Heme Oxygenase-1 Protects Gastric Mucosal Cells against Non-steroidal Anti-inflammatory Drugs*

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Gastric mucosal cell death by non-steroidal anti-inflammatory drugs (NSAIDs) is suggested to be involved in NSAID-induced gastric lesions. Therefore, cellular factors that suppress this cell death are important for protection of the gastric mucosa from NSAIDs. Heme oxygenase-1 (HO-1) is up-regulated by various stressors and protects cells against stressors. Here, we have examined up-regulation of HO-1 by NSAIDs and the contribution of HO-1 to the protection of gastric mucosal cells against NSAIDs both in vitro and in vivo. In cultured gastric mucosal cells, all NSAIDs tested up-regulated HO-1. In rats, orally administered indomethacin up-regulated HO-1, induced apoptosis, and produced lesions at gastric mucosa. An inhibitor of HO-stimulated NSAID-induced apoptosis in vitro and in vivo and also stimulated NSAID-produced gastric lesions, suggesting that NSAID-induced up-regulation of HO-1 protects the gastric mucosa from NSAID-induced gastric lesions by inhibiting NSAID-induced apoptosis. Indomethacin activated the HO-1 promoter and caused nuclear accumulation of NF-E2-related factor 2 (Nrf2), a transcription factor for the HO-1 gene. Examination of phosphorylation of p38 mitogen-activated protein kinase (MAPK) and experiments with its inhibitor strongly suggest that the nuclear accumulation of Nrf2 and resulting up-regulation of HO-1 by NSAIDs is mediated through NSAID-dependent activation (phosphorylation) of p38 MAPK. This is the first report showing the protective role of HO-1 against irritant-induced gastric lesions.

Non-steroidal anti-inflammatory drugs (NSAIDs) are a useful family of therapeutics, accounting for nearly 5% of all prescribed medications (1). The anti-inflammatory actions of NSAIDs are mediated through their inhibitory effects on cyclooxygenase (COX) activity. COX is an enzyme essential for the synthesis of prostaglandins (PGs), which have a strong capacity to induce inflammation. On the other hand, NSAID use is associated with gastrointestinal complications (2), with about 15–30% of chronic users of NSAIDs suffering from gastrointestinal ulcers and bleeding (3, 4).

Although PGs have a strong protective effect on gastrointestinal mucosa, the inhibition of COX by NSAIDs is not the sole explanation for the gastrointestinal side effects of NSAIDs (5). We have recently demonstrated that NSAIDs induce apoptosis in primary cultures of gastric mucosal cells in a manner independent of COX inhibition (6–9). As for the molecular mechanism governing this apoptosis, we recently proposed that permeabilization of cytoplasmic membranes by NSAIDs stimulates Ca2+ influx which in turn induces production of the C/EBP homologous transcription factor (CHOP), and activates calpain, a Ca2+-dependent cysteine protease, both of which have apoptosis-inducing ability (6). Furthermore, we suggested that both COX-inhibition and NSAID-induced cell death (such as apoptosis) in gastric mucosa are required for production of NSAID-induced gastric lesions in vivo (10). Cellular factors that suppress NSAID-induced apoptosis are therefore important for protection of gastric mucosa from NSAID-induced gastric lesions.

When cells are exposed to various stressors, including NSAIDs, they induce a number of proteins, so-called stress proteins, in order to protect themselves against such stressors. Molecular chaperons are representative stress proteins. Their up-regulation in cells confers resistance to various stressors as the chaperons re-fold or degrade denatured proteins produced by stressors (11). It has been shown that cytosolic molecular chaperons (such as heat shock proteins (HSPs)) and endoplasmic reticulum (ER) molecular chaperons (such as glucose-regulated proteins (GRPs)) are up-regulated by NSAIDs and make cells resistant to NSAIDs (12, 13). Furthermore, geranylgeranylacetone (GGA), the leading anti-ulcer drug on the Japanese market, has been reported to induce HSPs at

FBS, fetal bovine serum; GRP, glucose-regulated protein; HE, hematoxylin and eosin; HO-1, heme oxygenase-1; HSP, heat shock protein; IL, interleukin; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; MITT, 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide; NF-2, NF-E2-related factor 2; PG, prostaglandin; P3K, phosphatidylinositol 3-kinase; SnMP, Sn(IV) Mesoporphyrin; TUNEL, TdT-mediated dUTP-biotin end-labeling; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; PIPES, 1,4-piperazinediethanesulfonic acid.
gastric mucosa that protect gastric mucosal cells against NSAIDs and other gastric irritants (14–17).

