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Lansoprazole, a Proton Pump Inhibitor, Mediates Anti-Inflammatory Effect in Gastric Mucosal Cells through the Induction of Heme Oxygenase-1 via Activation of NF-E2-Related Factor 2 and Oxidation of Kelch-Like ECH-Associating Protein 1

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ABSTRACT

Induction of heme oxygenase-1 (HO-1) expression has been associated with cytoprotective and anti-inflammatory actions of lansoprazole, a proton pump inhibitor, but the underlying molecular mechanisms remain largely unresolved. In this study, we investigate the role of transcriptional NF-E2-related factor 2 (Nrf2), its phosphorylation/activation, and oxidation of Kelch-like ECH-associating protein 1 (Keap1) in lansoprazole-induced HO-1 up-regulation using cultured gastric epithelial cells (rat gastric mucosal cell line, RGM-1). HO-1 expression of RGM-1 cells was markedly enhanced in a time- and dose-dependent manner by the treatment with lansoprazole, and this up-regulation of HO-1 contributed to the inhibition of chemokine production from stimulated RGM-1 cells. Transfection of Nrf2-siRNA suppressed the lansoprazole-induced HO-1. An electrophoretic mobility shift assay showed increases in the nuclear translocation and stress-response elements (StRE) binding activity of Nrf2 proteins in RGM-1 cells treated with lansoprazole. Furthermore, in RGM-1 cells transfected with HO-1 enhancer luciferase reporter plasmid containing mutant StRE, lansoprazole-induced *HO-1* reporter gene activity was diminished. Lansoprazole promoted the phosphorylation of extracellular signal-regulated kinase (ERK), and lansoprazole-induced HO-1 up-regulation was suppressed by U0126, an ERK-specific inhibitor. Phosphorylated Nrf2 protein was detected in the phosphoprotein fraction purified by a Pro-Q Diamond Phosphoprotein Enrichment kit. Finally, an oxidative form of the Keap1 protein was detected in lansoprazole-treated RGM-1 cells by analyzing *S*-oxidized proteins using biotinylated cysteine as a molecular probe. These results indicate that lansoprazole up-regulates HO-1 expression in rat gastric epithelial cells, and the up-regulated HO-1 contributes to the anti-inflammatory effects of the drug. Phosphorylation of ERK and Nrf2, activation and nuclear translocation of Nrf2, and oxidation of Keap1 are all involved in the lansoprazole-induced HO-1 up-regulation.

Proton pump inhibitors (PPIs) such as lansoprazole and omeprazole are extensively used to treat acid-related disorders, including gastroesophageal reflux disease and peptic ulcer disease caused by stress, nonsteroidal anti-inflammatory drugs, and *Helicobacter pylori* infection. PPIs are strong

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ABBREVIATIONS: PPI, proton pump inhibitor; HO-1, heme oxygenase-1; HO, heme oxygenase; LPS, lipopolysaccharide; MAPK, mitogen-activated protein kinase; Nrf2, NF-E2-related factor 2; Keap1, Kelch-like ECH-associating protein 1; SnPP, tin-protoporphyrin; U0126, 1,4-diamino-2,3-dicyano-1,4-bis-(methylthio)butadiene; ERK, extracellular signal-regulated kinase; JNK, Jun N-terminal kinase; SP600125, anthra[1,9-*cd*] pyrazol-6 (2*H*)-one; IL, interleukin; CINC-1, cytokine-induced neutrophil chemoattractant-1; ELISA, enzyme-linked immunosorbent assay; PBS, phosphate-buffered saline; HRP, horseradish peroxidase; PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis; PVDF, polyvinylidene difluoride; TBS-T, Tris-buffered saline and 0.1% Tween 20; ECL, enhanced chemiluminescence; siRNA, small interfering RNA; EMSA, electrophoretic mobility shift assay; StRE, stress-responsive DNA element; bZIP, basic-leucine zipper; SB203580, 4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)-1*H*-imidazole; GRO, growth-regulated oncogene.

anti-secretory agents that act on gastric H^+/K^+-adenosine triphosphatase (H^+/K^+ ATPase) of parietal cells (Fellenius et al., 1981). In addition to their acid-suppressing effects, PPIs, especially lansoprazole, have been shown to modulate the inflammatory status, reduce oxidative stress, and ameliorate mucosal injuries in the esophagus (Yoshida et al., 2004), intestine (Ichikawa et al., 2004), and lung (Hendriks et al., 2001), in addition to the stomach (Becker et al., 2006). It has been demonstrated by in vitro studies that PPIs inhibit the increased expression of vascular adhesion molecules, the activation of neutrophils, and the production of proinflammatory cytokines (Yoshida et al., 2000; Handa et al., 2004). We recently demonstrated using in vivo models that lansoprazole inhibits acute inflammatory reactions as well as intestinal mucosal injuries induced by ischemia-reperfusion (Ichikawa et al., 2004). Our recent study using a DNA microarray clearly showed that lansoprazole induces several genes, including phase II detoxifying enzyme (NADPH-ubiquinone oxidoreductase, glutathione *S*-transferase) and antioxidant stress proteins [heme oxygenase-1 (HO-1), thioredoxin reductase, and superoxide dismutase) in gastric epithelial cells (Naito, JCBN2007, http://www2.kpu-m.ac.jp/%7Efirstmed/GeneChip.html]. Becker et al. (2006) also demonstrated that PPIs protect gastric epithelial cells against oxidative stress, and this protection is abrogated in the presence of an HO-1 inhibitor. These data indicate that lansoprazole-induced HO-1 induction might account for the cytoprotective and anti-inflammatory effects of lansoprazole independent of acid-secretion inhibition.

Heme oxygenase (HO) is involved in heme catabolism, a process in which the oxidation of heme leads to the production of iron, biliverdin, and carbon monoxide (Maines, 1997). Three mammalian HO isozymes have been identified, one of which, HO-1, is a stress-responsive protein. HO-1 is highly inducible by a vast array of stimuli, including oxidative stress, heat shock, ultraviolet radiation, ischemia-reperfusion, heavy metals, bacterial lipopolysaccharide (LPS), cytokines, nitric oxide, and its substrate, heme (Shibahara, 1988; Sassa, 2006). This strong adaptive response of HO-1 to various stimuli suggests an entirely new paradigm in which HO-1 could play a significant role in protection against inflammatory processes and oxidative tissue injury.

Recent studies have extensively investigated the transcriptional factors and regulatory regions that are responsible for induction of the *ho-1* gene. Several signaling molecules [e.g., mitogen-activated protein kinases (MAPKs)] and transcriptional regulators [activator protein-1, NF-E2-related factor 2 (Nrf2), hypoxia-inducible factor-1, Bach-1] participate in the regulation of the *ho-1* gene. In these molecules, accumulating data implicate Nrf2 as a key regulator of the adaptive response to oxidative stress (Alam et al., 1999; Itoh et al., 1999; Ishii et al., 2000; Itoh et al., 2003) and of the transcriptional activation of *ho-1* (Ryter and Choi, 2005).

Under normal conditions, Nrf2 localizes in the cytoplasm, where it interacts with the actin-binding protein, Kelch-like ECH-associating protein 1 (Keap1), and is rapidly degraded by the ubiquitin-proteasome pathway (Sekhar et al., 2002). Namely, Keap1 acts as negative regulator of Nrf2. Various stimuli, including electrophiles and oxidative stress, liberate Nrf2 from Keap1, allowing Nrf2 to translocate into the nucleus and to bind to stress- or antioxidant-response elements (He et al., 2001). Nuclear translocation of Nrf2 provides immediate transactivation of regulated encoding genes. In this sequence of Nrf2 activation, the phosphorylation of Nrf2 is an important event in the dissociation of Nrf2 from Keap1 (Bloom and Jaiswal, 2003; Numazawa et al., 2003). Furthermore, it has been demonstrated that the oxidation of Keap1 causes a change in the affinity of Keap1 with Nrf2, easily releasing Nrf2 (Dinkova-Kostova et al., 2002).

