cells from apoptosis in the presence of celecoxib. Furthermore, the stimulation of apoptosis and CHOP mRNA expression by transfection of siRNA for GRP78 in the absence of celecoxib suggests that constitutively expressed GRP78 under nonstress conditions contributes to cell survival.

We also tried to examine the effect of siRNA for GRP78 on apoptosis induced by chemotherapy drug without ability to induce ER stress response. As shown in Figure 10h, staurosporine, a chemotherapy drug, did not upregulate GRP78 at concentrations that are enough to induce apoptosis (Figure 10g). As shown in Figure 10g, transfection of siRNA for GRP78 slightly stimulated apoptosis induced by staurosporine. This slight stimulation can be explained by its effect on the spontaneous apoptosis (apoptosis in the absence of drugs). In other words, the extent of the stimulation was not so drastic as is the case of celecoxib (Figure 10e). Suppression of GRP78 through siRNA for GRP78 sensitized human breast cancer cells to etoposide-mediated cell death (Dong *et al*., 2005). These results suggested that the stimulatory effect of siRNA for GRP78 on apoptosis is apparent for apoptosis induced by chemotherapy drugs that induce ER stress response.

Discussion

In this study, we have shown that several NSAIDs upregulate ER chaperones not only in various types of cultured human gastric cancer cells but also in xenograft tumors in nude mice. We reported previously that a number of different NSAIDs upregulate GRP78 in guinea pig gastric mucosal cells in primary culture (Tsutsumi *et al*., 2004). Furthermore, we recently found that NSAIDs upregulate ER chaperones in HEK293 cells (human embryonic kidney cells) (Hoshino T *et al*., unpublished results). Therefore, it seems that NSAIDs upregulate ER chaperones as a part of their general mechanism of action. Concentrations of NSAIDs required for induction of GRP78 *in vitro* (60–100 μM) are relatively higher than clinical available concentrations of celecoxib (1–10 μM) (Lopez-Parra *et al*., 2005; Patel *et al*., 2005). It was reported that oral administration of celecoxib (100–200 mg/kg/day, similar conditions to our experiments in Figure 3) caused serum concentrations of 8.6–11.3 μM (Kulp *et al*., 2004). Therefore, under *in vivo* conditions, lower concentrations of celecoxib may be able to induce GRP78 than under *in vitro* conditions. This induction is likely to be mediated by COX-independent mechanisms, given that NSAIDs

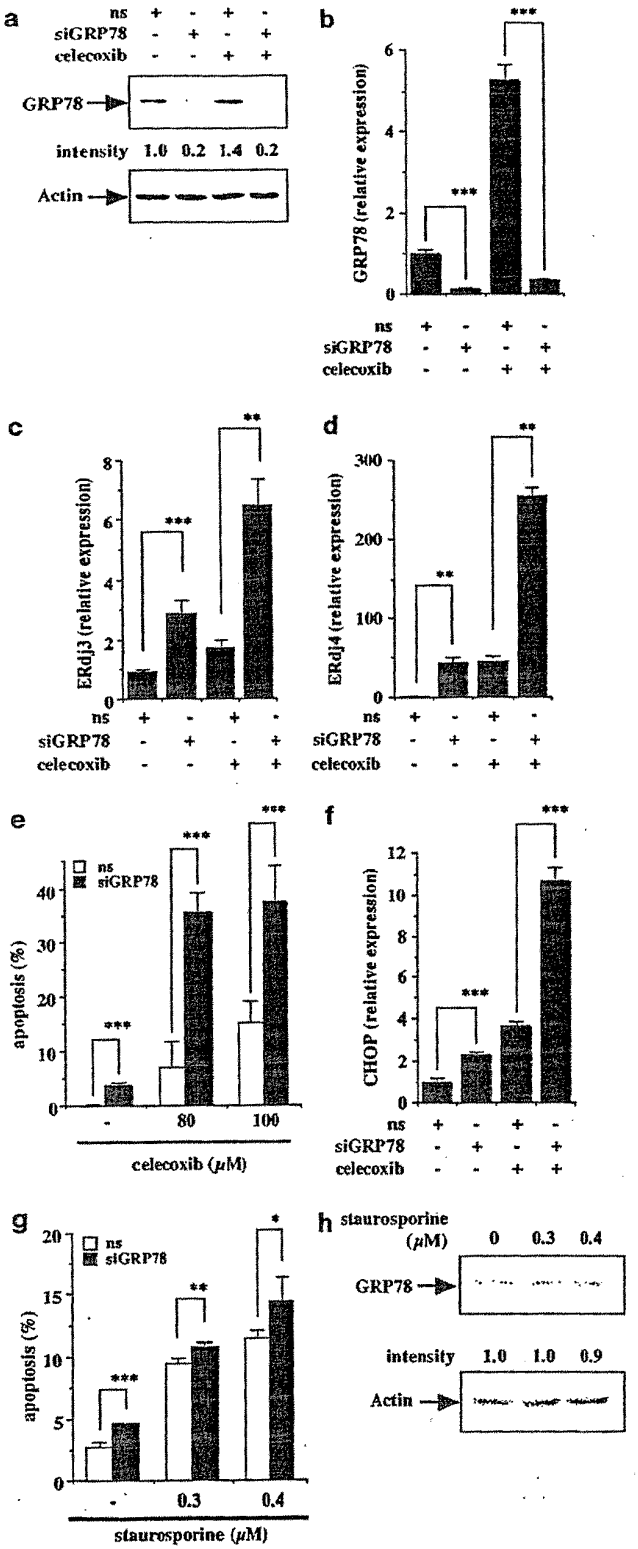

Figure 10 Effect of GRP78 siRNA on celecoxib-induced apoptosis. AGS cells were transfected with 5 μg of siRNA for GRP78 (siGRP78) or nonsilencing (ns) siRNA. After 48 h, cells were incubated with or without 80 μM celecoxib (**a**–**d** and **f**), indicated concentrations of celecoxib (**e**) or indicated concentrations of staurosporine (**g** and **h**) for 6 h. The levels of GRP78 protein (**a** and **h**), GRP78 mRNA (**b**), ERdj3 mRNA (**c**), ERdj4 mRNA (**d**) and CHOP mRNA (**f**) were estimated by immunoblotting or real-time RT–PCR experiments as described in the legends of Figures 1 and 2. Apoptotic cell numbers were determined by FACS as described in the legend of (Figure 7e and g). Values shown are mean ± s.d. ($n=3$). ***$P<0.001$; **$P<0.01$; *$P<0.05$.

increased GRP78 production irrespective of their COX-2 specificity and that COX-2-selective NSAIDs (celecoxib) upregulated GRP78 in cells that did not express COX-2. Furthermore, although indomethacin at a concentration of less than 1 μM inhibited both COX-1 and COX-2 (Kawai *et al.*, 1998), the upregulation of GRP78 required higher concentrations. The IC_{50} value of celecoxib for COX-2 inhibition is about 40 nM (Schroeder *et al.*, 2004; Ben-Chetrit *et al.*, 2005), which is much lower than the concentration required for the upregulation of GRP78 (about 60 μM).

