Adaptive HNE-Nrf2-HO-1 pathway against oxidative stress is associated with acute gastric mucosal lesions

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Ueda K, Ueyama T, Yoshida K, Kimura H, Ito T, Shimizu Y, Oka M, Tsuruo Y, Ichinose M. Adaptive HNE-Nrf2-HO-1 pathway against oxidative stress is associated with acute gastric mucosal lesions. Am J Physiol Gastrointest Liver Physiol 295: G460–G469, 2008. First published July 10, 2008; doi:10.1152/ajpgi.00204.2007.—Disturbance of the microcirculation and generation of reactive oxygen species are crucial in producing acute gastric mucosal lesions (AGML). To understand the protective mechanism against mucosal injury and oxidative stress in the stomach, we investigated sequential expression and localization of a product of lipid peroxidation and a chemical mediator of the oxidative response array, 4-hydroxynonenal (HNE), transcriptional factor, NF-E2-related factor (Nrf2), and the inducible heme oxygenase (HO-1) in the injured stomach. AGML was produced by intragastric administration of 0.6 N HCl in male rats. Expression and localization of HNE, Nrf2, and HO-1 were investigated by Western blotting, immunohistochemistry, real-time RT-PCR, and in situ hybridization histochemistry. Mucosal lesions and expression of HNE and HO-1 were assessed by pretreatment with the PGI2 analog beraprost or after sensory denervation by pretreatment with capsaicin. Mucosal lesions were assessed by pretreatment with a HO-1 inhibitor, zinc protoporphyrin (ZnPp). After AGML, increased generation of HNE was observed in the injured mucosa and the surrounding submucosa, followed by nuclear translocation of Nrf2 and upregulation of HO-1 in the macrophages located in the margin of the injured mucosa and in the submucosa. Pretreatment with beraprost attenuated AGML and downregulated the expression of HNE and HO-1, while sensory denervation aggravated AGML and upregulated the expression of HNE and HO-1. Pretreatment with ZnPp also aggravated AGML. The sequential HNE-Nrf2-HO-1 pathway in the gastric mucosal cells and the macrophages is involved in an adaptive mechanism against oxidative stress after AGML.

heat shock protein 32; reactive oxygen species; microcirculation; macrophages; 4-hydroxynonenal; heme oxygenase

Adaptive HNE-Nrf2-HO-1 pathway against oxidative stress is associated with acute gastric mucosal lesions. The costs of publication of this article were defrayed in part by the payment

PHYSICAL OR PSYCHOLOGICAL stress, or use of nonsteroidal anti-inflammatory drugs (NSAID) and alcohol are frequently associated with acute gastric mucosal lesions (AGML). Attention has been focused on the disturbance of gastric microcirculation and ischemia-reperfusion of the gastric mucosa (9, 19, 22, 36, 37). Gastric mucosal blood flow is regulated by many factors, including the autonomic nervous system, arteriovenular shunts, amines, and peptides. Capsaicin-sensitive spinal afferent neurons are also clearly involved in the mucosal blood flow and gastric protection (9, 22, 36, 37). Stimulation of the sensory receptors of these afferent neurons by gastric application of capsaicin or acid enhances gastric mucosal blood flow by releasing calcitonin gene-related peptide from the nerve endings, while functional denervation by pretreatment with high doses of capsaicin reduces the mucosal blood flow and worsens the gastric damages.

AGML is accompanied with bleeding and infiltration of inflammatory cells in the gastric mucosa. Pulverized erythrocytes release heme, a prooxidant. Neurotrophils produce the superoxide radical anion, one of the reactive oxygen species (ROS). The superoxide radical anion reacts with cellular lipids, leading to the breakdown of the polyunsaturated fatty acids to yield a broad array of the reactive short chain aldehydes such as malondialdehyde (MDA) and 4-hydroxynonenal (HNE; Refs. 41, 47). In fact, generation of MDA and HNE was observed in animal models of AGML (20, 21). HNE is known to be a major aldehyde produced during peroxidation of ω6-polyunsaturated fatty acids such as linoleic acid and arachidonic acid. HNE reacts with proteins and enzymes, resulting in their modification and inactivation. The major effects of HNE produced during oxidative stress are believed to be damage to cellular components, while recent studies (2, 10, 11, 12, 13, 15, 27) have shown that HNE at nontoxic levels can potently activate stress response pathways such as MAPK and nuclear translocation of a redox-sensitive transcriptional factor, NF-E2-related factor (Nrf2). The development of specific antibodies against protein-bound HNE has made it possible to obtain highly probable evidence for the occurrence of oxidative stress in vivo (40).