Heme oxygenase-1 (HO-1) is another type of stress protein. Not only its substrate, heme, but also various stressors such as oxidative stressors, ultraviolet irradiation, inflammatory cytokines, and heavy metals, have been reported to induce HO-1 production (18–20). HO-1 degrades heme to carbon monoxide (CO), free iron, and biliverdin. Biliverdin is subsequently converted into bilirubin by biliverdin reductase (18–20). Bilirubin and biliverdin are potent antioxidants and CO has anti-apoptotic activity. Therefore, up-regulation of HO-1 in cells makes cells resistant to apoptosis induced by various stressors (19–21).

HO-1 is also known as HSP32; however, the mechanism governing regulation of its expression is different from that of other HSPs (22). HO-1 is a phase II drug detoxifying enzyme. Such enzymes are regulated in a coordinated manner through a consensus cis-element and transcription factors, such as NF-E2-related factor 2 (Nrf2). HO-1-inducing stressors, such as reactive oxygen species, translocate Nrf2 from the cytoplasm into the nucleus where it binds to the consensus cis-element (Maf-recognition element (MARE)) to stimulate the transcription of genes located downstream (23–25). A number of kinases are involved in this activation (translocation) of Nrf2. They are mitogen-activated protein kinases (MAPKs) (extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK) and p38 MAPK) and phosphatidylinositol 3-kinase (PI3K). It has been suggested that the kinases involved in HO-1 up-regulation are different from each other depending on stressor and cell species (26–28).

It was recently reported that certain NSAIDs up-regulate HO-1 production in some types of cells (29–33). In this study, we show that all NSAIDs tested up-regulate HO-1 in cultured gastric mucosal cells, possibly through the p38 MAPK-dependent nuclear accumulation of Nrf2. The results of experiments with a specific inhibitor of HO (Sn(IV) Mesoporphyrin, SnMP) suggest that this up-regulation contributes to the suppression of NSAID-induced apoptosis and NSAID-induced gastric lesions.

EXPERIMENTAL PROCEDURES

Chemicals, Plasmids, and Animals—RPMI 1640 medium was obtained from Nissui Pharmaceutical Co. Pronase E and type 1 collagenase were purchased from Kaken Pharmaceutical Co. and Nitta Gelatin Co., respectively. Pluronic F127, fluo-3/AM, 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid (BAPTA-AM) was obtained from Dojindo Co. Flurbiprofen was from Cayman Chemicals and Loxoprofen was kindly provided by Sankyo Co. Fetal bovine serum (FBS), heme, β-nicotinamide adenine dinucleotide phosphate (β-NADP), glucose-6-phosphate dehydrogenase, glucose 6-phosphate, dicrofanc, anyosyomycin, ibuprofen, parafomaldehyde, probe-necid, proteinase K, and 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) were obtained from Sigma. SP600125, indomethacin and aspirin were obtained from Wako Co. Mayer’s hematoxylin, 1% eosin alcohol solution and Malinol were from MUTO pure chemical Co. Terminal deoxynucleotidyl transferase (TdTase) was from TOYOBO Co.

Biotin 14-ATP, Alexa Fluor 488 goat anti-rabbit immunoglobulin G, Alexa Fluor 488 conjugated with streptavidin and Lipofectamine (TM2000) were from Invitrogen. VECTASHIELD was from Vector Laboratory. SnMP was from Frontier Scientific Inc. Celecoxib was from LKT Laboratories Inc. Antibodies against HSP72, Nrf2, lamin B, GRP78 and actin were purchased from Santa Cruz Biotechnology Inc. Antibodies against HO-1 and p38 MAPK were from Stressgen and Cell Signaling Technology Inc., respectively. Acetyl-DEVD-methylcoumarin amide was from Peptide Institute Inc. O.C.T. compound was from Sakura Fintech. PD98059, SB203580, LY294002, and the Dual Luciferase Assay System, including a control plasmid harboring the Renilla reniformis luciferase gene (pRL-SV40), were from Promega. A plasmid containing the Photinus pyralis luciferase gene under control of the HO-1 gene promoter (pHO15luc) (34) was a gift kindly donated by J. Alam (Alton Ochsner Medical Foundation). This plasmid contain 15 kbp of mouse HO-1 5′-flanking region. A plasmid expressing enhanced green fluorescent protein (EGFP) (pEGFP-C1) was obtained from Clontech Laboratories Inc. Male guinea pigs weighing 200–300 g and male Wistar rats weighing 160–200 g were purchased from Kyudo Co. The experiments and procedures described here were carried out in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the National Institute of Health, and were approved by the Animal Care Committee of Kumamoto University.