The results in this study suggest that the upregulation of ER chaperones by celecoxib involves the PERK-eIF2α-ATF4 pathway. Other recent papers also suggest the importance of this pathway in the upregulation of ER chaperones by other ER stressors (Luo *et al.*, 2003; Roybal *et al.*, 2004). However, siRNA for ATF4 did not completely inhibit the celecoxib-dependent GRP78 upregulation, suggesting that pathways other than the PERK–eIF2α–ATF4 pathway are involved in this mechanism. In addition to PERK, two other ER transmembrane proteins (ATF6 and IRE1) are involved in the ER stress response. Activated ATF6 translocates to the nucleus where it specifically binds to the promoter of ER chaperone genes, while IRE1 splices X box-binding protein XBP-1 mRNA, to convert it into a potent activator for the transcription of ER chaperone genes.(Yoshida *et al.*, 2000; Kaufman, 2002; Ron, 2002) We suggest that both ATF6 and IRE1 are activated in the presence of NSAIDs in gastric mucosal cells in primary culture (Tsutsumi *et al.*, 2004), and we here showed that ATF6 is activated by celecoxib even in AGS cells.

It is well known that an increase in the intracellular Ca^{2+} concentration induces ER chaperone; Ca^{2+} ionophores, for example, induce ER chaperone (Drummond *et al.*, 1987; Wooden *et al.*, 1991). We suggested that an increase in intracellular Ca^{2+} concentration is involved in the celecoxib-dependent upregulation of ER chaperones based on the following observations: celecoxib increased the intracellular Ca^{2+} concentration and expression of ER chaperones simultaneously; and the intracellular Ca^{2+} chelator, BAPTA-AM, inhibited the celecoxib-dependent upregulation of ER chaperones. BAPTA-AM also inhibited the GRP78 upregulation caused by nimesulide or diclofenac but not by indomethacin. We recently showed that all of NSAIDs tested (including nimesulide and diclofenac) increased the intracellular Ca^{2+} level (Tomisato *et al.*, 2004a; Tanaka *et al.*, 2005). Since indomethacin absorbed fluo-3 fluorescence (530 nm), we could not measure the intracellular Ca^{2+} level in the presence of indomethacin by the assay system using fluo-3 (Tanaka *et al.*, 2005). Therefore, it seems that not only celecoxib but also most of NSAIDs induce GRP78 through increasing the intracellular Ca^{2+} level and that indomethacin induces GRP78 through different unknown mechanism. As for the mechanism for the increase in the intracellular Ca^{2+} level, both inhibition of sarcoplasmic/ER Ca^{2+} ATPase (sarco endoplasmic reticulum Ca^{2+} ATPase; an ER-located Ca^{2+} pump that is responsible for accumulation of Ca^{2+} in the ER) and stimulation of the influx of extracellular Ca^{2+} have been proposed (Johnson *et al.*, 2002; Wang *et al.*, 2004). We recently found that all of the NSAIDs tested permeabilize cellular membranes and this capacity of NSAIDs is closely related to their activity for increasing intracellular Ca^{2+} concentration, suggesting that NSAIDs stimulate the influx of extracellular Ca^{2+} by permeabilizing cytoplasmic membranes (Tomisato *et al.*, 2004a; Tanaka *et al.*, 2005). Since the suppression of celecoxib-dependent GRP78 upregulation by BAPTA-AM was partial, a further mechanism other than an increase in the intracellular Ca^{2+} level may be involved, in the upregulation of GRP78. Accumulation of unfolded proteins in the ER by inhibition of the proteasome system may be involved in this upregulation; it was suggested that NSAIDs inhibit the proteasome system and that specific inhibitors of the proteasome system, such as MG132, induce the ER stress response (Bush *et al.*, 1997; Huang *et al.*, 2002).

Overproduction of GRP78 in cells was reported to make them resistant to apoptosis induced by topoisomerase inhibitors, tunicamycin or Ca^{2+} ionophores (Morris *et al.*, 1997; Reddy *et al.*, 2003). We showed here that this phenomenon can be applied to celecoxib-induced apoptosis and that the antiapoptotic effect of GRP78 is stimulated by coexpression of its cochaperones, ERdj3 and ERdj4. This effect of ERdj4 depends on its J domain, as was the case for stimulation of the antiapoptotic effect of HSP70 by HSP40 (Gotoh *et al.*, 2001). Since the J domain of ERdj4 is essential for its interaction with GRP78 and its activation of GRP78 ATPase activity, the stimulation by ERdj4 (and also ERdj3 presumably) of the antiapoptotic activity of GRP78 should be mediated by this physical and functional interaction between them.

We previously used CHOP-deficient mice or a dominant-negative form of CHOP to show that CHOP, a transcription factor with apoptosis-inducing activity, is essential for NSAID-induced apoptosis (Tsutsumi *et al.*, 2004). Since induction of CHOP by celecoxib is partially suppressed by the overproduction of GRP78, it is reasonable to speculate that this suppression is involved in the antiapoptotic effect of GRP78. It was suggested that GRP78 binds to PERK (and also IRE1 and ATF6) and inactivates PERK's ER stress response inducing capabilities under nonstress conditions; when ER stressors accumulate unfolded proteins in the ER, PERK (and also IRE1 and ATF6) is activated to induce the ER stress response by releasing GRP78 from PERK, as a result of the binding of unfolded proteins to GRP78 (Bertolotti *et al.*, 2000; Shen *et al.*, 2002a). Induction of GRP78 by proteasome inhibitors has been reported (Hong *et al.*, 2004). It was reported that overexpression of GRP78 inhibited the phosphorylation of PERK in the presence of an ER stressor (thapsigargin) (Bertolotti *et al.*, 2000). Therefore, we consider that by this mechanism, overproduced GRP78 suppressed the celecoxib-dependent CHOP induction and resulting apoptosis. It was also suggested that GRP78 inhibits topoisomerase inhibitor-induced apoptosis by inhibiting

caspase-7 (Reddy *et al*., 2003), which may also be involved in the antiapoptotic effect of GRP78 against NSAIDs.

Resistance to chemotherapy is one of the major obstacles of cancer therapy. Owing to poor vascularization, solid tumors usually exist under conditions of glucose starvation and hypoxia, which causes induction of the ER stress response. In fact, overproduction of ER chaperones was reported in various types of tumors and artificial overproduction of ER chaperones stimulated tumor progression (Jamora *et al*., 1996; Koomagi *et al*., 1999; Fernandez *et al*., 2000; Song *et al*., 2001; Huo *et al*., 2004). Therefore, the finding in this study that overproduction of GRP78 makes cancer cells resistant to celecoxib is very important in considering the use of celecoxib in chemotherapy; it seems that not only constitutively overproduced ER chaperones in tumors but also ER chaperones induced by NSAIDs can make them resistant to chemotherapy by NSAIDs. In fact, we showed that orally administered celecoxib caused both inhibition of xenograft tumor growth and induction of GRP78 simultaneously in nude mice. We consider that GRP78 upregulation by celecoxib decreases the activity of this drug for the suppression of tumor growth and thus propose that an inhibitor of ER chaperones may be of clinical benefit by making tumor cells more responsive to NSAIDs.

Materials and methods

Chemicals, plasmids and animals

RPMI 1640 medium was obtained from Nissui Pharmaceutical Co. Fetal bovine serum (FBS) was obtained from Gibco Co. Pluronic F127, fluo-3/AM and BAPTA-AM were from Dojindo Co. Thapsigargin, staurosporine, diclofenac, RNaseA and propidium iodide (PI) were obtained from Sigma Co. PGE_2 and indomethacin were obtained from Wako Co. Celecoxib was from LKT Laboratories Inc. Nimesulide was from Cayman Chemical Co. Antibodies against GRP78, ATF6, lamin, ATF4 and actin were purchased from Santa Cruz Biotechnology Inc., and those against phosphorylated PERK and phosphorylated eIF2α were from Cell Signaling Technology Inc. The RNeasy kit, siRNAs and RNAiFect transfection reagent were from Qiagen. A first-strand cDNA synthesis kit was purchased from Amersham. Lipofectamine (TM2000) and pcDNA3.1 plasmid were obtained from Invitrogen. SYBR GREEN PCR Master Mix was from ABI. A plasmid of pEGFP-N1 was obtained from Clontech. Plasmids of pcDNA3.1/GRP78 and pCR3.1/ERdj3 were gifts from Drs Austin R and Haslam D, respectively. Plasmids of pcDNA3.1/ERdj4 and pcDNA3.1/ERdj4ΔJ were a gift from Dr K Imaizumi. Female ICR nude mice (5 weeks of age) were obtained from the Kyudoh 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.