The involvement of heat shock protein (HSP) families in many pathological conditions including gastric lesions has been extensively studied (31, 35, 39, 42). HSPs are classified into several families according to their apparent molecular weights and respective inducers. The involvement of HSP70 has been specifically studied in AGML. Some studies (1, 3, 7, 8, 28, 45) showed the involvement of HSP32, one of the small HSP families and regarded as heme oxygenase (HO)-1 (29) in gastrointestinal diseases. HO-1 is the inducible isoform of HO that catalyzes the first and rate-limiting step in heme degradation to produce equimolar quantities of biliverdin, carbon monoxide (CO), and free iron. Biliverdin is subsequently converted to bilirubin via the action of biliverdin reductase, and free iron is promptly sequestered into ferritin. CO and other toxic agents at low concentrations exert distinctly different effects on physiological and cellular functions. CO leads to
vasodilation and inhibition of platelet aggregation (33). In
addition, bilirubin exhibits the highest endogenous antioxidant activity among the constituents of normal human serum (6). Sequestration of free iron by ferritin lowers the prooxidant state of the cell. HO-1-dependent release of iron also resulted in the upregulation of ferritin (43). Therefore, the induction of HO-1 can provide cytoprotection against oxidative stress.

In this study, we focused on the involvement and localization of HNE, Nrf2, and HO-1 in an animal model of AGML, as a means of examining possible protective mechanisms against mucosal injury and oxidative stress in the stomach. The results obtained indicated an increase in HNE in the injured mucosal cells and the macrophages surrounding the lesions, followed by translocation of Nrf2 into the nucleus and upregulation of HO-1 in the macrophages located in the margin of the injured mucosa and in the submucosa. Capsaicin-sensitive afferent neurons and alterations of the microcirculation were found to be deeply involved in this process. Inhibition of HO-1 activity was aggravated the lesion, suggesting that HO-1 induced by primary injury and oxidative stress scavenges the prooxidant heme and promotes the regeneration of mucosal tissues and enhances the resistance against further oxidative stress.

MATERIALS AND METHODS

Tissue preparation. Wistar male rats, 5 wk old, were purchased from Kiwa Laboratories (Wakayama, Japan). Animals were housed in a temperature-controlled environment. Experiments were performed after the rats were allowed free access to food and water for 1 wk. The rats were fasted overnight before each study in individual wire-bottom cages. The rats were administered intrastrally (by gastric intuba-

tion) 0.6 N HCl (0.4 ml/100 g) without anesthesia according to the methods of Tsuji et al. (38). The rats were immediately decapitated at 15, 30, 60, and 90 min and 2, 3, 4, 6, and 24 h after application of HCl (n = 5 at each time point). Five fasted rats served as controls. The stomach was rapidly removed and gently rinsed with saline, and several tissue sections were punched out and immediately frozen using powdered dry ice within 1 min after decapitation. The rest of the tissues were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4, overnight at 4°C and then cryoprotected in PBS containing 30% sucrose for 3 days at 4°C. The tissue samples were mounted in optimal cutting temperature compound (Tissue-Tek, Sakura Finetechical, Tokyo, Japan) and frozen using powdered dry ice. The frozen samples were stored at −80°C until sectioned and assayed. All animal manipulations were approved by the Wakayama Medical College Animal Care and Use Committee.

Estimation of gastric mucosal lesions. The stomach was opened along the greater curvature, gently rinsed with saline, and then pinned open to expose the gastric mucosa. The gross appearance of the gastric mucosa was recorded by digital camera (FinePix30i, Fuji Film, Tokyo, Japan). Mucosal lesions were assessed with the aid of anatomical mapping and tracing software NeuroLucida with NeuroExplorer Ver. 5.05.4 (MicroBright Field, Williston, VT). In short, the digitized images were transferred to a Dell personal computer, and the border of lesions and the total surface area of the gastric mucosa were traced on a computer by a single observer who was unaware of the treatments. The injured area per total mucosal surface area was calculated.

Pharmacological treatment with the PGL analog beraprost. Beraprost sodium (a gift from TORAY, Tokyo, Japan and Kaken Pharmaceutical, Tokyo, Japan) was dissolved in saline. The dose of beraprost sodium was chosen according to our previous study (32) in which pretreatment with beraprost prevented the water immersion-restraint stress induced gastric lesions in rats. The drugs or an equivalent volume of saline as control was administered intragastrically (by gastric intubation). The fasted rats (n = 20) were divided into four groups. In group A (n = 5), saline was administered; 30 min later saline was again administered. In group B (n = 5), beraprost sodium (0.1 mg/kg) was administered; 30 min later saline was again administered. In group C (n = 5), saline was administered; 30 min later 0.6 N HCl (0.4 ml/100 g) was administered. In group D (n = 5), beraprost sodium (0.1 mg/kg) was administered; 30 min later 0.6 N HCl (0.4 ml/100 g) was administered. The rats were decapitated at 2 h after application of HCl or saline.