Gastric Damage Assay—Gastric damage assays were performed as described previously (10). Rats, which had been fasted for 24 h, were intraperitoneally injected with SnMP (dissolved in 0.1 N NaOH, adjusted to pH 7.6 with HCl). One hour later, indomethacin in 1% methylcellulose was orally administered. Three hours after the oral administration, the rats were sacrificed by decapitation under light anesthesia with ethyl ether, and the stomachs were removed and scored for hemorrhagic damage by an observer unaware of the treatment the rats had received. Calculation of the scores involved measuring the area of all lesions in millimeters squared and summing the values to give an overall gastric lesion index.

Cell Culture, Transfection, and Cell Viability Assay—Gastric mucosal cells were isolated from guinea pig fundic glands, as described previously (17, 35). Isolated gastric mucosal cells were cultured for 12 h in RPMI 1640 containing 0.3% v/v FBS, 100 units/ml penicillin, and 100 µg/ml streptomycin in type-I collagen-coated plastic culture plates in 5% CO2, 95% air at 37 °C. After removing non-adherent cells by washing with RPMI 1640, cells that were attached to the plate at ~50% confluence were used. Guinea pig gastric mucosal cells prepared under these conditions have been previously characterized, with the majority (about 90%) of such cells being identified as pit cells (17, 35).

Human gastric carcinoma cells (AGS) were cultured in RPMI1640 medium supplemented with 10% FBS, 100 units/ml penicillin and 100 µg/ml streptomycin in 5% CO2, 95% air at 37 °C. Unless otherwise noted, cells (0.8 × 10⁴ cells per well in 24-well plates, 4 × 10⁴ cells per well in 6-well plates, 6 × 10⁵ cells in 100-mm plates) were cultured for 24 h and then used in the experiments. Transfection of cells with plasmid was carried out using Lipofectamine (TM2000) and/or F127.
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out using Lipofectamine (TM2000) according to the manufacturer’s instructions. Transfected cells were used for experiments after a 24-h recovery period. Transfection efficiency was determined in parallel plates by transfection of cells with the pEGFP-C1 control vector. Transfection efficiency was more than 80% in all experiments.

NSAIDs were dissolved in Me₂SO or Na₂CO₃ (for indomethacin only) and control experiments (without NSAIDs) were performed in the presence of the same concentrations of Me₂SO or Na₂CO₃. Cells were exposed to NSAIDs by changing the medium. Cell viability was determined by the MTT method.

**Immunoblotting Analysis**—Whole cell extracts and nuclear extracts were prepared as described previously (36, 37). The protein concentration of samples was determined by the Bradford method. Samples were applied to 8% (HSP72 and GRP78), 10% (lamin B, Nrf2, p38 MAPK, and actin) or 12% (HO-1) polyacrylamide SDS gels, subjected to electrophoresis, and proteins then immunoblotted with appropriate antibodies.

**Luciferase Assay**—The luciferase assay was performed as described previously (7). Cells were transfected with 0.375 μg of each of the P. pyralis luciferase reporter plasmids (pHO15luc or its vector) and 0.125 μg of the internal standard plasmid bearing the R. reniformis luciferase reporter (pRL-SV40). P. pyralis luciferase activity in cell extracts was measured using the Dual Luciferase Assay System and then normalized for R. reniformis luciferase activity.

**Histological and Immunohistochemical Analysis**—Gastric tissue samples were fixed in 4% buffered paraformaldehyde, embedded in O.C.T. compound and cryosectioned. Sections were stained first with Mayer’s hematoxylin and then with 1% eosin alcohol solution for histological examination (hematoxylin and eosin (HE) staining). Samples were mounted with Malinol and inspected using microscopy (Olympus IX70).

For immunohistochemical analysis, sections were blocked with 2.5% goat serum for 10 min and then incubated for 12 h with antibody against HO-1 (1:500 dilution) in the presence of 2.5% bovine serum albumin, and finally incubated for 1 h with Alexa Fluor 488 goat anti-mouse immunoglobulin G. Samples were mounted with VECTASHIELD and inspected using fluorescence microscopy (Olympus IX70).

**TdT-mediated dUTP-biotin End-labeling (TUNEL) Assay**—Gastric tissue samples were fixed in 4% buffered paraformaldehyde, embedded in O.C.T. compound and cryosectioned. Sections were first incubated with proteinase K (10 μg/ml) for 15 min at 37 °C, then with TdTase and biontin 14-ATP for 1 h at 37 °C and finally with Alexa Fluor 488 conjugated with streptavidin for 1 h. Samples were mounted with VECTASHIELD and inspected using fluorescence microscopy (Olympus IX70).