Cell culture and overexpression of ER chaperones

Cells were cultured in RPMI1640 medium supplemented with 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin in a humidified atmosphere of 95% air with 5% CO_2 at 37°C. NSAIDs were dissolved in DMSO and control experiments (without NSAIDs) were performed in the presence of the same concentrations of DMSO. Cells were exposed to NSAIDs by changing the medium. Unless otherwise noted, cells (0.8×10^4 cells per well in 24-well plates, 4×10^4 cells per well in six-well plates, 6×10^5 cells in 100-mm plates) were cultured for 24 h and used in experiments. For transient expression of each gene, cells were seeded 24 h before transfection in six-well plates at a density of 5×10^5/well. The transfection with pcDNA3.1 containing each gene was carried out using Lipofectamine (TM2000) according to the manufacturer's instructions. Cells were used for experiments after a 48 h recovery period. Transfection efficiency was determined in parallel plates by transfection of the pEGFP-N1 control vector. Transfection efficiency was more than 80% in all experiments.

Analysis of apoptosis by fluorescence-activated cell sorting (FACS)

Apoptosis was monitored by FACS analysis as described previously (Alves da Costa *et al*., 2002). Briefly, cells were cultured in 100-mm plates and collected by centrifugation. Pellets were fixed with 70% ethanol for 4 h at −20°C and centrifuged again. Pellets were resuspended in phosphate–citrate buffer (0.2 M Na_2HPO_4 and 4 mM citric acid) and incubated for 20 min at RT. After centrifugation, the pellets were resuspended in DNA staining solution (50 mg/ml PI and 10 μg/ml RNaseA) and incubated for 20 min at RT. Samples were scanned with a FACSCalibur (Becton Dickinson) cell sorter under conditions to measure only specific PI-mediated fluorescence. The signal threshold was determined based on the fluorescence of nontransfected cells. Apoptotic cells appeared as a hypodiploid peak due to nuclear fragmentation and loss of DNA.

Real-time RT–PCR analysis

Total RNA was extracted from cells using an RNeasy kit according to the manufacturer's protocols. Samples (10 μg RNA) were reverse-transcribed using a first-strand cDNA synthesis kit according to the manufacturer's instructions. Synthesized cDNA was applied to real-time RT–PCR (ABI PRISM 7700) using SYBR GREEN PCR Master Mix and analysed with ABI PRISM 7700 Sequence Detection Software according to the manufacturer's instructions. Real-time cycle conditions were 2 min at 50°C, followed by 10 min at 90°C and then for 45 cycles at 95°C for 30 s and 63°C for 60 s. Specificity was confirmed by electrophoretic analysis of the reaction products and by inclusion of template- or reverse transcriptase-free controls. To normalize the amount of total RNA present in each reaction, the actin gene was used as an internal standard.

Primers were designed using the Primer3 website (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). Primers are listed as follows: gene name: forward primer, reverse primer. ATF4: 5′-tcaaacctcatgggttctcc-3′, 5′-gtgtcatccaacgtggtcag-3′; ATF2: 5-ctccagctcacacaactcca-3′, 5′-gtttcagctgtgccacttca-3′; ATF3: 5′-atgatgcttcaacacccaggc-3′, 5′-ttagctctgcaatgttccttcc-3′; CHOP: 5′-tgcctttctcttcggacact-3′, 5′-tgtgacctctgctggttctg-3′; GRP78: 5′-tag cgtatggtgctgctgtc-3′, 5′-tttgtcaggggtctttcacc-3′; ERdj3: 5′-cggt tccgaatcaaagttgt-3′, 5′-cttccatagcttcgctcctg-3′; ERdj4: 5′-aaaataag agcccggatgct-3′, 5′-cgcttcttggatccagtgtt-3′; actin: 5′-ggacttcgagcaagagatgg-3′, 5′-agcactgtgttggcgtacag-3′.

Immunoblotting analysis

Whole-cell extracts were prepared as described previously (Tsutsumi *et al*., 2002). The protein concentration of samples

was determined by the Bradford method. Samples were applied to 8% (for PERK and GRP78) or 10% (for eIF2α and actin) polyacrylamide SDS gels, subjected to electrophoresis, and proteins then immunoblotted with respective antibodies.

Xenograft tumor growth
Effect of celecoxib on xenograft tumor growth was examined as described previously (Kulp *et al*., 2004). Each nude mouse was inoculated s.c. in the right hind footpad with 2×10^6 cells of MKN-45 suspended in 0.1 ml of serum-free medium. When tumors reached a mean volume of $116 \pm 34\,mm^3$, mice started to receive single daily oral administration of celecoxib (100 or 200 mg/kg (body weight)) in 1% methylcellulose and this administration was continued for the duration of the study. Control mice received vehicle (1% methylcellulose only). Tumors were measured weekly using calipers and their volumes calculated using the following standard formula: $width^2 \times length \times 0.5$. Body weights were measured daily. For examination of GRP78 expression in tumors, tumor xenografts (dissected into <1-mm pieces) were solubilized with RIPA buffer (50 mM Tris-HCl (pH7.2), 150 mM NaCl, 1% NP-40, 1% Sodium deoxycholate and 0.05% SDS) and subjected to immunoblotting analysis.

Measurement of intracellular Ca^{2+} levels
The intracellular Ca^{2+} levels were monitored according to manufacture's protocols (Kao *et al*., 1989). Cells were washed with assay buffer (115 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgCl_2$, 20 mM HEPES and 13.8 mM glucose). Cells were then incubated with 4 μM fluo-3/AM in the assay buffer also containing 0.1% BSA, 0.04% Pluronic F127 and 2 mM probenecid for 40 min at 37°C. After washing twice with the assay buffer, cells were suspended in assay buffer also containing 2 mM probenecid. Fluo-3 fluorescence of cells in a water-jacketed cuvette (1.6×10^6 cells/cuvette) was measured with a HITACHI F-4500 spectrofluorophotometer by recording excitation signals at 490 nm and the emission signal at 530 nm at 1-second intervals. Maximum and minimum fluorescence values (F_{max} and F_{min}) were obtained by adding 10 μM ionomycin and 10 μM ionomycin plus 5 mM EGTA (in Ca^{2+}-free medium), respectively. The intracellular Ca^{2+} level was calculated according to the equation $[Ca^{2+}]_i = K_d(F-F_{min})/(F_{max}-F)$, where K_d is the apparent dissociation constant (400 nM) of the fluorescent dye–Ca^{2+} complex.

SiRNA targeting of genes
We used siRNA of 5′-ggagcgcauugauacuagadTdT-3′ and 5′-ucuaguaucaaugcgcuccdTdT-3′ or 5′-gccuaggucucuuagaugadTdT-3′ and 5′-ucaucuaagagaccuaggcdTdT-3′ as annealed oligonucleotides for repressing GRP78 or ATF4 expression, respectively. AGS cells were transfected with siRNA using RNAiFect transfection reagent according to the manufacturer's instructions. Nonsilencing siRNA (5′-uucuccgaacgugu cacgudTdT-3′ and 5′-acgugacacguucggagaadTdT-3′) was used as a negative control.