Functional denervation of capsaicin-sensitive afferent neurons. The rats with functional denervation of capsaicin-sensitive afferent neurons were prepared according to the method of Horie et al. (9). Capsaicin (Sigma, St. Louis, MO) was dissolved in vehicle containing 10% ethanol, 10% Tween 80, and 80% saline. Rats received capsaicin (125 mg/kg sc) over 3 days, with a dose of 25 mg/kg on the first day and 50 mg/kg on the second and third day. Control rats received an equal volume of vehicle in the same way. The rats were pretreated intramuscularly with terbutaline (0.1 mg/kg) and aminophylline (10 mg/kg) to counteract the respiratory impairment associated with capsaicin injection. To check the effectiveness of the treatment, a drop of 0.1% solution of capsaicin was instilled into each eye of a rat and its protective wiping movements were counted. Rats with functional denervation (n = 10) were administered intragastrically (by gastric intubation) saline (n = 5; group E) or 0.6 N HCl (0.4 ml/100 g; n = 5; group F). The rats were decapitated at 2 h after application of HCl or saline.

Pharmacological treatment with zinc protoporphyrin IX. Zinc protoporphyrin IX (ZnP; BIOMOL Research Laboratories, Plymouth Meeting, PA), an HO-1 inhibitor (4, 24, 46), was dissolved in 100% ethanol and 10-fold diluted in 7% NaHCO3. The fasted rats intraperitoneally received vehicle (n = 10) or ZnP (50 µg/kg; n = 10), 60 min before administration of 0.6 N HCl (0.4 ml/100 g). The rats were decapitated at 3 and 6 h (n = 5 for the saline group and ZnP group, respectively) after application of HCl.

Western blotting. The frozen tissues were minced, homogenized in buffer containing 0.01 M Tris·HCl pH 7.6, 0.15 M NaCl, 1% Triton X-100, and protease inhibitor cocktail (25), the frozen tissues were homogenized in STE buffer containing the following (in mmol/l): 320 sucrose, 10 Tris HCl pH 7.4, 1 EGTA, 5 NaN3, 10 NaF, and 0.05% Tween 20. The homogenates were centrifuged at 1,000 g for 15 min at 4°C. For subcellular fractionation (25), the frozen tissues were homogenized in STE buffer containing the following (in mmol/l): 320 sucrose, 10 Tris·HCl pH 7.4, 1 EGTA, 5 NaN3, 10 β-mercaptoethanol, 50 NaF, and protease inhibitor cocktail. The homogenates were centrifuged at 1,000 g for 10 min at 4°C, and the supernatant was designated as the cytosolic fraction. Nuclear extract was obtained by treating the pellets with STE buffer containing 1% Triton X-100 followed by centrifugation at 10,000 g for 10 min at 4°C. Protein concentrations were determined by Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, CA).

The samples were subjected to SDS-polyacrylamide gel (12.5%) electrophoresis and then immunoblotted. After blocking with skim milk, the blots were incubated with the primary antiserum against HNE (mouse monoclonal, J2; NOF, Tsukuba, Japan) at a concentration of 1 µg/ml, the primary antiserum against Nrf2 (rabbit polyclonal, C-20; Santa Cruz Biotechnology, Santa Cruz, CA) diluted 1:1,000, or HO-1 (rabbit polyclonal, SPA-895; Stressgen, Victoria, Canada) diluted 1:20,000, or antiserum against actin (rabbit polyclonal, A5060; Sigma) diluted 1:200, or antiserum against histone H1 (rabbit polyclonal, FL-219; Santa Cruz) diluted 1:200 with TBST (0.01 M Tris·HCl pH 7.6, 0.15 M NaCl, and 0.05% Tween 20) containing 1% BSA overnight at 4°C. After the samples were washed in TBST, they were incubated with peroxidase-labeled anti-mouse antibody (NA931; Amersham Biosciences UK limited, Buckinghamshire, UK) or peroxidase-labeled anti-rabbit antibody (NA934; Amersham) diluted 1:5,000 in TBST containing 1% BSA for 1 h at 37°C. After being washed with TBST, the reaction was visualized by an ECL Western blotting detection kit (Amersham). The signals on the immunoblots were measured by Lumino analyzer LAS-1000 plus.
(Fuji Film, Tokyo, Japan). The protein levels of targets were determined from a relative standard curve constructed by plot of the band densities and normalized by those of actin.