Thus, the Nrf2-Keap1 system is considered as a major defense mechanism that plays a key role in the induction of HO-1. However, it remains unclear whether lansoprazole is involved in the Nrf2-Keap1 system, or in the subsequent induction of HO-1, which is a potent anti-inflammatory enzyme. In the present study, we investigated the molecular mechanism of the anti-inflammatory action of lansoprazole mainly by using cultured gastric epithelial cells. In the analysis of the up-regulation of HO-1 by lansoprazole, we focused on the involvement of lansoprazole in the activation and phosphorylation of Nrf2 and oxidation of Keap1.

Materials and Methods

Materials. Lansoprazole (2-([3-methyl-4-(2,2,2-trifluoroethoxy)-pyridin-2-yl]methylsulfinyl)-1*H*-benzo[d]imidazole), a kind gift from Takeda Chemical Industries (Osaka, Japan), was dissolved in ethanol and the following experiments were performed as final concentration of ethanol was 0.5%. Omeprazole was obtained from Wako Pure Chemical (Osaka, Japan) and famotidine was obtained from Sigma-Aldrich Japan K.K. (Tokyo, Japan). The following inhibitors were used: Tin-protoporphyrin as an HO-1 inhibitor [tin-protoporphyrin (SnPP); Sapphire Bioscience Pty., Ltd., Redfern, Australia], mitogen-activated protein kinase kinase 1/2 inhibitor as an extracellular signal-regulated kinase (ERK) inhibitor (U0126; BIOMOL International LP, Plymouth Meeting, PA), p38 MAPK inhibitor (SB203580; BIOMOL International LP), and Jun N-terminal kinase (JNK) inhibitor (SP600125; BIOMOL International LP). Rabbit polyclonal anti-HO-1 antibody was purchased from Assay Designs (Ann Arbor, MI). The antibodies of p44/42, phosho-p44/42, p38, phosho-p38, JNK, and phosho-JNK were products of Cell Signaling Technology Inc. (Danvers, MA). Rabbit polyclonal anti-Keap1 antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). All other chemicals were of the highest quality commercially available.

Gastric Epithelial Cell Line. The rat gastric mucosal cell line RGM-1 (RCB-0876 at the Riken Cell Bank, Tsukuba, Japan), established by Matsui and Ohno, was used (Kobayashi et al., 1996). RGM-1 cells have the characteristics of gastric mucous-producing cells, and they have prostaglandin EP_4 receptors (Kobayashi et al., 1996) and do not express parietal cell-specific H^+, K^+-ATPase (Shimokawa et al., 2007). RGM-1 cells were grown in a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F12 medium supplemented with 10% heat-inactivated fetal bovine serum, 2 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin. The cells were incubated at 37°C in a humidified atmosphere with 5% CO_2. For the experiments, RGM-1 cells were trypsinized and seeded into 96-well cell culture plates or 8-well chamber glass slides. Experiments were performed when the cells were confluent.

Enzyme-Linked Immunosorbent Assay. RGM-1 cells were cultured in 96-well plates until confluent and stimulated with or without interleukin (IL)-1β (1 ng/ml; Genzyme-Techne, Cambridge, MA) and/or lansoprazole (10 μM) and/or an HO-1 inhibitor, SnPP (1 μM), for 6 h. Cytokine-induced neutrophil chemoattractant-1 (CINC-1) production (release in culture supernatants) was assessed by using an enzyme-linked immunosorbent assay (ELISA) kit (Immunobiological Laboratories, Gunma, Japan) according to the manufacturer's instructions. In brief, 100 μl of cell supernatants was placed into 96-well plates coated with rabbit anti-rat growth-regulated oncogene

(GRO)/CINC-1 IgG and incubated for 1 h at room temperature. The supernatants were then removed, and the wells were washed with 1% bovine serum albumin and 0.05% Tween 20 in phosphate-buffered saline (PBS). Subsequently, horseradish peroxidase (HRP)-conjugated rabbit-anti rat GRO/CINC-1 antibody (Fab fragments) was added, and the amount of CINC-1 was determined colorimetrically 30 min later (490-nm wavelength; MPR-A4i microplate reader; Tosoh, Tokyo, Japan) using tetra methyl benzidine as a substrate.

RNA Analysis. The mRNA expression of *ho-1*, *nrf2*, and β-*actin* as an internal control was determined by real-time polymerase chain reaction (PCR). Total RNA was isolated from intestinal mucosal tissue by the acid guanidinium phenol chloroform method with an Isogen kit (Nippon Gene Co., Ltd., Toyama, Japan), and the concentration of RNA was determined by absorbance at 260 nm in relation to that at 280 nm. The isolated RNA was stored at 70°C until used in real-time PCR. For the real-time PCR, 1 μg of extracted RNA was reverse-transcribed into first-strand cDNA at 42°C for 40 min, using 200 U of M-MLV reverse-transcriptase (Promega, Madison, WI) and 0.5 μg of oligo(dT) 15 primer (Takara Bio Inc., Shiga, Japan) in a 20-μl reaction mixture. Real-time PCR for *ho-1*, *nrf2*, and β-*actin* was carried out with a 7300 Real-Time PCR system (Applied Biosystems, Foster City, CA) using the DNA-binding dye SYBR Green I for the detection of PCR products. The reaction mixture contained 12.5 μl of Premix Ex Taq and 0.5 μl of ROX reference dye (Code RRO41A; Takara Bio Inc.), 1 μl of custom-synthesized primers, and 2 μl of cDNA (equivalent to 20 ng of total RNA) to give a final reaction volume of 25 μl. The PCR settings were as follows: the initial denaturation for 15 s at 95°C was followed by 40 cycles of amplification for 15 s at 95°C and 31 s at 60°C, with subsequent melting curve analysis in which the temperature was increased from 60 to 95°C. The primers had the following sequences: for *ho-1*, sense 5′-CAACCCCACCAAGTTCAAACA-3′ and antisense 5′-AGGCGGTCTTAGCCTCTTCTG-3′; for *nrf2*, sense 5′-CCGCCTGGGTTCAGTGACT-3′ and antisense 5′-GCTTTAGGTCCATTCTGTTTGACA-3′; and for β-*actin*, sense 5′-GAGCAAACATCCCCCAAAGTT-3′ and antisense 5′-GCCGTGGATACTTGGAGTGACT-3′. Relative quantification of gene expression with real-time PCR data were calculated relative to β-*actin*.

Western Blotting. Total proteins were mixed with SDS sample buffer. The samples were subjected to a 12% SDS-polyacrylamide gel electrophoresis (PAGE) and blotted onto a polyvinylidene difluoride (PVDF) membrane (Atto Corporation, Tokyo, Japan). The membrane was blocked with 2% bovine serum albumin in TBS-T (TBS and 0.1% Tween 20) at room temperature for 30 min. Western blotting was carried out using rabbit polyclonal anti-HO-1 antibody (1:1000 dilution in TBS-T), rabbit polyclonal anti-p44/42, phosho-p44/42, p38, phosho-p38, JNK, and phosho-JNK (1:1000) at room temperature for 1 h. After three washes with TBS-T, the membrane was incubated with anti-rabbit IgG-HRP (1:3000; GE Healthcare UK, Ltd., Chalfont St. Giles, UK) at room temperature for 45 min. The signals were visualized using an enhanced chemiluminescence (ECL) kit (GE Healthcare UK, Ltd.) according to the manufacturer's instructions. The band intensities were determined using CS Analyzer software, version 2.0 (Atto Corporation).

The Localization of HO-1. Confluent RGM-1 cultured on Lab Tek chamber slides were preincubated with or without lansoprazole (10 μM) for 6 h. The cells were then washed with PBS and stained with rabbit polyclonal anti-HO-1 antibody (Stressgen Bioreagents) and AlexaFluoro 488-labeled goat anti-rabbit IgG antibody (Invitrogen, Carlsbad, CA). The expression of HO-1 was observed under a confocal laser scanning microscope (FV1000; Olympus, Tokyo, Japan) using sequential activation. Photographic images were taken from four random fields.