Statistical analysis
All values are expressed as the mean ± standard deviation (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 unpaired results was used for the evaluation of differences between two groups. Differences were considered to be significant for values of $P < 0.05$.

Abbreviations

ATF, activating transcription factor; BAPTA-AM, 1,2-bis(2-aminophenoxy)ethane-*N,N,N′N′*-tetraacetic acid; CHOP, C/EBP homologous transcription factor; COX, cyclooxygenase; eIF2α, eukaryotic initiation factor-2α; ER, endoplasmic reticulum; FACS, fluorescence-activated cell sorting; FBS, fetal bovine serum; IRE1, protein-kinase and site-specific endoribonuclease; NSAIDs, non-steroidal anti-inflammatory drugs; PERK, eukaryotic translation initiation factor 2 kinase; PG, prostaglandin; PI, propidium iodide; SERCA, sarco endoplasmic reticulum Ca^{2+} ATPase; siRNA, small interfering RNA; XBP-1, X box binding protein.

Acknowledgements
We thank Drs R Austin (McMaster University), D Haslam (Washington University) and K Imaizumi (NAIST) for the pcDNA3.1/GRP78, pCR3.1/ERdj3 and pcDNA3.1/ERdj4 (ERdj4ΔJ) plasmids, respectively. This work is supported by Grants-in-Aid for Scientific Research from the Ministry of Health, Labour and Welfare of Japan; Suzuken Memorial Foundation; Japan Research Foundation for Clinical Pharmacology.

References

Alves da Costa C, Paitel E, Mattson MP, Amson R, Telerman A, Ancolio K *et al*. (2002). *Proc Natl Acad Sci USA* **99**: 4043–4048.
Ben-Chetrit E, Fischel R, Hinz B, Levy M. (2005). *Rheumatol Int* **25**: 332–335.
Bertolotti A, Zhang Y, Hendershot LM, Harding HP, Ron D. (2000). *Nat Cell Biol* **2**: 326–332.
Bush KT, Goldberg AL, Nigam SK. (1997). *J Biol Chem* **272**: 9086–9092.
Dong D, Ko B, Baumeister P, Swenson S, Costa F, Markland F *et al*. (2005). *Cancer Res* **65**: 5785–5791.
Drummond IA, Lee AS, Resendez Jr E, Steinhardt RA. (1987). *J Biol Chem* **262**: 12801–12805.
Eberhart CE, Coffey RJ, Radhika A, Giardiello FM, Ferrenbach S, DuBois RN. (1994). *Gastroenterology* **107**: 1183–1188.
Elder DJ, Halton DE, Hague A, Paraskeva C. (1997). *Clin Cancer Res* **3**: 1679–1683.
Fan XM, Wong BC, Lin MC, Cho CH, Wang WP, Kung HF *et al*. (2001). *J Gastroenterol Hepatol* **16**: 1098–1104.
Farrow DC, Vaughan TL, Hansten PD, Stanford JL, Risch HA, Gammon MD *et al*. (1998). *Cancer Epidemiol Biomarkers Prev* **7**: 97–102.
Fernandez PM, Tabbara SO, Jacobs LK, Manning FC, Tsangaris TN, Schwartz AM *et al*. (2000). *Breast Cancer Res Treat* **59**: 15–26.
Gotoh T, Terada K, Mori M. (2001). *Cell Death Differ* **8**: 357–366.
Gupta RA, Dubois RN. (2001). *Nat Rev Cancer* **1**: 11–21.
Hanif R, Pittas A, Feng Y, Koutsos MI, Qiao L, Staiano-Coico L *et al*. (1996). *Biochem Pharmacol* **52**: 237–245.