**Measurement of HO Activity**—Enzymatic activity of HO was determined as described previously (38), with some modifications.

Sample preparation from cultured cells: Cells were lysed by freeze–thawing and sonication in the 0.1 mM potassium phosphate buffer (pH 7.4) and centrifuged at 1000 × g for 10 min. The supernatants were applied to the HO assay system (see below).

Sample preparation from gastric mucosa: Gastric mucosal cells prepared from rats were homogenized in the 0.1 mM potassium phosphate buffer (pH 7.4) containing 0.25 mM sucrose, and centrifuged at 15,000 × g for 10 min. The supernatants were further centrifuged at 105,000 × g for 60 min. The precipitates were resuspended with the 0.1 mM potassium phosphate buffer (pH 7.4) containing 0.15 mM KCl and applied to the HO assay system (see below).

**HO Assay System**—After determination of the protein concentration, samples were incubated for 60 min at 37 °C in the dark with the following reagents: heme (17 μM), rat liver cytosol (10 mg/ml), MgCl₂ (2 mM), glucose-6-phosphate dehydrogenase (4 units), glucose-6-phosphate (0.85 mM), and β-NADP (2 mM) in 0.6 ml of 0.1 mM potassium phosphate buffer (pH 7.4). The reaction was stopped by placing the tubes on ice. The amount of bilirubin generated was estimated with a scanning spectrophotometer and was defined as the difference between 452 and 530 nm. The HO activity is expressed as pmol of bilirubin per milligram of protein per hour.

**Caspase Activity Assay**—The activity of caspase-3 was determined as described previously (39). Briefly, cells were collected by centrifugation and suspended in extraction buffer (50 mM PIPES (pH 7.0), 50 mM KCl, 5 mM EGTA, 2 mM MgCl₂, and 1 mM dithiothreitol). Suspensions were sonicated and centrifuged, after which the supernatants were incubated with fluorogenic peptide substrates (acetyl-DEVD-methylcoumarin amide) in reaction buffer (100 mM HEPES-KOH (pH 7.5), 10% sucrose, 0.1% CHAPS, and 1 mg/ml bovine serum albumin) for 15 min at 37 °C. The release of aminomethylcoumarin (AMC) was determined using a fluorescence spectrophotometer. One unit of protease activity was defined as the amount of enzyme required to release 1 pmol of AMC/min.

**Measurement of the Intracellular Ca²⁺ Level**—Intracellular Ca²⁺ levels were monitored as described (6). Briefly, cells were incubated with 4 μM fluo-3/AM in assay buffer supplemented with 0.1% bovine serum albumin, 0.04% Pluronic F127 and 2 mM probenecid, for 40 min at 37 °C. After washing twice with assay buffer, cells were suspended in assay buffer supplemented with 2 mM probenecid. Cells were transferred to a water-jacketed cuvette and the fluo-3 fluorescence was then measured with a HITACHI F-4500 spectrofluorophotometer. The intracellular Ca²⁺ level was calculated according to the equation 

\[ [Ca^{2+}]_i = K_d(F - F_{\text{min}})/(F_{\text{max}} - F), \]

where \( K_d \) is the apparent dissociation constant (400 nM) of the fluorescent dye-Ca²⁺ complex.

**Statistical Analysis**—All values are expressed as the mean ± S.D. One-way analysis of variance (ANOVA) followed by Scheffe’s multiple comparison test was used for evaluation of differences between groups. The Student’s t test for unequal results was used for the evaluation of differences between two groups. Differences were considered to be significant for values of \( p < 0.05 \).

**RESULTS**

**NSAIDs Up-regulate HO-1**—Up-regulation of HO-1 production by NSAIDs was examined in primary cultures of guinea pig gastric mucosal cells. This type of cell has been used as an in vitro model for physiological and pathological studies of gastric mucosa, because various characteristic features of gastric mucosal cells in vivo (such as vigorous secretion of mucin) are
reproduced in this system (17). As shown in Fig. 1A, treatment of cells with indomethacin up-regulated HO-1 very rapidly (within 3 h of the addition of indomethacin) and transiently (HO-1 levels returned to pre-treatment levels 24 h after the addition). Indomethacin is known to up-regulate other stress proteins (HSPs and GRPs) (12, 13). The results in Fig. 1A show that up-regulation of HO-1 by indomethacin occurs prior to that of HSP72 and GRP78. Fig. 1B shows the effects of different doses of indomethacin on HO-1 up-regulation. Up-regulation of HO-1 was just apparent at 25–50 μM indomethacin and was distinct at 200–400 μM indomethacin. These concentrations of indomethacin did not affect cell viability (Fig. 1C), showing that up-regulation of HO-1 by indomethacin is not the result of indomethacin-induced cell damage. On the other hand, up-regulation of HSP72 and GRP78 required much higher concentrations of indomethacin (Fig. 1B); in other words, up-regulation of these proteins occurs simultaneously with cell damage (Fig. 1C).