Blocking of Nrf2. Nrf2-small interfering RNA (siRNA) and control-siRNA (a nontargeting siRNA; Santa Cruz Biotechnology) were transfected using the siRNA transfection reagent according to the manufacturer's protocol. The sequences of Nrf2-siRNA oligonucleotides were as follows: sense, 5′-CGCUCAGAACUGUAGGAAAAGGAAG-AG-3′ and antisense, 5′-UA-GCGAGUCUUGACAUCCUUUUCCUUC-5′. In RGM-1 transfected with Nrf2-siRNA or control-siRNA, the expression of the HO-1 gene and protein by lansoprazole treatment were investigated using real-time PCR and Western blotting. Nrf2(−/−) and Nrf2(+/+) (wild type) fibroblasts were described previously (Wakabayashi et al., 2003). As stated above, the expression of the HO-1 gene and protein by lansoprazole treatment were checked.

Electrophoretic Mobility Shift Assay for Nrf2. By using electrophoretic mobility shift assay (EMSA), we assessed lansoprazole-induced Nrf2 binding activity in RGM-1 cells. Cells were cultured in 10-cm cell culture dishes. RGM-1 cells were preincubated with or without lansoprazole for 2 h. The medium was then removed and the cells were washed twice with PBS. Nuclear protein fractions were extracted as described previously (Cepinskas et al., 2003). For EMSA, 5 μg of total extracted nuclear proteins was incubated with 1 pmol double-stranded [^{32}P]ATP end-labeled oligonucleotide probe containing the core stress-responsive DNA element (StRE) sequences (Alam and Cook, 2003) (sense strand, 5′-TTTTCTGCTGAGTCAAGGTCCG-3′; antisense strand, 3′-AAAAGACGACTCAGTTCCAGGC-5′) in binding buffer (10 mM HEPES, pH 7.9, 80 mM NaCl, 3 mM MgCl2, 0.1 mM EDTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and 10% glycerol). After electrophoresis under nondenaturing conditions (0.5× TBE buffer), the gels were dried and radioactive bands were visualized on X-ray films. In the supershift assays, nuclear extracts were incubated with 1 μg of rabbit polyclonal anti-Nrf2 antibody (Santa Cruz Biotechnology) for 1 h after addition of probe.

Plasmid Preparation and Transient Transfection Assay. A total of 188 base pairs of mouse HO-1 E2 enhancer was PCR-amplified with the primers (5′-AAA GGT ACC GCA GCC AGG GCA GTC-3′ and 5′-AAA GAG CTC CCA GCA CGT CCG CTC-3′) and cloned into KpnI and SacI sites of pRBGP3 plasmid (Igarashi et al., 1994). To make mutant HO-1 enhancer luciferase reporter, all three StRE elements (5′-GCTGAGTCA-3′) in E2 enhancer were mutated to 5′-AATGAGTCA-3′ by PCR-based mutagenesis using PfuUltra High-Fidelity DNA polymerase (Stratagene, La Jolla, CA). For reporter assay, RGM-1 cells were transfected using TransFast Transfection Reagent (Promega) according to the manufacturer's instructions. The luciferase assay was performed with the Dual-Luciferase Reporter Assay Kit (Promega) following the supplier's protocol and measured in a Biolumat Luminometer (Berthold Technologies GmbH and Co. KG, Bad Wildbad, Germany). Transfection efficiencies were routinely normalized to the activity of a cotransfected *Renilla* luciferase. The means of five independent experiments were presented with the S.E.M.

Isolation of Phosphoproteins from Cellular Extracts. RGM-1 cells were preincubated with or without lansoprazole for 2 h. Isolation of phosphoproteins from cellular extracts was carried out using a Pro-Q Diamond Phosphoprotein Enrichment Kit (Invitrogen Detection Technologies) according to the manufacturer's protocol. In brief, 1 mg of cellular extracts was prepared using lysis buffer (8 M Urea, 30 mM Tris-HCl, 4% CHAPS, pH 8.5), then supplemented with a Protease Inhibitor and Endonuclease Module (20 μl/1 ml protease inhibitor, 10 μl/1 ml endonuclease; Invitrogen Detection Technologies). After 30 min on ice, the cellular extracts were diluted with 5 ml of washing buffer and applied to a column containing 1 ml of resin, and the flow-through was saved for analysis of the unphosphorylated proteins. After washing the column with washing buffer, bound proteins were eluted with 250 μl of elution buffer. The elution step was repeated five times. The eluate and the flow-through were concentrated until the sample volume was reduced to approximately 50 μl using Vivaspin filtration concentrators with a 10 kDa cutoff polyethersulfone membrane. A solution of 25 mM Tris, pH 7.5, and 0.25% CHAPS was then added to the retentate reservoir, and the sample was concentrated to a volume of approximately 50 μl. The samples were precipitated using the methanol-chloroform-water method. The precipitated samples were dissolved in 50 μl of lysis

buffer (8 M Urea, 30 mM Tris-HCl, 4% CHAPS, pH 8.5). The supernatants were recovered by centrifugation at 15,000*g* for 5 min and separated on a 12% SDS-PAGE, and the gel was stained with Pro-Q Diamond phosphoprotein gel stain (Invitrogen) and Sypro ruby gel stain (Invitrogen).

Analysis of *S*-Oxidized Proteins. The cells were incubated with 100 μM biotin-cysteine for 15 min as described previously (Ishii and Uchida, 2004). The cells were then washed twice with PBS and stimulated with 10 μM lansoprazole. Finally, the cells were lysed in lysis buffer and centrifuged at 10,000 rpm for 10 min at 4°C, and the supernatants were stored at −80°C until use. For detection of the *S*-oxidized proteins, these samples were mixed with SDS sample buffer, separated by 10% SDS-PAGE, and blotted onto a PVDF membrane. The membrane was blocked, washed, and incubated with the Streptavidin-HRP conjugate (GE Healthcare). This procedure was followed by the addition of ECL reagents, and then the membrane was visualized.

Subsequently, to confirm the oxidation of the Keap1 protein, the cells were incubated with 100 μM biotin-cysteine for 15 min, washed twice with PBS, and stimulated with 10 μM lansoprazole for 45 min. The cells were lysed in lysis buffer and centrifuged at 10,000 rpm for 10 min at 4°C, and the supernatants were stored. To isolate the biotin-cysteine modified protein, the samples were mixed with Streptavidin MicroBeads (Miltenyi Biotec K.K., Tokyo, Japan), and the MicroBeads-labeled proteins were separated using μ Columns (Miltenyi Biotec K.K.). The collected samples were mixed with SDS sample buffer and separated by 10% SDS-PAGE and then blotted onto a PVDF membrane. The membrane was blocked, washed, and incubated with rabbit polyclonal anti-Keap1 antibody (1:500; Santa-Cruz Biotechnology) at room temperature for 1 h. After three washes with TBS-T, the membrane was incubated with anti-rabbit IgG-HRP (GE Healthcare). This procedure was followed by the addition of ECL reagents, and then the membrane was visualized.

Statistical Analysis. The results are presented as the mean ± S.E.M. An overall difference between the groups was determined by one-way analysis of variance. If the one-way analysis of variance was significant, the differences between individual groups were analyzed by Tukey's multiple comparison test. Values of $P < 0.05$ were considered statistically significant. All analyses were performed using the program GraphPad Prism 4 (GraphPad Software Inc., San Diego, CA) on a Macintosh computer.

Results

Lansoprazole Up-Regulated HO-1. To test the expression of HO-1 induced by lansoprazole, we checked the HO-1 gene and protein expression using real-time PCR and Western blotting. Lansoprazole (10 μM) stimulation significantly increased the expression of the *ho-1* gene in RGM-1 cells in a time-dependent manner (Fig. 1A). In association with the increased *ho-1* gene expression, the expression of the HO-1 protein was also increased in a time-dependent manner with a peak at 6 h (Fig. 1B), and the increase in the HO-1 protein was dependent on the concentration of lansoprazole (Fig. 1C). The localization of HO-1 in RGM-1 cells in response to lansoprazole was also assessed using confocal microscopy. A strong expression of HO-1 in the cytoplasm of RGM-1 cells was induced 6 h after the stimulation of RGM-1 cells with lansoprazole (Fig. 1D). Furthermore, we checked whether another anti-acid secretory agent also induced the expression of HO-1. As a result, omeprazole, another PPI, and famotidine, H_2-receptor antagonists induced the faint expression of HO-1 compared to HO-1 expression induced by lansoprazole (Fig. 1E).