Harding HP, Novoa I, Zhang Y, Zeng H, Wek R, Schapira M *et al.* (2000). *Mol Cell* **6**: 1099–1108.
Hong M, Li M, Mao C, Lee AS. (2004). *J Cell Biochem* **92**: 723–732.
Hoshino T, Tsutsumi S, Tomisato W, Hwang HJ, Tsuchiya T, Mizushima T. (2003). *J Biol Chem* **278**: 12752–12758.
Hu PJ, Yu J, Zeng ZR, Leung WK, Lin HL, Tang BD *et al.* (2004). *Gut* **53**: 195–200.
Huang YC, Chuang LY, Hung WC. (2002). *Mol Pharmacol* **62**: 1515–1521.
Huo R, Zhu YF, Ma X, Lin M, Zhou ZM, Sha JH. (2004). *Cell Tissue Res* **316**: 359–367.
Husain SS, Szabo IL, Tamawski AS. (2002). *Am J Gastroenterol* **97**: 542–553.
Jamora C, Dennert G, Lee AS. (1996). *Proc Natl Acad Sci USA* **93**: 7690–7694.
Jiang HY, Wek SA, McGrath BC, Lu D, Hai T, Harding HP *et al.* (2004). *Mol Cell Biol* **24**: 1365–1377.
Johnson AJ, Hsu AL, Lin HP, Song X, Chen CS. (2002). *Biochem J* **366**: 831–837.
Kao JP, Harootunian AT, Tsien RY. (1989). *J Biol Chem* **264**: 8179–8184.
Kaufman RJ. (2002). *J Clin Invest* **110**: 1389–1398.
Kawai S, Nishida S, Kato M, Furumaya Y, Okamoto R, Koshino T *et al.* (1998). *Eur J Pharmacol* **347**: 87–94.
Kismet K, Akay MT, Abbasoglu O, Ercan A. (2004). *Cancer Detect Prev* **28**: 127–142.
Koki AT, Masferrer JL. (2002). *Cancer Control* **9**: 28–35.
Koomagi R, Mattern J, Volm M. (1999). *Anticancer Res* **19**: 4333–4336.
Kulp SK, Yang YT, Hung CC, Chen KF, Lai JP, Tseng PH *et al.* (2004). *Cancer Res* **64**: 1444–1451.
Kurisu J, Honma A, Miyajima H, Kondo S, Okumura M, Imaizumi K. (2003). *Genes Cells* **8**: 189–202.
Landry SJ. (2003). *Biochemistry* **42**: 4926–4936.
Leahy KM, Ornberg RL, Wang Y, Zweifel BS, Koki AT, Masferrer JL. (2002). *Cancer Res* **62**: 625–631.
Lee AS. (2001). *Trends Biochem Sci* **26**: 504–510.
Lim JW, Kim H, Kim KH. (2001). *Lab Invest* **81**: 349–360.
Lopez-Parra M, Claria J, Titos E, Planaguma A, Parrizas M, Masferrer JL *et al.* (2005). *J Hepatol* **42**: 75–81.
Luo S, Baumeister P, Yang S, Abcouwer SF, Lee AS. (2003). *J Biol Chem* **278**: 37375–37385.
Mima S, Tsutsumi S, Ushijima H, Takeda M, Fukuda I, Yokomizo K *et al.* (2005). *Cancer Res* **65**: 1868–1876.
Morris JA, Dorner AJ, Edwards CA, Hendershot LM, Kaufman RJ. (1997). *J Biol Chem* **272**: 4327–4334.
Okada K, Sugihara H, Bamba M, Bamba T, Hattori T. (2000). *Cancer Genet Cytogenet* **118**: 99–107.
Patel MI, Subbaramaiah K, Du B, Chang M, Yang P, Newman RA *et al.* (2005). *Clin Cancer Res* **11**: 1999–2007.
Piazza GA, Rahm AL, Krutzsch M, Sperl G, Paranka NS, Gross PH *et al.* (1995). *Cancer Res* **55**: 3110–3116.
Reddy RK, Mao C, Baumeister P, Austin RC, Kaufman RJ, Lee AS. (2003). *J Biol Chem* **278**: 20915–20924.
Riendeau D, Charleson S, Cromlish W, Mancini JA, Wong E, Guay J. (1997). *Can J Physiol Pharmacol* **75**: 1088–1095.
Ristimaki A, Honkanen N, Jankala H, Sipponen P, Harkonen M. (1997). *Cancer Res* **57**: 1276–1280.
Ron D. (2002). *J Clin Invest* **110**: 1383–1388.
Roybal CN, Yang S, Sun CW, Hurtado D, Vander Jagt DL, Townes TM *et al.* (2004). *J Biol Chem* **279**: 14844–14852.
Schroeder CP, Yang P, Newman RA, Lotan R. (2004). *Cancer Biol Ther* **3**: 847–852.
Shen J, Chen X, Hendershot L, Prywes R. (2002a). *Dev Cell* **3**: 99–111.
Shen Y, Meunier L, Hendershot LM. (2002b). *J Biol Chem* **277**: 15947–15956.
Smalley WE, Ray WA, Daugherty JR, Griffin MR. (1995). *Am J Epidemiol* **141**: 539–545.
Song MS, Park YK, Lee JH, Park K. (2001). *Cancer Res* **61**: 8322–8330.
Sorensen HT, Friis S, Norgard B, Mellemkjaer L, Blot WJ, McLaughlin JK *et al.* (2003). *Br J Cancer* **88**: 1687–1692.
Tanaka K, Tomisato W, Hoshino T, Ishihara T, Namba T, Aburaya M *et al.* (2005). *J Biol Chem* **280**: 31059–31067.
Tomisato W, Tanaka K, Katsu T, Kakuta H, Sasaki K, Tsutsumi S *et al.* (2004a). *Biochem Biophys Res Commun* **323**: 1032–1039.
Tomisato W, Tsutsumi S, Hoshino T, Hwang HJ, Mio M, Tsuchiya T *et al.* (2004b). *Biochem Pharmacol* **67**: 575–585.
Tomisato W, Tsutsumi S, Rokutan K, Tsuchiya T, Mizushima T. (2001). *Am J Physiol Gastrointest Liver Physiol* **281**: G1092–G1100.
Tsutsumi S, Gotoh T, Tomisato W, Mima S, Hoshino T, Hwang HJ *et al.* (2004). *Cell Death Differ* **11**: 1009–1016.
Tsutsumi S, Tomisato W, Takano T, Rokutan K, Tsuchiya T, Mizushima T. (2002). *Biochim Biophys Acta* **1589**: 168–180.
Wang JL, Lin KL, Chen JS, Lu YC, Jiann BP, Chang HT *et al.* (2004). *Biochem Pharmacol* **67**: 1123–1130.
Williams CS, Watson AJ, Sheng H, Helou R, Shao J, DuBois RN. (2000). *Cancer Res* **60**: 6045–6051.
Wooden SK, Li LJ, Navarro D, Qadri I, Pereira L, Lee AS. (1991). *Mol Cell Biol* **11**: 5612–5623.
Xu W, Liu L, Charles IG, Moncada S. (2004). *Nat Cell Biol* **6**: 1129–1134.
Yoshida H, Okada T, Haze K, Yanagi H, Yura T, Negishi M *et al.* (2000). *Mol Cell Biol* **20**: 6755–6767.
Yu M, Haslam RH, Haslam DB. (2000). *J Biol Chem* **275**: 24984–24992.
Zhang X, Morham SG, Langenbach R, Young DA. (1999). *J Exp Med* **190**: 451–459.
Zweifel BS, Davis TW, Ornberg RL, Masferrer JL. (2002). *Cancer Res* **62**: 6706–6711.

THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 281, NO. 44, pp. 33422–33432, November 3, 2006

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

Received for publication, March 6, 2006, and in revised form, June 14, 2006 Published, JBC Papers in Press, August 31, 2006, DOI 10.1074/jbc.M602074200

Mayuko Aburaya‡, Ken-Ichiro Tanaka‡, Tatsuya Hoshino‡, Shinji Tsutsumi‡, Keitarou Suzuki‡, Masaki Makise‡, Reiko Akagi§, and Tohru Mizushima‡1

From the ‡Graduate School of Medical and Pharmaceutical Sciences, Kumamoto University, Kumamoto 862-0973, Japan and the §Faculty of Health and Welfare Science, Okayama Prefectural University, Soja 719-1197, Japan

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)[2] 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 Ca^{2+} influx which in turn induces production of the C/EBP homologous transcription factor (CHOP), and activates calpain, a Ca^{2+}-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 chaperones (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

* This work was supported by grants-in-aid for scientific research from the Ministry of Health, Labor, and Welfare of Japan, as well as by Japan Science and Technology Agency, the Daiwa Securities Health Foundation, the Mochida Memorial Foundation for Medical and Pharmaceutical Research, the Suzuken Memorial Foundation, and the Japan Research Foundation for Clinical Pharmacology. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] To whom correspondence should be addressed: Graduate School of Medical and Pharmaceutical Sciences, Kumamoto University, Kumamoto 862-0973, Japan. Tel.: 81-96-371-4323; E-mail: mizu@gpo.kumamoto-u.ac.jp.

[2] The abbreviations used are: NSAIDs, non-steroidal anti-inflammatory drugs; AMC, aminomethylcoumarin; BAPTA-AM, 1,2-bis(2-aminophenoxy)ethane-*N,N,N′N′*-tetraacetic acid; β-NA, β-nicotinamide adenine dinucleotide phosphate; CHOP, C/EBP homologous transcription factor; CO, carbon monoxide; COX, cyclooxygenase; EGFP, enhanced green fluorescent protein; ER, endoplasmic reticulum; ERK, extracellular signal-regulated kinase; 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; MTT, 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide; Nrf2, NF-E2-related factor 2; PG, prostaglandin; PI3K, 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 I 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, diclofenac, anysomycin, ibuprofen, paraformaldehyde, probenecid, 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 Fintechnical. 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% CO_2, 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% CO_2, 95% air at 37 °C. Unless otherwise noted, cells (0.8×10^4 cells per well in 24-well plates, 4×10^4 cells per well in 6-well plates, 6×10^5 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) 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 Me_2SO or Na_2CO_3 (for indomethacin only) and control experiments (without NSAIDs) were performed in the presence of the same concentrations of Me_2SO or Na_2CO_3. 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 protenase K (10 μg/ml) for 15 min at 37 °C, then with TdTase and biotin 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 M 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 M potassium phosphate buffer (pH 7.4) containing 0.25 M 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 M potassium phosphate buffer (pH 7.4) containing 0.15 M 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), $MgCl_2$ (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 M 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 $MgCl_2$, 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 Ca^{2+} Level—Intracellular Ca^{2+} 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 Ca^{2+} level was calculated according to the equation $[Ca^{2+}]_i = K_d(F - F_{min})/(F_{max} - F)$, where K_d is the apparent dissociation constant (400 nM) of the fluorescent dye-Ca^{2+} 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 unpaired 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