**Immunohistochemistry.** Frozen sections of 6 μm in thickness were cut in a cryostat and thaw mounted onto silane-coated slides. The sections were incubated with 3% H2O2 in distilled water for 20 min to quench the endogenous peroxidase activity. After being rinsed twice with PBS, the sections were incubated with the primary monoclonal antibody against HNE (mouse monoclonal, J2) at a concentration of 1 μg/ml, diluted with 0.1 M PBS containing 5% normal horse serum and 0.3% Triton X-100, or primary antiserum against Nr2 (rabbit polyclonal, C-20), or primary antiserum against HO-1 (rabbit polyclonal, SPA-895) diluted 1:1,000 with 0.1 M PBS containing 5% normal goat serum and 0.3% Triton X-100 for 48 h at 4°C. The omission of the primary or secondary antibody completely eliminated all immunoreactive staining. After being washed in PBS, the sections were incubated with the secondary antibody (biotinylated horse anti-mouse IgG or biotinylated goat anti-rabbit IgG, respectively; Vector Laboratories, Burlingame, CA) diluted 1:200 with 0.1 M PBS containing 5% normal horse serum and 0.3% Triton X-100 for 1 h at 37°C. After being rinsed twice with PBS, they were incubated with avidin-biotin-horseradish peroxidase complex (ABC Elite kit, Vector Laboratories) for 1 h at 37°C. After being washed in 0.05 M Tris-HCl buffer pH 7.6, immunoreactions were visualized by incubation in 0.05 M Tris-HCl buffer pH 7.6 containing 0.02% 3,3′-diaminobenzidine tetrahydrochloride and 0.005% H2O2 for 2–5 min. The sections were counter stained with hematoxylin.

For double fluorescence immunohistochemistry, sections were incubated simultaneously with the primary antiserum against HO-1 (rabbit polyclonal, SPA-895) diluted 1:1,000 and monoclonal antibody against macrophages ED1 (CD68) and ED2 (CD163) (mouse monoclonal; Affinity Bioreagents, Golden, CO) diluted 1:200 with 0.1 M PBS containing 5% normal goat serum and 0.3% Triton X-100 or with the antibody against HNE (mouse monoclonal, J2) at a concentration of 1 μg/ml for 48 h at 4°C. After being rinsed twice with PBS, the sections were incubated with the secondary antibody (biotinylated goat anti-rabbit IgG; Vector Laboratories) diluted 1:200 in PBS for 1 h at 37°C. Finally, they were incubated in 1:100 dilution of Texas red avidin (Vector Laboratories) simultaneously with 1:100 dilution of FITC-conjugated goat anti-mouse IgG antibody (Jackson ImmunoResearch Laboratories, West Grove, PA) in 0.1 M PBS containing 5% normal goat serum and 0.3% Triton X-100 for 1 h at 37°C. In some cases, nuclear staining by DAPI (KPL; Gaithersburg, MD) was performed. They were rinsed twice with PBS and coverslipped with an anti-fade solution (VECTASHIELD; Vector Laboratories).

**Semiquantitative estimation of immunohistochemical signals for HNE.** Immunohistochemical signals for HNE were semiquantitatively assessed with the aid of the anatomical mapping and tracing software NeuronLucida with NeuroExplorer Ver. 5.05.4 (MicroBrightField). In short, the digitized images were transferred to a Dell personal computer, and the border of the HNE-immunopositive area and the total area of the gastric mucosa were traced on a computer by a single observer who was unaware of the treatments. The immunopositive area per total mucosal area was calculated.

**Real-time RT-PCR.** Total RNA was extracted from punch-out gastric tissues were extracted by RNasey mini kit (QIAGEN, Tokyo, Japan) and digested with RNase free-DNase (QIAGEN). Expression of Nr2 and HO-1 mRNAs was determined by real-time RT-PCR. Primers were made using the following sequences based on the nucleotides 718-979 of rat HO-1 mRNA (34): 5′-aagagctgacagccgcc-3′ (forward) and 5′-gcataattccactgaccc-3′ (reverse); the nucleotides 556-751 of rat Nr2 mRNA (27) and 5′-GAAGCCGCATTGACTGAT-3′ (forward), 5′-GGGATCGATGAGTAA-3′ (reverse). As an internal control, we also estimated the expression of rat GAPDH mRNA using the following sequences based on the nucleotides 904-1,034 of rat GAPDH mRNA (29); 5′-AGGTGTTGTCCTCCGTGACTCTC-3′ (forward) and 5′-CTGTTGGCTGTACCATTC-3′ (reverse). Total RNA (0.1 μg) was converted into cDNA by reverse transcription using random primer p(dN)6 and pOligo-p(T)15 primers and AMV reverse transcriptase (Roche Diagnostics, Indianapolis, IN) in a total reaction volume of 20 μl. PCR amplification using a LightCycler instrument was carried out in 20 μl of reaction mixture consisting of LightCycler FastStart DNA Master SYBR Green I (Roche Diagnostics, Indianapolis, IN) in a total reaction volume of 20 μl. PCR amplification using a LightCycler instrument was carried out in 20 μl of reaction mixture consisting of LightCycler FastStart DNA Master SYBR Green I (Roche Diagnostics, Indianapolis, IN). Each probe, and 2 μl of template cDNA in a LightCycler capillary. The relative mRNA in each sample was then quantified automatically by reference to the standard curve constructed each time according to the LightCycler software. The levels of mRNA were calculated with reference to external standard curves constructed by plotting the log number of 10-fold serial diluted cDNA samples against the respective threshold cycle with second derivative maximum method. Each product showed a single band corresponding to the estimated molecular size (196-bp Nr2 mRNA, 262-bp HO-1 mRNA, and 309-bp GAPDH). The expression of mRNA level in each sample was normalized against its GAPDH mRNA level.