Lansoprazole-Induced HO-1 Up-Regulation Contributed to the Inhibition of Chemokine Production from Gastric Mucosal Cells. Our previous report demonstrated that rat gastric epithelial cells (RGM-1 cells) produced CINC-1 in response to various proinflammatory cytokines, such as tumor necrosis factor-α, IL-1β, and bacterial LPS. To confirm the anti-inflammatory effect of lansoprazole-induced HO-1 on RGM-1 cells, we measured the production of CINC-1 on RGM-1 cells using ELISA. As shown in Fig. 2, proinflammatory cytokines (IL-1β, 1 ng/ml) significantly increased the production of CINC-1 by RGM-1 cells after 6 h of stimulation.

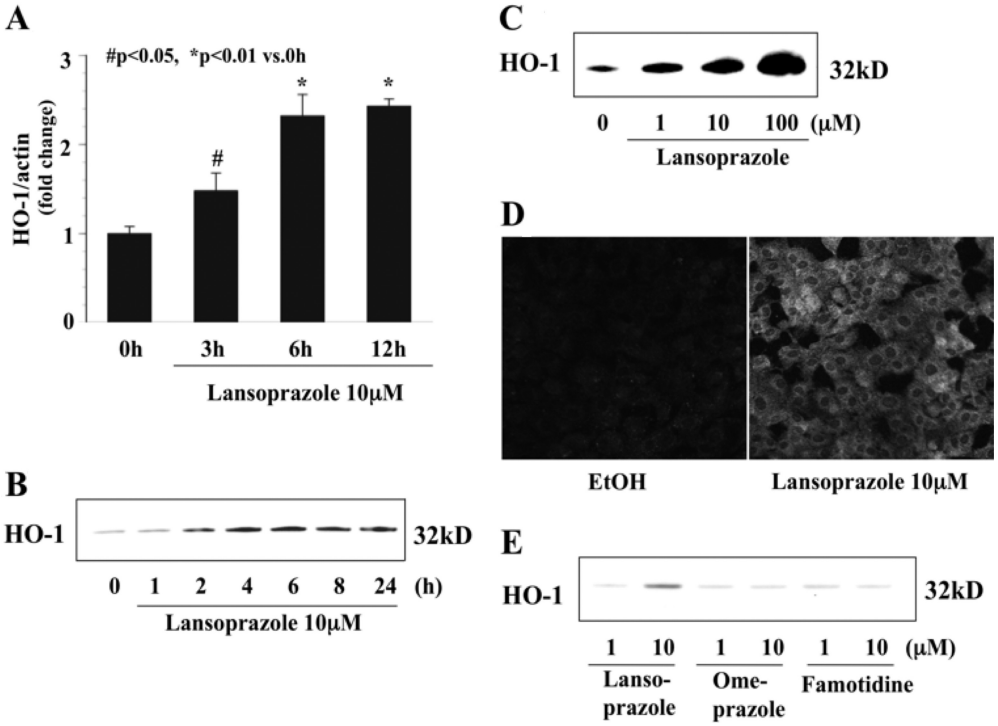

Fig. 1. Up-regulation of HO-1 by lansoprazole in RGM-1 cells. RGM-1 cells were incubated with lansoprazole. A, *ho-1* gene expression was measured using real-time PCR. The mRNA level of β-*actin* was determined simultaneously, and *HO-1*/β-*actin* ratio was calculated for each sample. Values represent the mean ± S.E.M. ($n = 6$). #, $p < 0.05$ and *, $p < 0.01$ compared to no stimulation (0 h). B and C, whole-cell extracts were prepared and analyzed by immunoblotting with an antibody against HO-1. D, the expression and the localization of HO-1 in RGM-1 cells 6 h after lansoprazole treatment was investigated using confocal microscopy. E, omeprazole, another PPI, and famotidine, H_2-receptor antagonists induced the faint expression of HO-1 compared to HO-1 expression induced by lansoprazole.

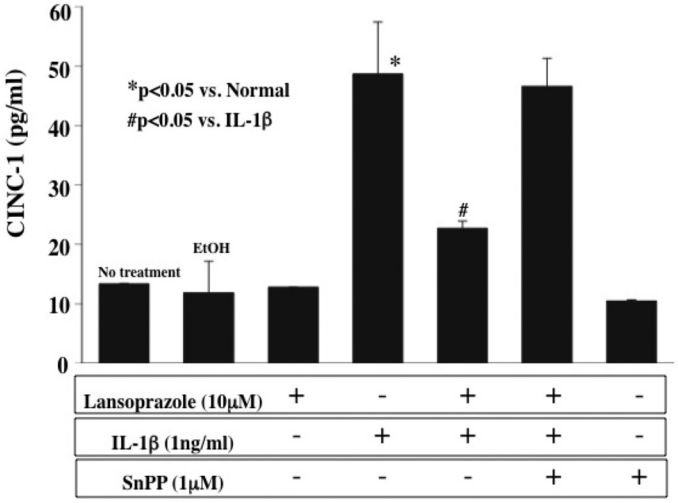

Fig. 2. The inhibition of CINC-1 production in RGM-1 cells treated with lansoprazole. RGM-1 cells were stimulated with or without IL-1β (1 ng/ml), and/or lansoprazole (10 μM) and/or an HO-1 inhibitor, SnPP (1 μM) for 6 h. The concentration of CINC-1 in culture supernatant was determined by an ELISA kit specific for rat CINC-1. Data represent the mean ± S.E. ($n = 3$). ∗, $p < 0.05$ compared to the ethanol group. #, $p < 0.05$ compared to the IL-1β stimulation group.

Unstimulated RGM-1 cells produced a small amount of CINC-1 during the same period of time. The increased production of CINC-1 by IL-1β was significantly inhibited with lansoprazole (10 μM) treatment. This inhibition was diminished by simultaneous treatment with SnPP, an HO-1 inhibitor (Fig. 2). The concentration of SnPP used in the experiment did not affect the CINC-1 production in unstimulated cells. Based on a previous report (Shokawa et al., 2006; Canas et al., 2007), the concentration used was sufficient to specifically inhibit HO activity. On this basis, the results in Fig. 2 show that up-regulation of HO-1 by lansoprazole contributes to the inhibition of chemokine production from stimulated gastric mucosal cells.

Role of Nrf2 in HO-1 Up-Regulation by Lansoprazole. An siRNA approach was used to determine whether lansoprazole-mediated up-regulation of HO-1 was dependent on Nrf2. RGM-1 cells were transiently transfected with either control siRNA or siRNA directed against Nrf2. Thirty hours after transfection, cells were exposed to lansoprazole for 6 h, and then *ho-1* mRNA and HO-1 protein expression were examined by real-time PCR and Western blotting, respectively. Real-time PCR analysis revealed that treatment with siRNA oligonucleotides led to a 51% reduction in the *nrf2* mRNA level in RGM-1 cells 30 h after transfection (Fig. 3A). Under these conditions, *ho-1* mRNA expression by lansoprazole stimulation was significantly decreased in comparison with that in the control siRNA (Fig. 3B). In cells transfected with control siRNA, lansoprazole also produced an increase in the HO-1 protein expression. The intensity of the HO-1-immunoreactive band was measured and expressed relative to the expression of actin (Fig. 3D). Transfection of siRNA directed against Nrf2 suppressed the lansoprazole-induced expression of HO-1 by 71%. Moreover, to reaffirm that Nrf2 plays a role in the HO-1 expression induced by lansoprazole, we tested the effects of lansoprazole on the expression of HO-1 in Nrf2(+/+) and Nrf2(−/−) fibroblasts, because the efficacy of *nrf2* mRNA reduction by Nrf2 siRNA in RGM-1 cells was not as good as expected. In Nrf2(+/+) fibroblasts, lansoprazole up-regulated *ho-1* mRNA and HO-1 protein expression at the concentration of 100 μM (Fig. 4A). As shown in Fig. 4B, *ho-1* mRNA expression was faint in Nrf2(−/−) fibroblasts with or without lansoprazole incubation. Immunoblotting showed that the expression of HO-1 protein was