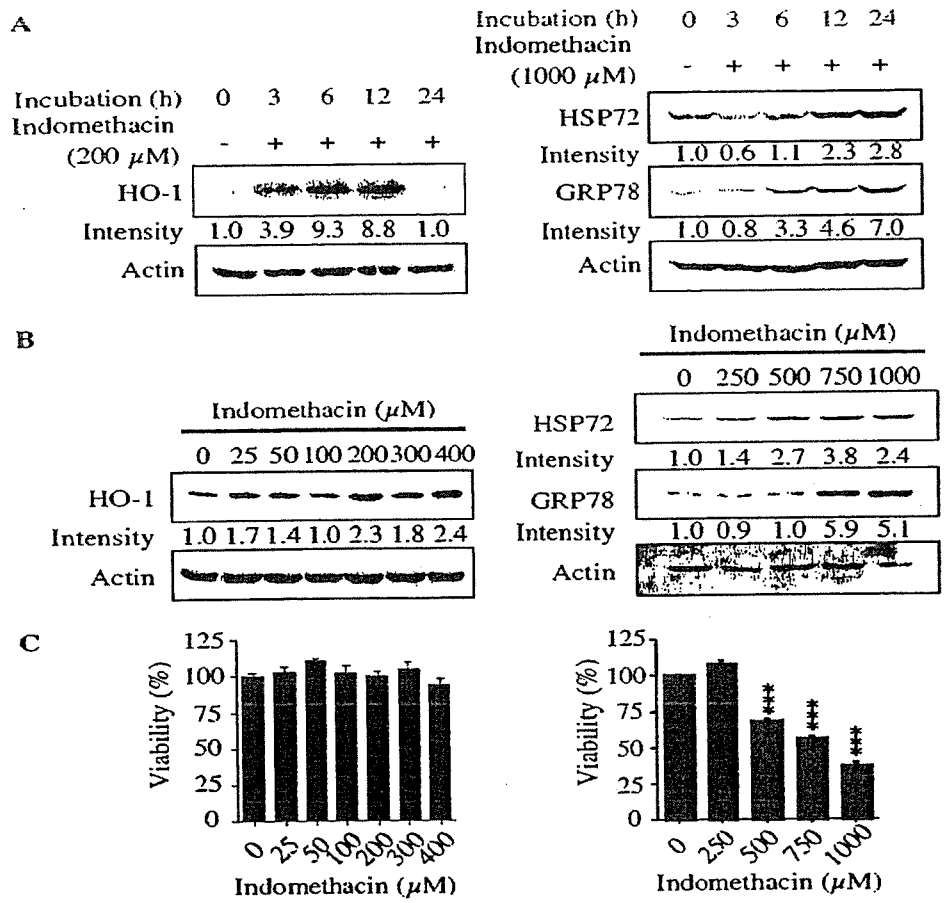

FIGURE 1. **Up-regulation of HO-1 by indomethacin in gastric mucosal cells in primary culture.** Guinea pig gastric mucosal cells in primary culture were incubated with the indicated concentrations of indomethacin for indicated periods (*A*), 6 h (HO-1 in *B* and *C*) or 24 h (HSP72 and GRP78 in *B* and *C*). Whole cell extracts were prepared and analyzed by immunoblotting with an antibody against HO-1, HSP72, GRP78, or actin. The band intensity was determined and expressed relative to the control (*A* and *B*). Cell viability was determined by the MTT method. Values shown are relative to the control (in the absence of indomethacin) and are given as the mean ± S.D. ($n = 3$). ***, $p < 0.001$ (*C*).

reproduced in this system (17). As shown in Fig. 1*A*, 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. 1*A* show that up-regulation of HO-1 by indomethacin occurs prior to that of HSP72 and GRP78. Fig. 1*B* 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. 1*C*), 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. 1*B*); in other words, up-regulation of these proteins occurs simultaneously with cell damage (Fig. 1*C*).

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 (diclofenac and flurbiprofen) 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. 6*B*).

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. 3*A*, 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. 3*A* did not affect cell viability in the absence of NSAIDs (Fig. 3*A*). 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. 3*A* 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. 3*C*). Cell death, as highlighted in Fig. 3*A*, appears to be mediated by apoptosis given that we observed NSAID-dependent activation of caspase-3 under the same experimental conditions as in Fig. 3*A* (data not shown) and treatment of cells with SnMP stimulated the activity of caspase-3 in the presence of each NSAID (Fig. 3*B*). 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.

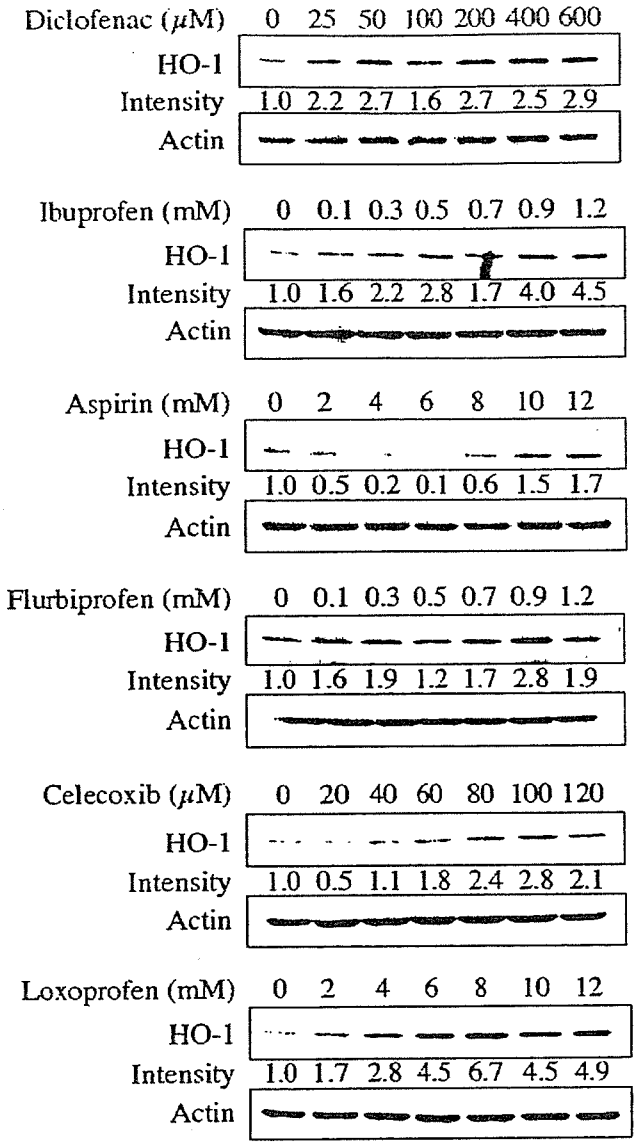

FIGURE 2. **Up-regulation of HO-1 by various NSAIDs.** Guinea pig gastric mucosal cells in primary culture were incubated with the indicated concentration of each NSAID for 6 h. Up-regulation of HO-1 was monitored as described in the legend to Fig. 1.

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. 5*A*) 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. 4*A*). 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. 4*A*).