**Fig. 1.** Expression and localization of 4-hydroxyynonenal (HNE) in acute gastric mucosal lesions (AGML). Western blotting showed that the intensities of HNE-immunoreactive bands were increased after administration of HCl (A). B, C: light photomicrographs of gastric mucosa in 2 h after HCl administration. Immunoreactivities for HNE were observed on scattered cells in damaged and surrounding areas. Omission of the primary antibody completely eliminated immunoreactivity (C). Scale bars = 20 μm (B and C).
In situ hybridization histochemistry. Frozen sections of 6 μm in thickness were cut in a cryostat and thaw mounted onto silane-coated slides. The probe for detection of HO-1 mRNA was complementary to the nucleotides 276-315 of rat HO-1 mRNA (34; gctctatctcctcttc-cagggccgtatagatatggtacaa). A computer-assisted homology search revealed no identical sequences in any rat genes in the database (GenBank). In situ hybridization histochemistry (ISH) was performed as we described previously (32). The slides were coated with K-5 emulsion (Ilford, Knutsford, UK) diluted 1:2 with water for autoradiography and then exposed for 6–10 wk at 4°C. Slides were developed in D-19 (Kodak, Rochester, NY), and the sections were counter stained with hematoxylin-eosin for morphological examinations.

Data analysis. Statistical analysis was performed by one-way and two-way ANOVA followed by Fisher’s protected least significant difference test, or Student’s t-test, and Pearson’s correlation coefficient using StatView software (Abacus Concepts, Berkeley, CA).

RESULTS

At 15 min after administration of HCl, bloody clots and linear hemorrhages were present on the surface of the damaged mucosa. They reached a maximal level by 1 h and remained at the same severity until 6 h. At 24 h, the gastric mucosa appeared almost normal. Microscopically, desquamation of the luminal cells and deep necrotic lesions were observed. Deep necrotic lesions progressed into the gastric gland cells beyond the gastric pits and were seen as early as 15 min after injury. After 6 h, disrupted superficial epithelial cells were mostly restored to normal, except for those with deep mucosal defects. Twenty-four hours later, mucosal lesions were nearly restituted. Edema of the submucosa persisted from 15 min to 6 h. These findings corresponded with the previous studies (42).

As shown in Fig. 1A, the intensities of HNE-immunoreactive bands were increased after administration of HCl. The molecular mass of the major band was ~35 kDa. Immunohistochemical study showed that HNE-immunoreactivity was observed in the scattered cells in the injured mucosa (Fig. 1B). The omission of the primary antibody completely eliminated the immunoreactivity (Fig. 1C). These data indicate an increase of

Fig. 2. Expression and localization of NF-E2-related factor (Nrf2) in AGML. Real-time RT-PCR showed that relative Nrf2 mRNA levels after administration of HCl were not altered (A). Western blotting showed that the intensities of Nrf2 signals in the nuclear fraction (histone H1 fraction) were increased at 1 and 3 h, while those in the cytosolic fraction were decreased at 3 h (B), indicating translocation of Nrf2 from the cytosolic fraction to the nuclear fraction. C1, C2: light photomicrographs of gastric mucosa at 2 h after HCl administration. Nrf2 immunoreactivities were observed on scattered cells in damaged and surrounding areas (C1). Omission of the primary antibody completely eliminated immunoreactivity (C2). D, E, F, G, H, I, J, and K: double fluorescence immunohistochemistry of gastric mucosa. In area surrounding lesion, Nrf2 immunoreactivities (D, E, G1, and H1, colored in red) were colocalized with ED1 (D and G2, colored in green) or ED2 immunoreactivity (E and H2, colored in red), suggesting some of Nrf2-positive cells are macrophage (indicated with arrow in D, E, or merged in G3 and H3). Nrf2-positive cells (F and H1, colored in red) are also HNE-positive (F and H2 colored in green), suggesting a possible interaction between HNE and Nrf2 (indicated with arrow in F or merged in I3). Nrf2 immunoreactivity (J1, colored in red) did not overlap with DAPI in cell (J2, colored in blue) taken from control rats (J3), while it (K1) did overlap with DAPI (K2, colored in blue) in cells at 3 h (K3), indicating that Nrf2 was translocated into nucleus. Scale bars = 20 μm (C–F). Scale bars = 2 μm (G–K).
HNE-binding proteins in these cells. HNE can also activate stress response pathways such as translocation of the redox-sensitive transcriptional factor Nrf2. Next, we focused on the expression and localization of Nrf2.