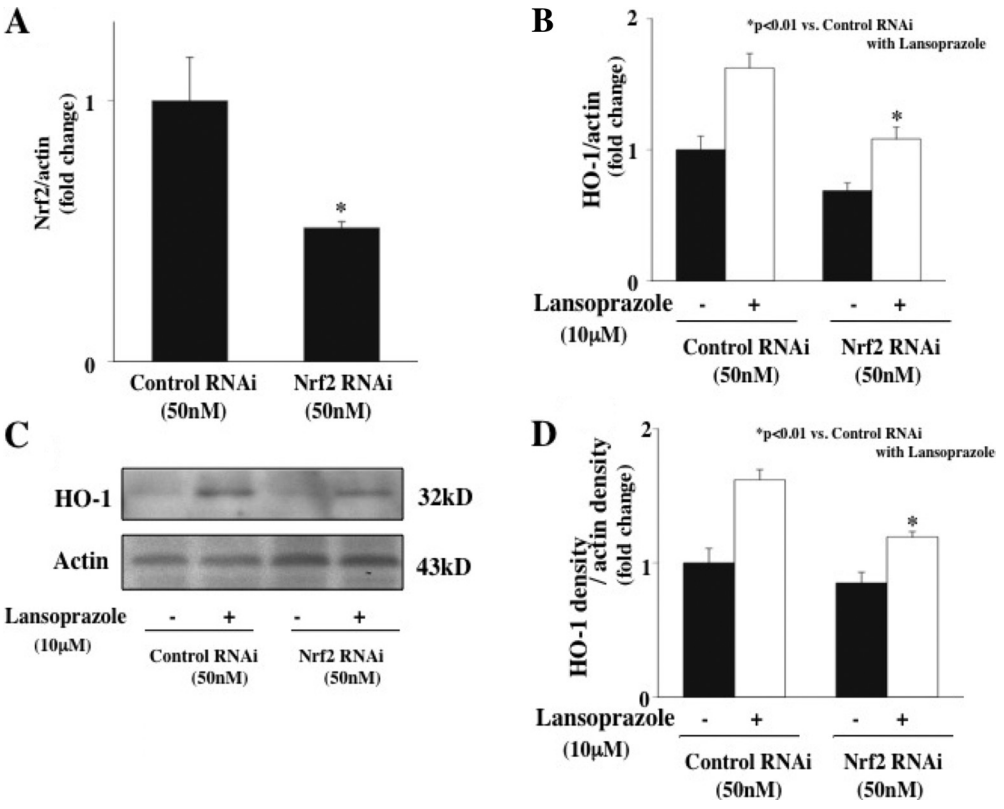

Fig. 3. The effect of Nrf2-siRNA on HO-1 expression. RGM-1 cells were transfected with Nrf2-specific or control siRNA oligonucleotides by using the RNAi transfection reagent. Thereafter, the cells were stimulated with lansoprazole for 6 h. The mRNA was extracted from the cells, and the expression of Nrf2 (A) and HO-1 (B) was monitored using real-time PCR. Values are the mean ± S.E.M. -fold change, considering 1 as the value for control siRNA samples ($n = 6$). ∗, $p < 0.05$ compared to control-siRNA (A) and control-siRNA incubated with lansoprazole (B). Moreover, the protein was also isolated, immunoblotted using anit-HO-1 antibody and actin as internal control (C), and densitometric analysis (D) was carried out using the ImageJ software (Wayne Rashband; National Institutes of Health, Bethesda, MD).

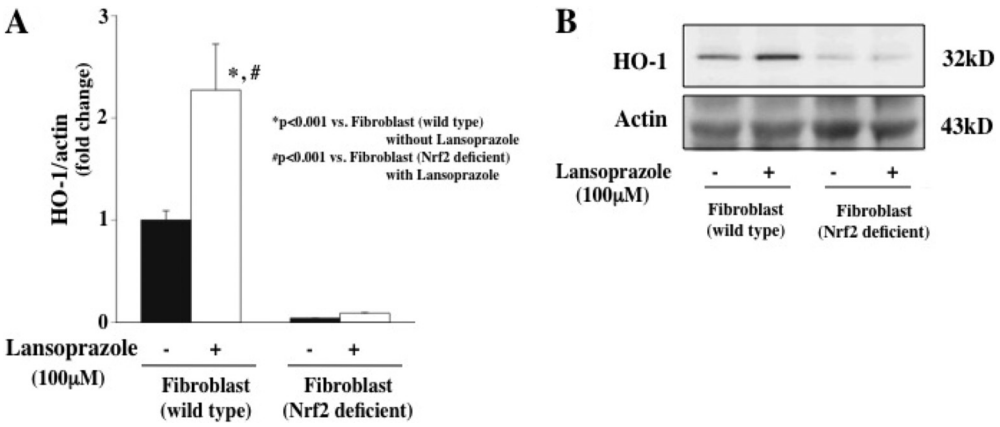

Fig. 4. The expression of HO-1 in Nrf2(−/−) fibroblasts treated with lansoprazole. Nrf2(+/+) and Nrf2(−/−) fibroblasts were incubated with or without lansoprazole (100 μM) for 6 h. The mRNA was extracted from the cells, and the expression of HO-1 (A) was monitored using real-time PCR. Values are the mean ± S.E.M. -fold change, considering 1 as the value for Nrf2(+/+) fibroblasts without lansoprazole. Furthermore, the protein was also isolated, immunoblotted using anit-HO-1 antibody and actin as internal control (B).

not detectable in Nrf2(−/−) fibroblasts (Fig. 4B). These experiments demonstrate a direct correlation between Nrf2 and HO-1 expression and support the contention that lansoprazole-mediated up-regulation of HO-1 is Nrf2-dependent.

StRE-Binding Activities in RGM-1 Cells Treated with Lansoprazole. To analyze the DNA binding activity of Nrf2, we carried out EMSA with the use of nuclear extracts from RGM-1 cells with or without lansoprazole treatment. As shown in Fig. 5A, StRE-binding proteins were detected and increased with lansoprazole treatment in a dose-dependent manner (Fig. 5A, arrow). In addition, the complex band that increased with lansoprazole treatment disappeared with the addition of anti-Nrf2 polyclonal antibody (Fig. 5B, arrowhead). These results indicated that one of the StRE-binding proteins increased by lansoprazole was Nrf2. Lansoprazole translocated Nrf2 to the nuclear fraction and promoted Nrf2-DNA binding activities.

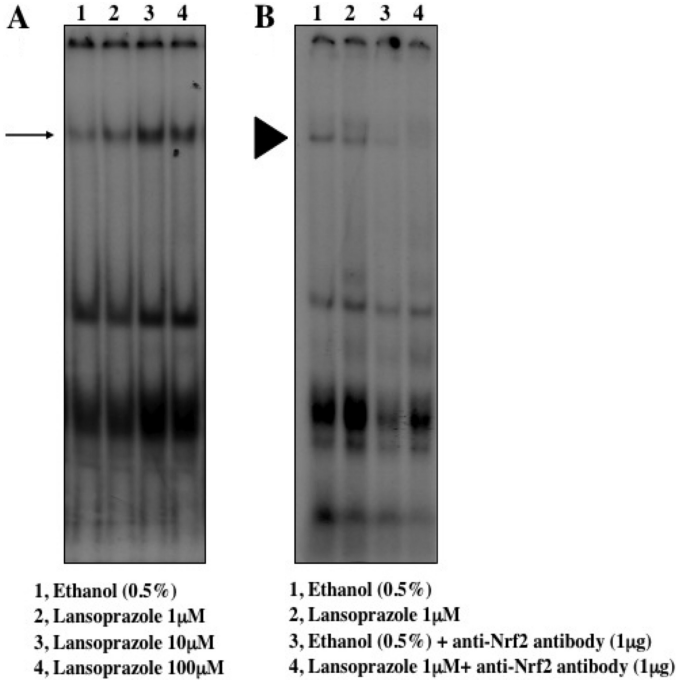

Fig. 5. Nuclear translocation and DNA-binding activity of Nrf2 in RGM-1 cells by lansoprazole stimulation. RGM-1 cells were treated with 10 μM lansoprazole for 2 h. Preparation of cell extracts and EMSA reactions were carried out as described under *Materials and Methods*. The DNA-protein complexes were observed (arrow), and the intensity of this band was increased in a dose-dependent manner (A). Identification of StRE-binding proteins was carried out by the supershift assay using anti-Nrf2 antibody (B). The complex band that increased with lansoprazole treatment disappeared by the addition of anti-Nrf2 antibody (arrowhead).