We also examined 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

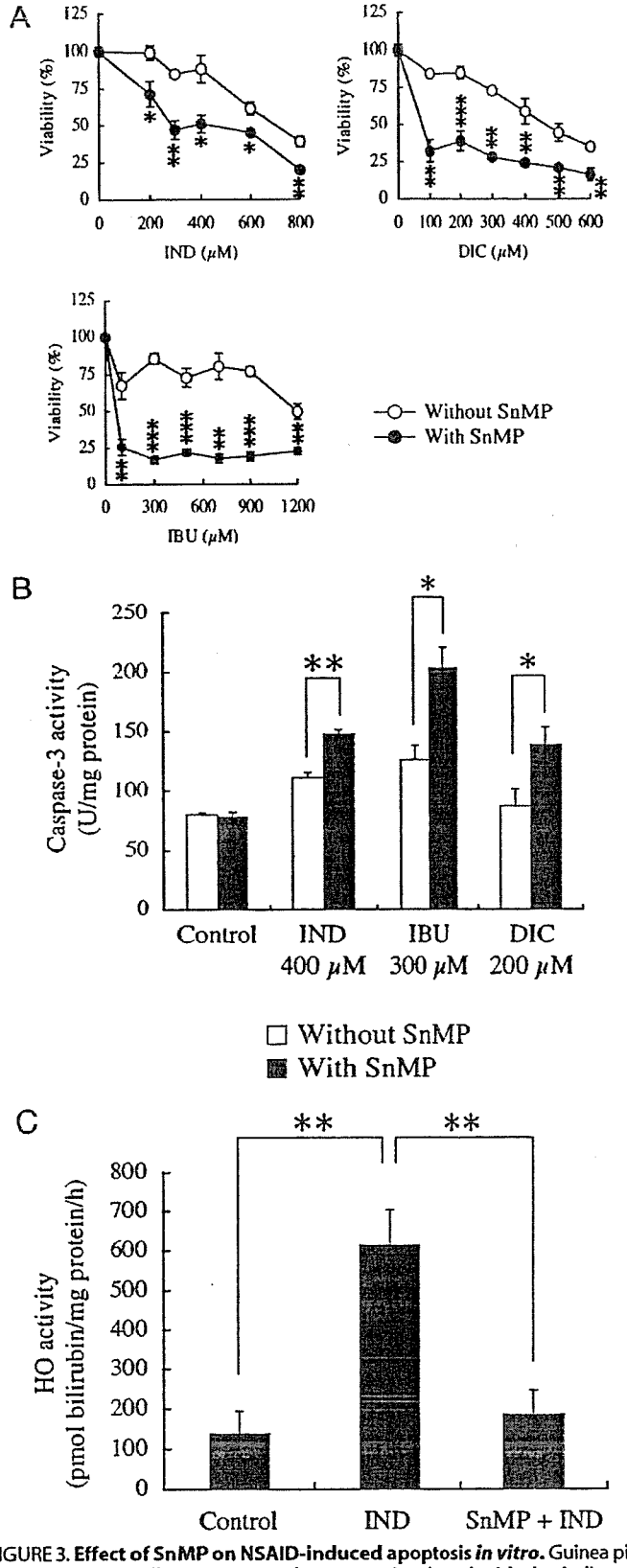

FIGURE 3. **Effect of SnMP on NSAID-induced apoptosis *in vitro*.** Guinea pig gastric mucosal cells in primary culture were incubated with the indicated concentrations of indomethacin (*IND*), diclofenac (*DIC*), or ibuprofen (*IBU*) in the presence or absence of 50 μM SnMP for 16 h (*A* and *B*) or 6 h (*C*), as indicated. Cell viability was determined using the MTT method and shown are 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. ($n = 3$). ***, $p < 0.001$; **, $p < 0.01$; *, $p < 0.05$.

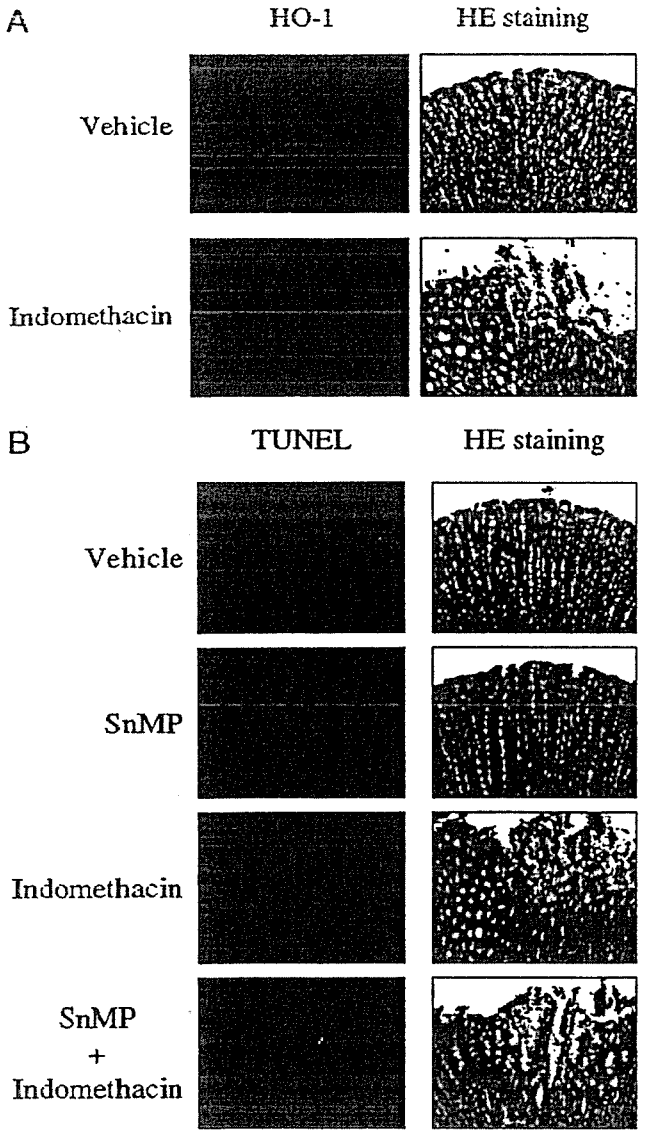

FIGURE 4. **Up-regulation of HO-1 and induction of apoptosis by indomethacin at gastric mucosa *in vivo*.** Rats were intraperitoneally pre-administered with 1 μmol/kg SnMP or vehicle 1 h before the administration of indomethacin (*B*). Rats were orally administered with 10 mg/kg indomethacin (*A* and *B*). After 4 h, sections of gastric tissues were prepared and subjected to histological examination (HE staining) and immunohistochemical analysis with an antibody against HO-1 (*A*) or TUNEL assay (*B*).