We also examined up-regulation of HO-1 by other NSAIDs (diclofenac, ibuprofen, aspirin, flurbiprofen, celecoxib, and loxoprofen). All of the NSAIDs tested up-regulated HO-1 (Fig. 2) at concentrations that did not affect cell viability (data not shown). As was the case for indomethacin, some NSAIDs showed two peaks in their dose response profile of HO-1 up-regulation (Fig. 2). COX exists as two subtypes, COX-1 and COX-2, for which celecoxib and flurbiprofen are COX-2-selective in their action. Results in Fig. 2 show that all NSAIDs tested increased cellular HO-1, irrespective of their COX-2 specificity. IC_{50} values for COX inhibition of each NSAID (40–42) are not related to the concentration required for HO-1 up-regulation (Figs. 1 and 2). Furthermore, loxoprofen is a pro-drug, meaning that its active metabolite but not itself has COX inhibitory activity (43). Therefore, it seems that NSAIDs up-regulate HO-1 independently of COX inhibition (see Fig. 6B).

**Contribution of HO-1 Up-regulation by NSAIDs to Protection of Gastric Mucosal Cells in Vitro and in Vivo**—Because up-regulation of HO-1 in cells protects cells against various stressors (19, 21), it is possible that up-regulation of HO-1 by NSAIDs protects gastric mucosal cells against NSAIDs. To test this idea, we examined the effect of an inhibitor of HO on NSAID-induced cell death in vitro. SnMP is a representative inhibitor of HO, which inhibits the enzymatic activity of HO by acting as a substrate analogue (44). As shown in Fig. 3A, SnMP stimulated cell death in the presence of various concentrations of NSAIDs (indomethacin, diclofenac, and ibuprofen), lowering the concentrations of NSAIDs required for induction of cell death. The concentration of SnMP used in the experiments pertaining to Fig. 3A did not affect cell viability in the absence of NSAIDs (Fig. 3A). Based on a previous report (45), the concentration used is enough to specifically inhibit HO activity. In fact, we measured the activity of HO under the same conditions as in Fig. 3A and confirmed that the activity of HO was stimulated by treatment of cell with indomethacin and this stimulation was diminished by simultaneous treatment with SnMP (Fig. 3C). Cell death, as highlighted in Fig. 3A, appears to be mediated by apoptosis given that we observed NSAID-dependent activation of caspase-3 under the same experimental conditions as in Fig. 3A (data not shown) and treatment of cells with SnMP stimulated the activity of caspase-3 in the presence of each NSAID (Fig. 3B). On this basis, the results in Fig. 3 show that SnMP stimulates NSAID-induced apoptosis and, therefore, suggest that up-regulation of HO-1 by NSAIDs contributes to protection of gastric mucosal cells from NSAID-induced apoptosis.
To address the in vivo relevance of the in vitro result (HO-1 up-regulation by NSAIDs), we tested whether orally administered NSAIDs up-regulate HO-1 in the gastric mucosa of rats. Oral administration of 10 mg/kg indomethacin produced gastric lesions in rats (see Fig. 5A) as described previously (10). Sections were prepared from the gastric tissues of these rats and were subjected to histological and immunohistochemical analysis. HE staining showed the presence of lesions in the gastric mucosa of indomethacin-administered rats but not in that from vehicle-administered rats (Fig. 4A). Furthermore, immunohistochemical analysis with an antibody against HO-1 showed that HO-1 is up-regulated in the gastric mucosa of indomethacin-administered rats relative to that from vehicle-administered rats (Fig. 4A).

We also examined the effect of indomethacin on the level of apoptosis at gastric mucosa that was monitored by TUNEL assay. Accompanying the production of gastric lesions, an increase in

**FIGURE 2. Up-regulation of HO-1 by various NSAIDs.** Guinea pig gastric mucosal cells in primary culture were incubated with the indicated concentration of each NSAID for 6 h. Up-regulation of HO-1 was monitored as described in the legend to Fig. 1.