HO-1 Enhancer Activity in RGM-1 Cells Treated with Lansoprazole. To further examine the role of Nrf2 in lansoprazole-induced HO-1 gene expression, we performed luciferase reporter assay using HO-1 E2 enhancer-luciferase reporter gene in which HO-1 E2 enhancer containing three StREs was placed upstream of rabbit β-globin TATA box and luciferase gene. We also prepared a mutant enhancer reporter gene where all of the three Nrf2 binding sites, but not overlapping AP-1 binding sites, were mutated (see *Materials and Methods*). As shown in Fig. 6A, lansoprazole-induced HO-1 reporter gene activity was markedly diminished by the mutation, indicating that Nrf2 was involved at least in part in the lansoprazole-induced HO-1 enhancer activity. Furthermore, lansoprazole-induced reporter activity was diminished by the cotransfection of Keap1 or dominant-negative Nrf2 expression plasmids (Fig. 6B). Together, these results indicated that lansoprazole activates HO-1 enhancer activity via Nrf2.

Lansoprazole-Induced MAPK Activation Contributed to the HO-1 Up-Regulation in RGM-1 Cells. Various MAPKs have been reported to be involved in HO-1 up-regulation (Elbirt et al., 1998). In this study, we tried to identify the kinase involved in the lansoprazole-induced up-regulation of HO-1 by Western blots using phosphorylation status-dependent and -independent antibodies against p38, ERK1/2 (44 and 42 kDa), and JNK kinases (54 and 46 kDa) at 0, 5, 10, 30, and 60 min after lansoprazole treatment. Western blots showed the phosphorylation of ERK in RGM-1 cells 5 min after lansoprazole treatment, but not that of p38 MAPK and JNK (Fig. 7A).

Moreover, RGM-1 cells were stimulated by lansoprazole in the presence of a specific inhibitor for each of the MAPKs (SP600125 for JNK, U01263 for ERK, and SB203580 for p38 MAPK) for 6 h. As shown in Fig. 7B, the inhibitor of ERK (U01263), but not the inhibitors of the other two kinases (p38 MAPK and JNK), suppressed the up-regulation of HO-1 by lansoprazole.

Identification of Phosphorylated Nrf2 Protein in RGM-1 Cells Treated with Lansoprazole. To isolate phosphorylated proteins from RGM-1 cell lysate, we used the Pro-Q Diamond Phosphoprotein Enrichment kit. The cell lysate (L fraction) was loaded on a column containing a phosphoprotein-binding resin. The unphosphorylated proteins were passed through the column (F fraction), and the

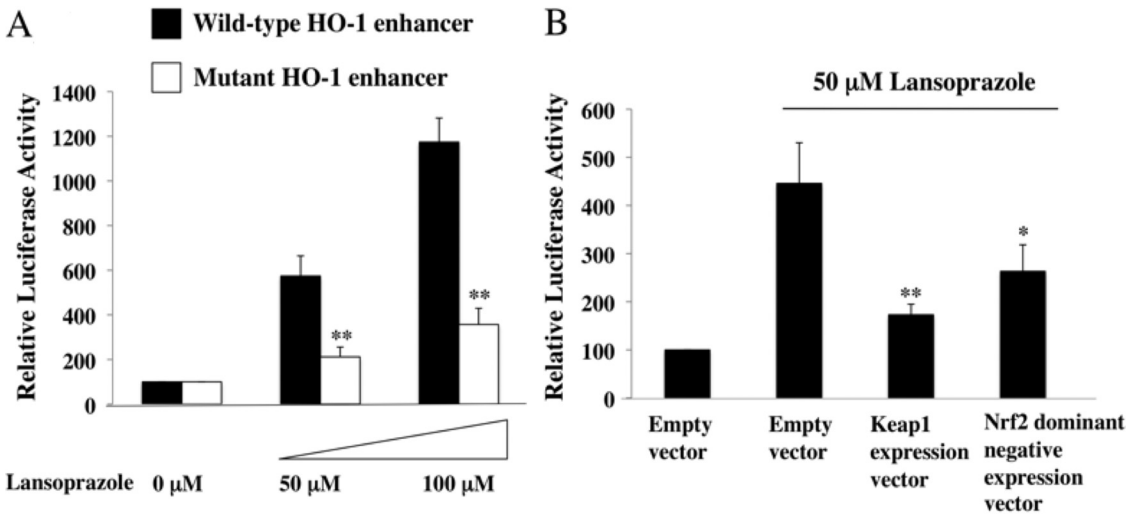

Fig. 6. Lansoprazole activates HO-1 enhancer activity via Nrf2 in RGM-1 cells. A, wild-type or mutant HO-1 enhancer luciferase reporter plasmids (100 ng) were transfected into RGM-1 cells and treated with lansoprazole for 12 h. Firefly luciferase activities were normalized with *Renilla* luciferase activities expressed from a cotransfected pRL-TK plasmid. Luciferase activity treated with vehicle (dimethyl sulfoxide) in the absence of lansoprazole was arbitrarily set as 100, and the mean values of at least five independent experiments are shown as relative luciferase units with the S.E.M. **, significantly different from the activity of wild-type reporter-transfected cells in the same dose of lansoprazole (**, $p < 0.01$). B, wild-type HO-1 enhancer luciferase plasmids (100 ng) were transfected into RGM-1 cells in the presence of 50 μM lansoprazole for 12 h together with either Keap1 expression vector, Nrf2 dominant-negative expression vector, or empty vector. * and **, significantly different from the activity of empty vector-transfected cells in the presence of 50 μM lansoprazole (*$p < 0.05$, **, $p < 0.01$).

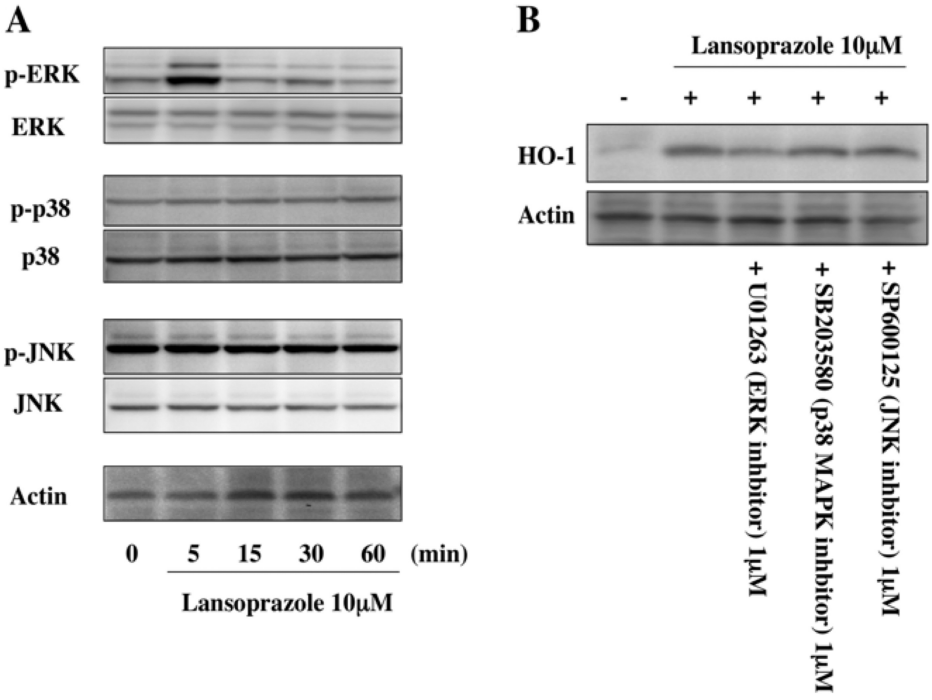

Fig. 7. Involvement of MAPK in the up-regulation of HO-1 by lansoprazole. A, the expression of p44/42, phosho-p44/42, p38, phosho-p38, JNK, and phosho-JNK in RGM-1 cells incubated with lansoprazole was measured using Western blotting. B, RGM-1 cells were treated by lansoprazole with indicated concentrations of each inhibitor (SP600125 for JNK, U01263 for ERK, and SB203580 for p38 MAPK) for 6 h. The expression of HO-1 was monitored using Western blotting.

phosphorylated proteins were subsequently eluted (E fraction). These proteins were precipitated from each fraction, dissolved, and separated by SDS-PAGE. Then, the gel was stained with Pro-Q Diamond phosphoprotein stain for the detection of the phosphorylated proteins (Fig. 8A), followed by Sypro ruby gel stain for the detection of total proteins (Fig. 8B).

