes (7). The increase in HO-1 expression could be a protective response against the ROS-originated injury induced by TNBS. HO-1 immunolocalization was observed in the vascular endothelium and smooth muscle layers of the colon as early as 2 h (Fig. 4, B and C). Hayashi et al. (11) have reported that HO-1 induction in endothelial cells can suppress oxidant-induced microvascular leukocyte adhesion through the action of bilirubin. The present study demonstrated that changes in HO activity caused by SnMP affected MPO activity, which is an index of neutrophil infiltration. As the colonic damage advanced, colitis was characterized by increasing inflammatory infiltrates in the colonic lamina propria. HO-1-immunoreactive cells were found to be in both polymorphonuclear and monomorphonuclear cells (Fig. 4D). ROS in the insulted mucosa cause an adverse reaction with tissue macromolecules, e.g., DNA, protein, and lipid, resulting in pathophysiological changes. ROS production within the colonic interstitium therefore represents an important pathway by which TNBS mediates the pathogenesis in IBD. The increase in oxidant-induced HO activity can increase cellular capacity to catalyze free heme, which generates into bilirubin and its precursor biliverdin to scavenge ROS (36). HO inhibition of SnMP treatment enhanced chemiluminescence response in TNBS-induced colonic tissue injury, suggesting that HO-1 induction is a physiological defense against oxidant damage in colonic tissue.

Experimental data, including our previous study (8), have demonstrated that TNBS-induced colitis is associated with increased expression and activity of iNOS in infiltrated inflammatory cells, especially neutrophils and macrophages. High NO concentrations from activated iNOS are toxic and can damage tissues, because NO reacts with superoxide anion to form peroxynitrite in the course of inflammation (3, 8, 31). Peroxynitrite, a highly cytotoxic oxidant, promotes oxidative and nitrative injury of lipids, proteins, DNA, and other cellular biomolecules, leading to changes of cellular structure and function. Thus excess NO may be important in the pathogenesis of IBD. Indeed, aminoguanidine (an iNOS inhibitor) treatment ameliorated colonic damage (23); however, HO-1 expression was not significantly reduced (unpublished observations). These findings suggest that unlike a high NO level in tissues (5), a subthreshold NO concentration could not modulate HO-1 expression.

The exact mechanism whereby upregulation of HO-1 attenuates iNOS expression requires exploration. Because iNOS contains heme, a substrate for HO, in its
active sites, it is postulated that catabolism of cellular heme by HO would limit iNOS synthesis (1, 4). It has been shown that inducible macrophage NOS is a cytochrome P-450-type hemoprotein and contains a protoheme IX in its active site (44). HO degrades protoheme IX by cleaving its \( \alpha \)-methene bridge into CO, iron, and biliverdin IX\( \alpha \) (40). In addition, both HO and NOS systems require NADPH as an electron donor, thus competition to consume NADPH by increasing HO-1 molecules might impair NO synthesis in the NOS system. In the present study, SnMP administration enhanced mRNA and protein expression of iNOS in TNBS-induced colitis. To validate the possibility that alteration of HO activity affects iNOS mRNA expression, we examined the effect of an HO inducer on iNOS mRNA transcription. The administration of HO activity inducers such as hemin lowered iNOS mRNA and protein expression in the TNBS-inflamed colon (Fig. 9). HO-1 inducers such as hemin and SNP have been reported (1, 4) to abolish iNOS mRNA and protein expression both in vitro and vivo, and the action could be indirect through the substances released from heme metabolism. Iron released from the process of heme degradation would attenuate the NO level by down-regulating mRNA nuclear transcription and protein of iNOS initiated by inflammatory factors (13, 43). The improper overexpression of HO-1 would, however, reverse the HO-1-related protective effect, because large amounts of iron released from heme are harmful and are recognized to increase cellular susceptibility to oxidative stress (38). It is crucial to determine the concentration of HO-1 inducer needed to promote protective HO-1 expression. Therefore, appropriate HO-1 induction should be important in providing protection in tissues. This phenomenon requires further investigation.

CO released in the process of heme catabolism was also shown to inactivate existing iNOS by interacting with its heme iron moiety (21). iNOS expression is primarily from activated macrophages that increase their infiltration from 6 h after injury and reach the maximum at 72 h (32). The significant positive correlation between iNOS activity and colonic lesion area indicated that increased iNOS activity and expression contributed to colonic lesion formation induced by TNBS in rats. Furthermore, exogenous administration of L-arginine followed by SnMP pretreatment aggravated the colonic damage by the TNBS enema (Fig. 10). Thus iNOS downregulation by HO-1 induction may ameliorate the colonic lesion. It is likely that cells may possess an intrinsic system to attenuate NO-mediated cytotoxicity, and HO-1 may be a potential candidate. Excessive NO production originating from intrinsic...
endothelial cells and monocytes can induce HO-1 gene expression. Both transcriptional and posttranscriptional mechanisms have been implicated in this action (5, 46). To lessen the extent of NO-induced injury, cells can rapidly recruit HO-1 and defend against NO-mediated oxidative injury as a negative feedback regulation mechanism. Our results indicate that the negative modulation of iNOS possibly occurs at transcription and enzyme activity levels.

Experimental evidence has demonstrated that both biliverdin and bilirubin possess antimcomplement effects and inhibit the cytotoxic activity of human T lymphocytes (10, 26). CO generated from HO-1 expression inhibits the release of proinflammatory cytokines, such as tumor necrosis factor-α and interleukin-1β, and increases the release of anti-inflammatory cytokines such as tumor necrosis factor-α and interleukin-10 from macrophages (27). In addition, other inflammatory enzymes containing heme, such as cyclooxygenase, could also be modulated by HO activity. Thus modulation of HO-1 activity may represent a therapeutic strategy to ameliorate damages in IBD. However, it would be prudent to evaluate the beneficial effect of HO-1.

In conclusion, our data demonstrate that HO-1 expression is a self-defense mechanism against experimental colitis induced by TNBS. The increase of heme degradation by HO-1 induction could scavenge ROS products and decrease NO production by inhibiting iNOS expression and activity.

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