**FIGURE 3. Effect of SnMP on NSAID-induced apoptosis in vitro.** Guinea pig gastric mucosal cells in primary culture were incubated with the indicated concentrations of indomethacin (IND), diclofenac (DIC), or ibuprofen (IBU) in the presence or absence of 50 μM SnMP for 16 h (A and B) or 6 h (C), as indicated. Cell viability was determined using the MTT method and shown as relative to the control (in the absence of both NSAIDs and SnMP) (A). Activities of caspase-3 (B) or HO (C) in cells were measured and expressed as described under “Experimental Procedures” (B and C). Values are given as mean ± S.D. (n = 3). ***, p < 0.001; **, p < 0.01; *, p < 0.05.
TUNEL-positive cells (apoptotic cells) was observed with the indomethacin administration (Fig. 4B). Furthermore, pre-administration of SnMP stimulates indomethacin-induced apoptosis whereas this pre-administration did not induce apoptosis without subsequent indomethacin administration (Fig. 4B). These results suggest that up-regulation of HO-1 by indomethacin contributes to protection of gastric mucosal cells from NSAID-induced apoptosis also in vivo.

To examine the role of this NSAID-dependent HO-1 up-regulation in gastric mucosa, we examined the effect of SnMP on NSAID-induced gastric lesions in rats. As shown in Fig. 5A, pre-administration of SnMP (1 μmol/kg, intraperitoneally) stimulated the production of gastric lesions following oral administration of indomethacin. This administration of SnMP did not produce gastric lesions unless it was followed by the oral administration of indomethacin (data not shown). Based on a previous report (46), the concentration used should be adequate to specifically inhibit HO activity. In fact, we measured the activity of HO under the same conditions as in Fig. 5A and confirmed that the activity of HO at gastric mucosa was stimulated by the oral administration of indomethacin and this stimulation was diminished by the intraperitoneal pre-administration of SnMP (Fig. 5B). These results strongly suggest that the indomethacin-induced up-regulation of HO-1 in gastric mucosa contributes to the protection of gastric mucosa from the formation of indomethacin-induced gastric lesions.

**Mechanism for Indomethacin-induced Up-regulation of HO-1**—To investigate the molecular mechanism governing the up-regulation of HO-1 by NSAIDs, instead of using guinea pig gastric mucosal cells in primary culture, we used AGS cells in which various molecular biology techniques can be used. First, we reproduced HO-1 up-regulation by indomethacin in AGS cells (Fig. 6A). In this cell type, the slight up-regulation of HO-1 seen at relatively low concentrations of indomethacin in primary cultures of guinea pig gastric mucosal cells (Fig. 1) was not
observed. We next examined the effect of exogenously added PGE$_2$, a major PG in gastric mucosa, on indomethacin-induced up-regulation of HO-1. As shown in Fig. 6B, the addition of 1 $\mu$M PGE$_2$ to the culture medium did not attenuate the indomethacin-induced up-regulation of HO-1. We previously determined the level of PGE$_2$ in the culture medium of AGS cells to be about 10 nM (47). Therefore, inhibition of PGE$_2$ synthesis by indomethacin (COX inhibition) does not seem to be involved in the up-regulation of HO-1 by indomethacin. We recently reported that various NSAIDs, including indomethacin, increase intracellular Ca$^{2+}$ levels and that this increase is responsible for NSAID-dependent up-regulation of some proteins, such as claudin-4, GRP78, and CHOP (6, 13, 48). Here, we tested the contribution of this increase in the intracellular Ca$^{2+}$ level to HO-1 up-regulation by indomethacin. As shown in Fig. 6C, an intracellular Ca$^{2+}$ chelator (BAPTA-AM) did not affect HO-1 up-regulation by indomethacin. The concentrations of BAPTA-AM used in this experiment have been shown to inhibit the up-regulation of claudin-4 and GRP78 in AGS cells (13, 48) and we confirmed that the concentration of BAPTA-AM completely inhibited the indomethacin-dependent increase in the intracellular free Ca$^{2+}$ level (Fig. 6D) (because clear increase in the intracellular Ca$^{2+}$ level was not observed with 600 or 800 $\mu$M of indomethacin (maybe because of its inhibitory effect on fluo-3 fluorescence), we used 200 $\mu$M of indomethacin). Results suggest that increases in intracellular Ca$^{2+}$ levels are not involved in indomethacin-induced up-regulation of HO-1.

Up-regulation of HO-1 by heme and various other stressors is due to activation of its transcription; in other words, cis-elements in the promoter of the HO-1 gene and its specific transcription factors, such as Nrf2, are important for the up-regulation (23, 49). We measured the activity of the HO-1 gene promoter using a reporter plasmid where the promoter sequence of the HO-1 gene was inserted upstream of the $P$. pyralis luciferase gene (34). As shown in Fig. 7, treatment of cells with indomethacin-stimulated luciferase activity in cells in both a dose- and incubation period-dependent manner, suggesting that the up-regulation of HO-1 by indomethacin is regulated at the level of transcription.