Analysis by Pro-Q Diamond staining of SDS gels showed the obvious increases in phosphorylated proteins in the E fraction compared to the L or F fractions. Lansoprazole treatment increased the phosphorylated proteins in the E fraction detected by the Pro-Q Diamond staining (Fig. 8A). To detect Nrf2 phosphorylation, the cell lysate and eluted proteins were separated by SDS-PAGE and immunoblotted. The phosphorylated Nrf2 was increased in the E fraction of RGM-1 cells treated with lansoprazole. Thus, lansoprazole promoted the phosphorylation of various proteins, and at least one of these phosphorylated proteins was Nrf2.

Identification of Oxidation of the Keap1 Protein in RGM-1 Cells Treated with Lansoprazole. To detect cellular proteins that undergo cysteine-target oxidation by lansoprazole stimulation, biotin-cysteine was used as molecular probe. This probe rapidly crosses the plasma membrane and can be used to detect, quantify, purify, and identify proteins

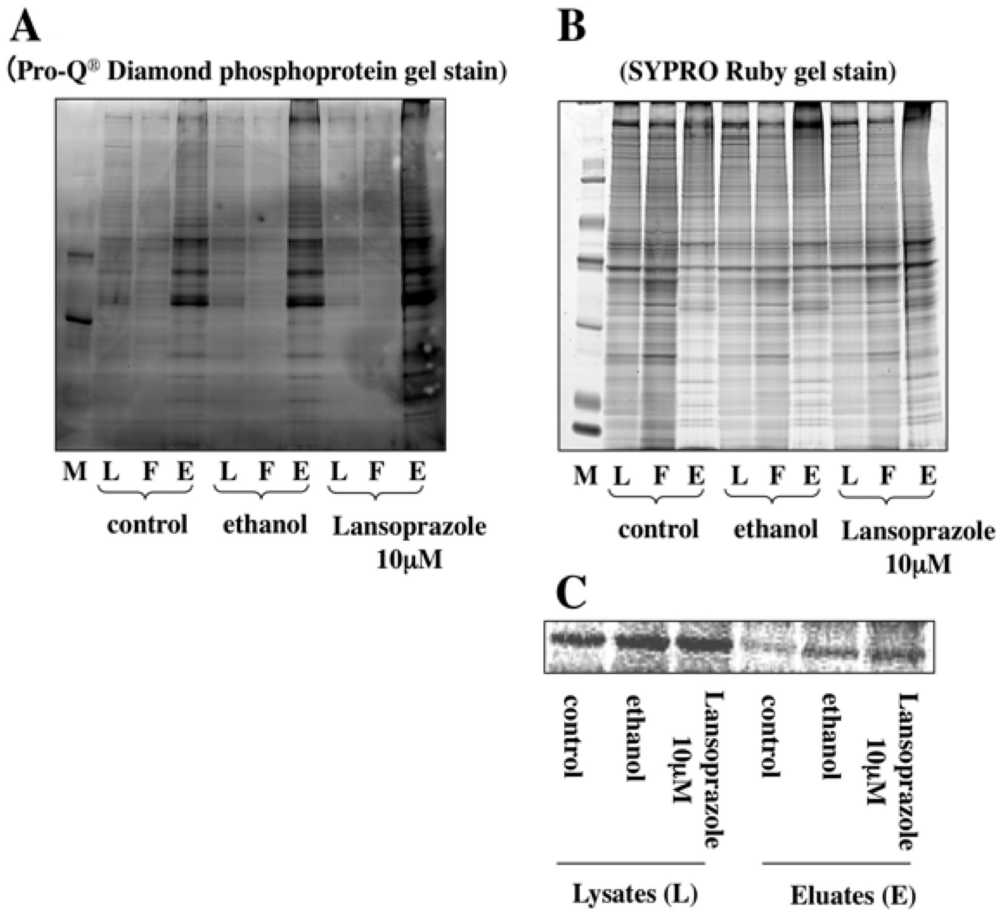

Fig. 8. The phosphorylation of protein in RGM-1 cells treated with lansoprazole. Lansoprazole-treated RGM-1 cells lysates (L), flow-through (F), and eluates (E) were obtained using the Pro-Q Diamond Phosphoprotein Enrichment kit. Proteins were precipitated from each fraction and dissolved, separated by SDS-PAGE. The gel was stained with Sypro ruby gel stain for the detection of total proteins (A) and subsequently Pro-Q Diamond phosphoprotein stain for the detection of the phosphorylated proteins (B). Furthermore, to detect the phosphorylation of Nrf2 in RGM-1 cells by lansoprazole, the cell lysates and eluates were immunoblotted using anti-Nrf2 antibody (C).

susceptible to oxidation in all compartments of cells (Ishii and Uchida, 2004). RGM-1 cells exposed to biotin-cysteine were treated with 10 μM lansoprazole, and the biotin-cysteine-modified proteins were analyzed by Western blotting with streptavidin-HRP. As shown in Fig. 9A, several proteins were found to be *S*-oxidized in response to lansoprazole in a dose-dependent manner. Moreover, the incorporation of biotin-cysteine into endogenous Keap1 was confirmed by the use of pull-down Streptavidin beads and μ Columns followed by Western blotting with an anti-Keap1 antibody. As shown in Fig. 9B, the Keap1 protein was detected among the biotin-cysteine-modified proteins, indicating that endogenous Keap1 was oxidatively modified during the lansoprazole stimulation.

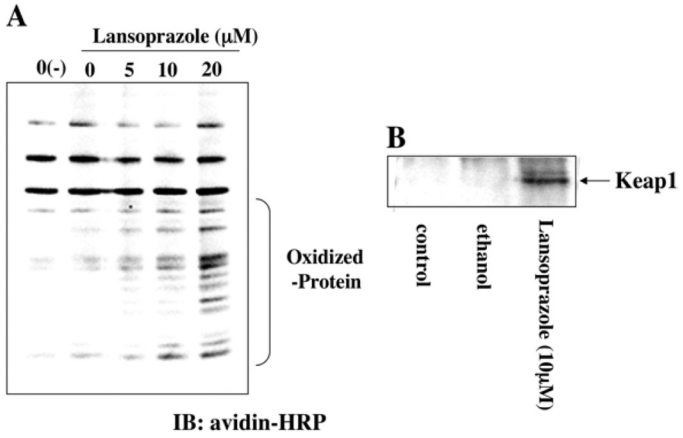

Fig. 9. The detection and identification of *S*-oxidized proteins in RGM-1 cells exposed to lansoprazole. A, biotin-cysteine-protein adducts were detected by Western blotting. RGM-1 cells were incubated with 100 μM biotin-cysteine for 15 min and then treated with 10 μM lansoprazole for 45 min. Total cell lysate was separated by 10% SDS-PAGE followed by immunoblotting analysis. B, to isolate the biotin-cysteine-modified protein, the cell lysates were mixed with Streptavidin MicroBeads, and subsequently the MicromBeads-labeled proteins were separated using μ Columns. The collected samples were analyzed by Western blotting using anti-Keap1 antibody.

Discussion

In the present study, we confirmed that lansoprazole induced HO-1 up-regulation in rat gastric epithelial cells. Incubation with lansoprazole (1–100 μM) induced expression of the *ho-1* gene in the early phase within 3 h of lansoprazole addition. In association with the induction of *ho-1* gene expression, the expression of the HO-1 protein was significantly increased in a time- and dose-dependent manner after lansoprazole treatment, and confocal microscopy revealed that the HO-1 protein was localized to the cytoplasm fraction. In this study, we could not observe HO-1 up-regulation induced by other anti-acid secretory agents such as omeprazole and famotidine. Although Becker et al. (2006) demonstrated that omeprazole as well as lansoprazole induced the expression of HO-1 on gastric epithelial cells, this effect might be based on the high concentration of omeprazole (300 μM) that they administrated.