The levels of Nrf2 mRNA were not changed after AGML (Fig. 2A). Western blotting showed that the intensities of Nrf2 signals in the nuclear fraction (confirmed by the signals for histone H1) were increased at 1 and 3 h, while those in the cytosolic fraction were decreased at 3 h, indicating the translocation of Nrf2 from the cytosol to the nucleus (Fig. 2B). Nrf2 immunoreactivity was observed in the scattered cells in the injured mucosa (Fig. 2C1). The omission of the primary antibody completely eliminated the immunoreactivity (Fig. 2C2). Double fluorescence immunohistochemistry showed that Nrf2-immunoreactive cells in the margin of the lesions (Fig. 2, D, E, G1, and H1, colored in red) were both ED1 (Fig. 2, D and G2, colored in green) and ED2 positive (Fig. 2, E and H2, colored in green), indicating that they were the macrophages (Fig. 2, G3 and H3). Nrf2-immunoreactive cells in the margin of the lesions (Fig. 2, F and I1, colored in red) were also colocalized with HNE immunoreactivity (Fig. 2, F and I2, colored in green), suggesting an interaction between HNE and Nrf2 (Fig. 2F3). Nrf2 immunoreactivity (Fig. 2J1, colored in red) did not overlap with DAPI in the cells (Fig. 2J2, colored in blue) taken from control rats (Fig. 2J3), while it (Fig. 2K1) did overlap with DAPI (Fig. 2K2, colored in blue) in the cells at 3 h (Fig. 2K3), indicating that Nrf2 had translocated into the nucleus of the macrophages. One of the downstream factors from Nrf2 is considered as HO-1 (2). Next, we investigated the expression and localization of HO-1 after AGML.

The levels of HO-1 mRNA were slightly increased at 90 min, and increased 15-fold at 3 h compared with control levels. They were gradually decreased and maintained at 24 h (Fig. 3A). ISH demonstrated that signals for HO-1 mRNA were observed in the scattered cells in the lamina propria and the surrounding submucosa and muscle layer (Fig. 3, B and C). Western blotting showed that immunoreactive signals for HO-1 were gradually increased, reaching significant levels at 6 and 24 h (Fig. 4A). Clear HO-1-immunoreactive cells (red arrows) were observed at 6 h in the corresponding cells as shown by ISH (Fig. 4B). The omission of the primary antibody completely eliminated the immunoreactivity (Fig. 4C). Double fluorescence immunohistochemistry showed that HO-1-immunoreactive cells (Fig. 4, D, F1, and G1, colored in red) were both ED1 (Fig. 4, D and F2, colored in green) and ED2 positive (Fig. 4G2, colored in green), indicating that they were the macrophages (Fig. 4, F3 and G3). Signals for HNE (Fig. 4E, colored in green) were observed in the injured mucosa, while signals for HO-1 (Fig. 4E) were observed in the surrounding area. In the marginal zone, colocalization of HNE (Fig. 4, E and H2, colored in green) and HO-1 (Fig. 4, E and H1, colored in red) was observed (Fig. 4H3). Taken together, these findings suggested the HNE was produced in the injured mucosa and the surrounding macrophages, followed by translocation of Nrf2 and induction of HO-1 in the macrophages.

To elucidate the contribution of mucosal microcirculation and of capsaicin-sensitive afferent neurons on the generation of AGML and expression of HO-1, we used two pharmacological approaches. First, the improvement of mucosal microcirculation by pretreatment with the PGl2 analog beraprost, which

![Image](http://ajpgi.physiology.org/)
the immunoreactive signals for HNE and HO-1 mRNA levels ($r = 0.625; n = 15; P < 0.01$; Fig. 5, E and F).

To elucidate the functional role of HO-1 induction after AGML, we used pharmacological treatment with the HO-1 inhibitor ZnPP. Pretreatment with ZnPP significantly exacerbated HCl-induced AGML at 3 and 6 h (Fig. 6).

**DISCUSSION**

Generation of ROS and oxidative stress are crucial in the pathogenesis of AGML. Antioxidative stress mechanisms have been investigated in HCl-induced AGML models. In this study, an increase of HNE-binding proteins was observed in the injured mucosa and the surrounding macrophages, followed by translocation of Nrf2 into the nucleus and upregulation of HO-1 in the macrophages. Capsaicin-sensitive afferent neurons and alteration of the microcirculation were deeply involved in this process. The lesion was aggravated by inhibition of HO-1, suggesting that HO-1 scavenges the prooxidant heme, protects the mucosal tissues, and promotes the regeneration of mucosal tissues.