We then examined the effect of indomethacin on the amount of nuclear Nrf2. Nuclear fractions were prepared from indomethacin-treated or control AGS cells and the amount of Nrf2
was monitored by immunoblotting analysis. As shown in Fig. 8A, indomethacin increased the amount of Nrf2 in nuclear fractions, suggesting that indomethacin stimulated the translocation of Nrf2 from the cytoplasm into the nucleus.

Various kinases have been reported to be involved in HO-1 up-regulation and Nrf2 nuclear accumulation (26, 27, 50). In this study, we tried to identify the kinase involved in the NSAID-induced up-regulation of HO-1 using a specific inhibitor for each kinase (PI3K and MAPKs (ERK, JNK, and p38 MAPK)). As shown in Fig. 9, an inhibitor of p38 MAPK (SB203580), but not inhibitors for other kinases (PI3K, ERK, and JNK), suppressed the up-regulation of HO-1 by indomethacin. SB203580 also suppressed indomethacin-dependent nuclear accumulation of Nrf2 (Fig. 8B). It is known that p38 MAPK is activated by its phosphorylation (51). We found that as well as anysomycin, an activator of p38 MAPK, indomethacin increased levels of the phosphorylated form of p38 MAPK in cells (Fig. 10). Furthermore, this phosphorylation was almost completely inhibited by SB203580 but not by SnMP (Fig. 10). SB203580 did not affect the expression of HO-1, the nuclear accumulation of Nrf2 or the phosphorylation of p38 MAPK in the absence of indomethacin (Figs. 8–10). None of these inhibitors used in experiments pertaining to Figs. 8–10 affected cell viability at the concentrations used (data not shown) which, based on previous reports (52–56), would have been sufficient to inhibit each target molecule specifically.

**DISCUSSION**

In this study we found that all of the NSAIDs tested up-regulate HO-1 in primary cultures of guinea pig gastric mucosal cells. Because the concentrations of NSAIDs and incubation periods required for the up-regulation of HO-1 were relatively low and short, respectively, when compared with that of HSPs and GRPs, the NSAID-induced up-regulation of HO-1 seems to be important for the pharmacological actions of NSAIDs *in vivo*. In fact, we have shown that orally administered indomethacin up-regulates HO-1 at gastric mucosa at doses that cause production of gastric lesions in rats.

Using a specific inhibitor for HO, SnMP, we examined the physiological role of NSAID-induced up-regulation of HO-1 both *in vitro* and *in vivo*. HO inhibition by SnMP stimulated NSAID-induced apoptosis both *in vitro* and *in vivo* and also stimulated NSAID-induced production of gastric lesions *in vivo*. Taking previous observations into consideration, we speculate that both of these phenomena (*in vitro* and *in vivo*) are related to each other. NSAIDs induce not only necrosis but also apoptosis in primary cultures of gastric mucosal cells (9). Furthermore, we suggested that both COX inhibition at the gastric mucosa and direct gastric mucosal cell damage (such as induction of apoptosis) by NSAIDs are required for the production of gastric lesions by NSAIDs *in vivo*. gastric lesions developed in a manner that depends on both an intravenously administered
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low dose of indomethacin (inhibition of COX activity at the gastric mucosa without direct gastric mucosal cell damage) and an orally administered cytotoxic COX-2-selective NSAID (direct gastric mucosal cell damage without inhibition of COX at gastric mucosa) (10). We consider that NSAID induced up-regulation of HO-1 contributes to the protection of gastric mucosa from the formation of gastric lesions by suppressing NSAID-induced apoptosis of gastric mucosal cells. From this point of view, we propose here that non-toxic HO-1 inducers are therapeutically beneficial as anti-ulcer drugs, by analogy to the non-toxic HSP inducer, GGA (clinically used anti-ulcer drug).

As for the mechanism of NSAID-induced up-regulation of HO-1, we have shown using a luciferase reporter assay that up-regulation occurs at the level of transcription and that the transcription factor for the HO-1 gene, Nrf2, is accumulated in the nucleus in the presence of indomethacin. These results show that the mechanism is similar for NSAIDs and other HO-1 inducers (19). Because the kinase involved in Nrf2 activation and the resulting HO-1 up-regulation differ according to the stressor and cell species, we tried to identify the kinase responsible, using specific inhibitors of various kinases. An inhibitor of p38 MAPK (SB203580) suppressed not only indomethacin-dependent HO-1 up-regulation but also nuclear accumulation of Nrf2, strongly suggesting that the nuclear accumulation of Nrf2 and resulting up-regulation of HO-1 by indomethacin is mediated through the activation (phosphorylation) of p38 MAPK.