To investigate the contribution of lansoprazole-induced HO-1 up-regulation to chemokine production by RGM-1 cells, we measured whether the production of CINC-1 induced by

IL-1β was inhibited by lansoprazole. CINC-1, a counterpart of the human GRO (a member of IL-8 family), has been suggested to play a critical role as a mediator of neutrophil infiltration in rats during inflammation. Pretreatment with lansoprazole significantly inhibited the production of CINC-1 from stimulated RGM-1 cells. In addition, the inhibition was reversed by cotreatment with the HO-1 inhibitor SnPP. These data indicate that the anti-inflammatory effect of lansoprazole is mediated through the induction of HO-1.

For the mechanism of lansoprazole-induced up-regulation of HO-1, we have shown using an siRNA approach and Nrf2-deficient fibroblasts that the transcriptional factor Nrf2 plays a critical role in the up-regulation of HO-1 in RGM-1 cells. The treatment of RGM-1 cells with Nrf2-siRNA decreased the constitutive *ho-1* mRNA level and abolished the lansoprazole-induced *ho-1* mRNA and HO-1 protein expression, suggesting that Nrf2 plays a pivotal role in the regulation of HO-1 in RGM-1 cells. The involvement of Nrf2 in the HO-1 up-regulation induced by lansoprazole was also confirmed by the findings obtained from Nrf2-deficient fibroblasts. The expression of *ho-1* mRNA and HO-1 protein was faint in Nrf2-deficient fibroblasts with or without lansoprazole incubation.

Extensive analysis has identified a common mechanism involving the StRE/Nrf2 transcription factor pathway for gene regulation in response to a diverse array of HO-1 inducers (He et al., 2001; Alam et al., 2003; Gong et al., 2003; Massrieh et al., 2006). The StREs mediate transcriptional activation in response to multiple agents, including heme, heavy metals, TPA, arsenite, hydrogen peroxide, hyperoxia, LPS, and various electrophiles (Choi and Alam, 1996). These motifs in StRE are targets for multiple members of the basic-leucine zipper (bZIP) superfamily of sequence-specific DNA-binding proteins, including the AP-1, cAMP response element-binding protein/ATF, Maf, and CNC-bZIP classes of transcription factors. Alam et al. (1999) have also described that the induction of HO-1 by StREs in L929 fibroblasts demonstrated potent *trans*-activation of the E1 enhancer by CNC-bZIP factors, particularly Nrf2. In this study, EMSA reactions using nuclear protein fractions from RGM-1 cells were carried out to identify DNA-binding proteins potentially responsible for lansoprazole-mediated *ho-1* up-regulation. Lansoprazole treatment of RGM-1 cells significantly increased the levels of the StRE-protein complex in a concentration-dependent manner. In addition, the increased complex band disappeared after the addition of anti-Nrf2 polyclonal antibody. These data indicate that activation of the *ho-1* gene by lansoprazole occurs, at least in part, as a consequence of increased binding of Nrf2 to the StREs. Moreover, in a luciferase reporter assay using HO-1 enhancer-luciferase reporter gene containing mutant StREs, lansoprazole-induced HO-1 reporter gene activity was markedly decreased by the mutation. On the other hand, lansoprazole-induced reporter activity was also diminished by the cotransfection of dominant-negative Nrf2 expression plasmids. Together, these results confirm that lansoprazole activates HO-1 enhancer activity via Nrf2.

Multiple protein kinase pathways, such as MAPK, protein kinase C, and p38 MAPK, have been proposed to play a role in HO-1 induction. The present study demonstrated that lansoprazole promoted the phosphorylation of ERK, but not that of p38 MAPK or JNK in RGM-1 cells, and that lansoprazole-induced HO-1 up-regulation was suppressed by U01263, a specific inhibitor for ERK. These data indicate that HO-1 up-regulation by lansoprazole was at least partly mediated by the ERK pathway. The role of the ERK pathway in the induction of HO-1 is controversial; ERK has been found to regulate HO-1 expression in some studies (Gong et al., 2003; Schwer et al., 2008) but not in others (Ning et al., 2002). This discrepancy may reflect cell type- and/or species-specific differences in the regulatory mechanism of HO-1.

It has recently been described that phosphorylated forms of Nrf2 modified after chemically induced oxidative stress play a potential role in Nrf2 activation and degradation (Pi et al., 2007). Furthermore, electrophilic- or reactive oxygen species-induced nuclear Nrf2 accumulation is shown to be highly dependent on protein kinase CK2 activity. In this study, the detection of phosphorylated protein was assayed using Pro-Q Diamond phosphoprotein stain. Consequently, the phosphoproteins in RGM-1 cells were markedly increased by treatment with lansoprazole. The purification of these phosphoproteins induced by lansoprazole was performed using a Pro-Q Diamond Phosphoprotein Enrichment kit, and Nrf2 was clearly detected in the purified phosphoproteins. The increased phosphorylated forms of Nrf2 might play a role in its activation or nuclear accumulation.

Keap1 is rich in cysteine residues, and this fact has indicated that Keap1 is a sensor protein of xenobiotic and oxidative stress. Early studies proposed that Keap1 oxidation released Nrf2, allowing it to enter the nucleus and bind to antioxidant-response element-containing genes (Dhakshinamoorthy and Jaiswal, 2001; Itoh et al., 2003). In agreement with these studies, in vitro oxidation of Keap1 has been shown to cause a change in the affinity of Keap1 for the Neh2 domain, releasing Nrf2 (Dinkova-Kostova et al., 2002). Recent in vivo studies have reported that oxidation does release the Nrf2/Keap1 complex (Zhang and Hannink, 2003). In the present study, lansoprazole promoted the oxidized protein in a dose-dependent manner. Like Ishi and Uchida (2004) and Sakurai et al. (2006), we used biotin-cysteine as a probe in our investigation of cysteine-targeted oxidation of proteins.

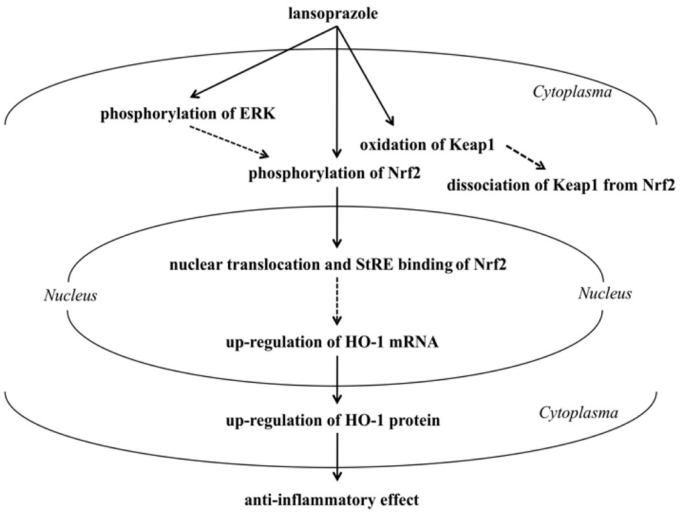

Fig. 10. Lansoprazole exerts anti-inflammatory effect through the up-regulation of HO-1 expression in rat gastric epithelial cells. Phosphorylation of ERK and Nrf2, activation and nuclear translocation of Nrf2, and oxidation of Keap1 are all involved in the lansoprazole-induced HO-1 up-regulation.

We also purified and blotted the oxidized proteins and confirmed the existence of Keap1 in these oxidized proteins.

In summary, our data showed that lansoprazole up-regulated HO-1 expression in rat gastric epithelial cells, and the up-regulated HO-1 had anti-inflammatory effects. It appears that lansoprazole-induced HO-1 production is mediated by the activation, phosphorylation, and nuclear translocation of Nrf2 in accompaniment with the dissection of oxidized Keap1 (Fig. 10). Further studies will be needed to clarify the mechanisms involved in this phenomenon in greater detail.

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