Generation of MDA and HNE was observed in animal models of AGML (21, 22). However, previous studies did not show the localization of these products and functional implications. By use of a HNE-specific monoclonal antibody, we demonstrated that HNE-binding proteins were increased after AGML. However, it is hard to detect nonbinding HNE in vivo because nonbinding HNE is unstable and rapidly binds to the proteins. Although we did not show the increase of nonbinding HNE, the increase of HNE-binding proteins suggests that the generation of HNE is increased in the injured stomach as in previous studies (21, 22). HNE immunoreactivity was observed in the injured mucosa and the surrounding cells, suggesting that HNE was mainly produced in these cells. HNE-immunoreactive scattered cells located in the margin of the lesion were identified as macrophages in successive experiments.

![Fig. 4. Expression and localization of immunoreactivity for HO-1 in AGML.](image)
The transcriptional factor Nrf2 is sequestered in the cytoplasm by Kelch-like ECH-associated protein, Keap1, under unstimulated conditions but translocates into the nucleus and transactivates the electrophilic response element/antioxidant response element (EpRE/ARE) upon exposure to oxidative insults (5, 14, 16, 18, 23, 44). In this AGML model, mRNA levels of Nrf2 were not altered, while nuclear translocation of Nrf2 in the macrophages was demonstrated by both Western blotting and immunohistochemistry. Immunoreactivities for HNE and Nrf2 were colocalized in the macrophages located in the margin of the lesion. An increase of HNE-binding proteins was observed at 15 min, and nuclear translocation of Nrf2 was observed at 1 h. These spatial and temporal data are in accord with the notion that HNE is generated in the macrophages and triggers the nuclear translocation of Nrf2 in the macrophages. Several signal pathways, including ERK, p38 MAPK, JNK, PKC, and phosphatidylinositol 3-kinase, were shown to be involved in EpRE/ARE-mediated gene induction. The two mechanisms that have been proposed for Nrf2-Keap1 dissociation are Nrf2 phosphorylation (27) and Keap1 modification (44). Phosphorylation of Nrf2 Ser40 by atypical PKC is involved in HNE-induced liberation from the complex with Keap1, followed by the nuclear translocation of Nrf2 and induction of HO-1 (27). HNE-induced nuclear translocation of Nrf2 and induction of $\gamma$-glutamyl transpeptidase (GGT), one of the antioxidant and phase II enzymes in the lung type II epithelial cells was mediated by both ERK and p38 MAPK but not PKC or phosphatidylinositol 3-kinase (48).

The expression of HO-1 was observed in gastrointestinal diseases such as inflammatory colitis and gastric ulcers (1, 3, 7, 8, 28, 45). HO-1 is highly induced in response to a variety of oxidative stressors, such as heme, metals, hypoxia, ischemia, hyperthermia, and inflammation, which facilitate the generation of ROS. ROS initiate chain reactions, resulting in lipid peroxidation and generation of biologically active aldehydes such as HNE. In this study, mRNA signals for HO-1 and immunoreactivity for HO-1 were observed in the ED1- and ED2-positive macrophages and HO-1 immunoreactivity was

Fig. 5. Evaluation of mucosal lesions, expression of HNE and HO-1 mRNA after pharmacological treatment with the PGI$_2$ analog beraprost, or functional denervation with a high dose of capsaicin. A: estimation of lesion. Pretreatment with beraprost significantly improved the HCl-induced lesion index, while functional denervation aggravated the mucosal lesion. $^*P < 0.01$, vehicle/HCl vs. PGI$_2$/HCl; $^P < 0.01$, vehicle/HCl vs. capsaicin/HCl. B: immunohistochemical signals for HNE in gastric mucosa. B1: vehicle/HCl. B2: vehicle/vehicle. B3: PGI$_2$/HCl. B4: PGI$_2$/vehicle. B5: capsaicin/HCl. B6: capsaicin/vehicle. Scale bar = 20 µm. C: semiquantitative analysis of immunohistochemical signals for HNE. Pretreatment with beraprost significantly reduced signals for HNE, while functional denervation increased signals. $^1P < 0.01$, vehicle/HCl vs. PGI$_2$/HCl; $^2P < 0.01$, vehicle/HCl vs. capsaicin/HCl. D: estimation of HO-1 mRNA levels by use of real-time RT-PCR. Expression of HO-1 mRNA on basal states was not influenced by pharmacological treatments, while a significant increase of HO-1 mRNA levels was observed on application of HCl. Pretreatment with beraprost attenuated HCl-induced upregulation of HO-1 mRNA levels, while functional denervation significantly augmented HCl-induced upregulation of HO-1 mRNA levels. $^*P < 0.01$, vehicle/vehicle vs. vehicle/HCl; $^1NS$, PGI$_2$/vehicle vs. PGI$_2$/HCl; $^2P < 0.01$, vehicle/HCl vs. capsaicin/HCl; $^3P < 0.01$, vehicle/HCl vs. capsaicin/HCl; $^4NS$, capsaicin/vehicle vs. capsaicin/HCl; $^5P < 0.01$, vehicle/HCl vs. capsaicin/HCl; $^6P < 0.01$, PGI$_2$/HCl vs. capsaicin/HCl. E: correlation between lesion index and immunoreactive signals for HNE. F: correlation between the immunoreactive signals for HNE and HO-1 mRNA levels. A significant positive correlation was noted between lesion indexes and immunoreactive signals for HNE (E) and between the immunoreactive signals for HNE and HO-1 mRNA levels (F). Pearson’s correlation coefficients are 0.542 (E) and 0.625 (F), respectively ($n = 15$; $P < 0.01$).
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of HO-1 was observed in the mucosal cells but not in the