Although HO-1 was reported to activate p38 MAPK through CO production (57), the idea that the activation of p38 MAPK by NSAIDs is the result of the HO-1 up-regulation was not supported by the observation that SnMP did not suppress the NSAID-stimulated phosphorylation of p38 MAPK (Fig. 10).

At present, it is unclear how NSAIDs activate p38 MAPK. That is to say, the direct target of NSAIDs that leads to HO-1 up-regulation has not been defined. COX is a target of NSAIDs, which accounts for their anti-inflammatory activity, because PGs, such as PGE₂, have a strong capacity to induce inflammation. Because the capacity of each NSAID to up-regulate HO-1 did not correlate with their ability to inhibit COX and given that exogenously added PGE₂ did not suppress the up-regulation of HO-1 by NSAIDs, COX does not seem to be involved in NSAID-induced up-regulation of HO-1. We recently proposed that the cytotoxicity of NSAIDs results from the interaction of these molecules with cell membranes. The ability of each NSAID to result in membrane permeabilization correlated well with their cytotoxicity. NSAIDs increase the intracellular level of Ca²⁺ by stimulating Ca²⁺ influx through permeabilization of cytoplasmic membranes, and BAPTA-AM, an intracellular Ca²⁺ chelator, suppressed NSAID-induced apoptosis (6). However, membrane permeabilization and the resulting increase in the intracellular Ca²⁺ level also do not appear to be involved in NSAID-induced up-regulation of HO-1. Higher concentrations of NSAIDs are required for membrane permeabilization and increased intracellular Ca²⁺ levels than are required for HO-1 up-regulation (6), and BAPTA-AM did not suppress the HO-1 up-regulation by indomethacin. It was recently reported that thapsigargin, a specific inducer of the ER stress response, up-regulates HO-1 (58). Moreover, we reported that NSAIDs induce the ER stress response (7). However, the idea that NSAID-dependent HO-1 up-regulation is mediated through the ER stress response was not supported by our observations. In particular, the time course and dose response properties of HO-1 up-regulation did not correlate with those of the ER stress response (up-regulation of GRP78), and BAPTA-AM suppressed the NSAID-induced ER stress response (up-regulation of GRP78) (13) but not the up-regulation of HO-1.

HO-1 up-regulation has been suggested to play a protective role in inflammation. HO-1 deficiency in humans is associated with susceptibility to inflammation (59) and HO-1 knock-out mice show higher mortality rates after exposure to endotoxin than wild-type mice (60). Furthermore, HO-1 up-regulation inhibits or stimulates production of tumor necrosis factor (TNF) α (a pro-inflammatory mediator) or interleukin (IL)-10 (an anti-inflammatory mediator), respectively, through CO production, and inhibits microvascular endothelial cell-leukocyte adhesion through bilirubin production (61–63). Therefore, up-regulation of HO-1 by NSAIDs may be involved in not only the protection of gastric mucosa from NSAID-induced gastric lesions but also in the anti-inflammatory activity of NSAIDs.

NSAIDs show various pharmacological activities other than their anti-inflammatory action (such as chemopreventive activity and anti-Alzheimer’s disease activity). The HO-1 up-regulation by NSAIDs may also be involved in these activities. Epidemiological studies have shown that prolonged use of aspirin or other NSAIDs reduces the risk of Alzheimer’s disease (64), although the mechanism for this activity is not fully understood. Tau protein plays a major role in the development of Alzheimer’s disease and high levels of reactive oxygen species are believed to promote the development of Alzheimer’s disease (65). Overexpression of HO-1 was reported to result in decreased levels of tau protein in cells (66). We consider that the NSAID-induced up-regulation of HO-1 is involved in the anti-Alzheimer’s disease activity of NSAIDs resulting from a decrease in the level of tau protein and reactive oxygen species. On the other hand, based on epidemiological and animal studies, it was proposed that inducers of phase II drug detoxifying enzymes, that include HO-1, can be useful as chemopreventive drugs for cancer, because they can metabolize (detoxify) endogenous and environmental carcinogens (67). Furthermore, in nrf2-disrupted mice the chemopreventive effect of dithiolethiones (an inducer of phase II drug detoxifying genes) was lost because of a defect in the expression of phase II drug detoxifying genes (68). Based on these observations, we consider that up-regulation of HO-1 by NSAIDs is involved in their chemopreventive activity through the induction phase II drug detoxifying enzymes such as HO-1.

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