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(45). In this AGML model, induction of inducible NOS mRNA

mRNA was observed at 3 h, and induction of HO-1

sulfonic acid-induced colitis in rats, induction of inducible

oxide (NO) is also an inducer of HO-1 in the endothelial cell

involved in HO-1 induction in gastric macrophages. Nitric

oxide (NO) is also an inducer of HO-1 in the endothelial cell and

vascular smooth muscle cells (30). In trinitrobenzene

sulfonic acid-induced colitis in rats, induction of inducible NOS mRNA was observed at 3 h, and induction of HO-1 mRNA was observed at 6 h and reached the maximum at 72 h (45). In this AGML model, induction of inducible NOS mRNA was not observed but generation of nitrotyrosine was observed (data not shown). Thus the NO-mediated pathway might likewise be involved in HO-1 expression. Precise analysis of the NO-mediated pathway is currently under investigation in our laboratories. In the NSAID-induced AGML model, induction of HO-1 was observed in the mucosal cells but not in the macrophages (1). HO-1 immunoreactivity was also observed partially in the injured mucosal cells in this HCl-induced AGML model. These discrepancies may be a consequence of differences in the type of the mucosal injury in the two models.

The importance of mucosal microcirculation and capsaicin-sensitive afferent neurons has been emphasized (9, 19, 22, 36, 37). Indeed, pretreatment with the PG1\textsubscript{2} analog beraprost provided effective protection against HCl-induced gastric mucosal lesioning, while denervation of capsaicin-sensitive afferent neurons exacerbated lesioning. In accordance with the severity of lesions, the levels of the immunoreactive signals for HNE were increased, as indicated by a significant positive correlation between the lesion indexes and immunoreactive signals for HNE. A significant positive correlation between immunoreactive signals for HNE and HO-1 mRNA levels was also observed. Although these positive correlations do not always confirm the causal relationship be-

tween the generation of HNE and HO-1 induction, these results suggest that the induction of HO-1 is dependent on the levels of severity of the lesion and generation of HNE.

Recent studies (17, 26) with HO-1 knockout mice have demonstrated that HO-1 ameliorates the tissue damage. In this study, we used a specific inhibitor of HO-1 (ZnPP) to evaluate the physiological significance of HO-1 in AGML. Inhibition of HO-1 aggravated HCl-induced lesioning, suggesting that HO-1 scavenges the prooxidant heme and promotes the regeneration of mucosal tissues. Here, a significant increase of HO-1 protein was observed at 6 h, while the effects of ZnPP treatment were already observed at 3 h. Hence, ZnPP inhibits the activity of HO-1, which is expressed in the normal mucosa. The increased level of HO-1 protein was observed at 24 h when mucosal lesions had mostly been repaired. From this, we concluded that in response to primary injury, the gastric mucosa acquires a resistance to successive insults by an upregulation of HO-1 particularly in the macrophages. However, further evidence will be required to propose a possible role for HO-1 in protecting against gastric mucosal injury.

The scheme in Fig. 7 summarizes the present results and the possible mechanism and functional significances of our data. Both heme released from pulverized erythrocytes and superoxide radical anions produced by neutrophils triggered the oxidative stress response and generation of HNE in the injured mucosal cells and the surrounding macrophages. Both HNE released from the injured mucosal cells and HNE produced in the macrophages upregulated HO-1 levels in the macrophages. Heme is metabolized by HO-1, resulting in the formation of equimolar quantities of biliverdin, CO, and free iron. CO improves the microcirculation via vasodilation and inhibition of platelet aggregation (33). Bilirubin has endogenous antiox-
oxidant activity (6). Ferritin traps free iron and HO-1-dependent release of iron also results in the upregulation of ferritin (43). Therefore, the induction of HO-1 can provide cytoprotection against oxidative stress. The disturbance of the microcirculation and generation of ROS are deeply implicated in the expression of HNE and HO-1 and the pathogenesis of AGML. In conclusion, our data demonstrate that the HNE-Nrf2-HO-1 pathway in macrophages is an adaptive defense mechanism against oxidative stress in response to AGML.

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