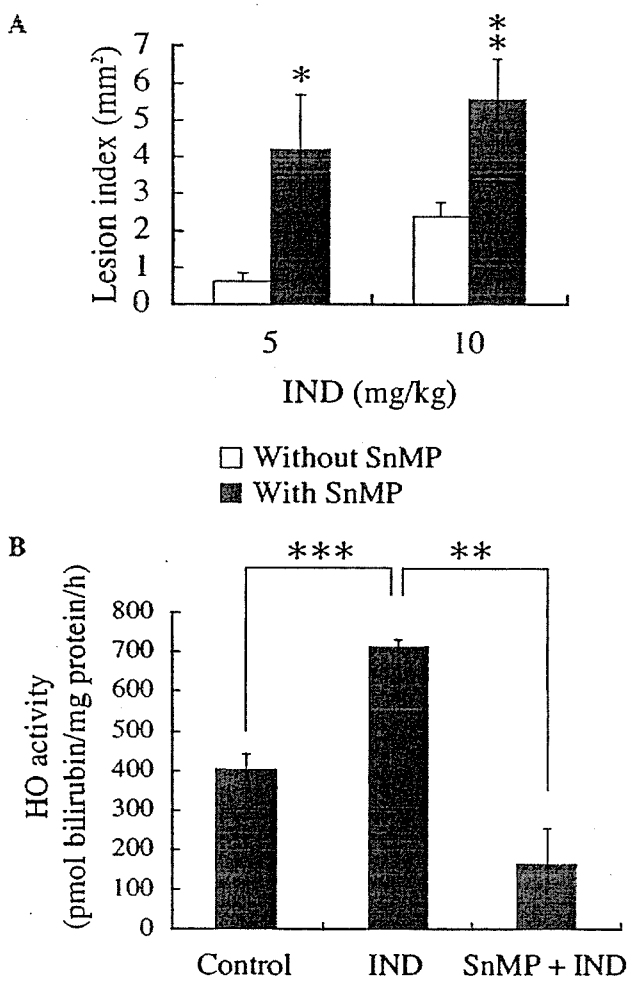

FIGURE 5. **Effect of SnMP on NSAID-induced gastric lesions.** Rats were intraperitoneally administered with 1 μmol/kg SnMP or vehicle. After 1 h, animals were administered orally with the indicated doses of indomethacin. After 3 h, the stomach was removed. The stomach was scored for hemorrhagic damage (*A*). Activities of HO in the stomach were measured and expressed as described under "Experimental Procedures" (*B*). Values are given as mean ± S.D. ($n = 3$–6). ***, $p < 0.001$; **, $p < 0.01$; *, $p < 0.05$.

TUNEL-positive cells (apoptotic cells) was observed with the indomethacin administration (Fig. 4*B*). Furthermore, pre-administration of SnMP stimulates indomethacin-induced apoptosis whereas this pre-administration did not induce apoptosis without subsequent indomethacin administration (Fig. 4*B*). 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. 5*A*, 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. 5*A* 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. 5*B*). 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. 6*A*). 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

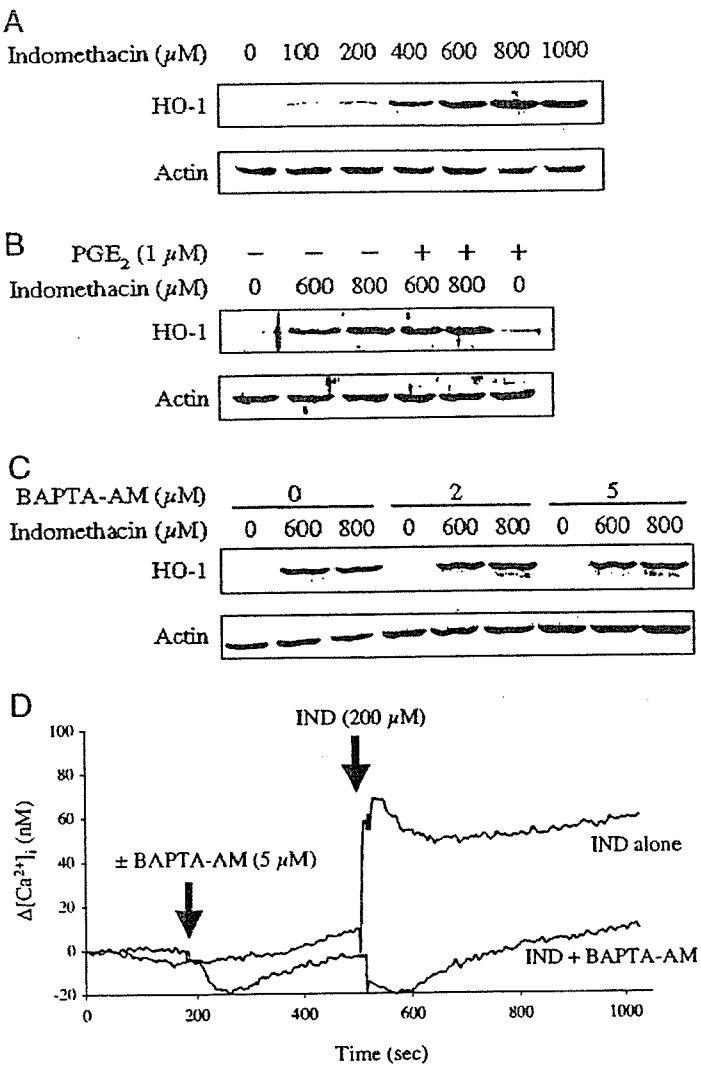

FIGURE 6. **Up-regulation of HO-1 by indomethacin in AGS cells.** AGS cells were preincubated with the indicated concentrations of BAPTA-AM for 1 h (*C*). Cells were incubated with the indicated concentrations of indomethacin for 6 h in the absence (*A* and *C*) or presence of 1 μM PGE_2. Up-regulation of HO-1 was monitored as described in the legend of Fig. 1. The intracellular Ca^{2+} level was monitored using a fluo-3/AM assay system. Fluo-3/AM-loaded cells were first treated with or without BAPTA-AM and then with indomethacin (*IND*). The time course of fluo-3 fluorescence change was monitored and increase in the intracellular Ca^{2+} level ($\Delta[Ca^{2+}]_i$) is shown (*D*).

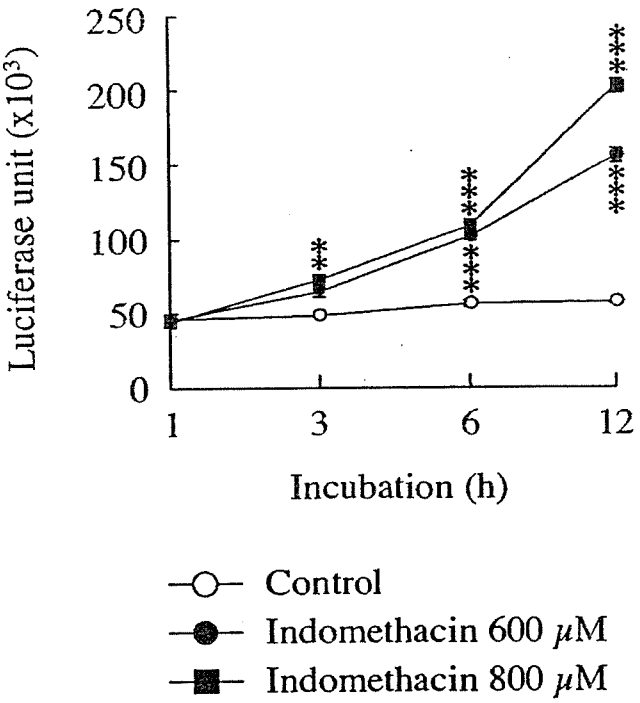

FIGURE 7. **Activation of the promoter activity of the *HO-1* gene by indomethacin.** AGS cells were co-transfected with pRL-SV40 (internal control plasmid carrying the *R. reniformis* luciferase gene) and pHO15luc (the promoter sequences of the *HO-1* gene were inserted upstream of the *P. pyralis* luciferase gene) or its vector. After 24 h, cells were incubated with the indicated concentrations of indomethacin for the indicated periods. *P. pyralis* luciferase activity was measured, and normalized for *R. reniformis* luciferase activity. Values were calculated by subtracting the background level (values from vector control experiments). Values are mean ± S.D. ($n = 3$). ***, $p < 0.001$; **, $p < 0.01$.

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. 6*B*, the addition of 1 μM 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. 6*C*, 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. 6*D*) (because clear increase in the intracellular Ca^{2+} level was not observed with 600 or 800 μM of indomethacin (maybe because of its inhibitory effect on fluo-3 fluorescence), we used 200 μM 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, 24, 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 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

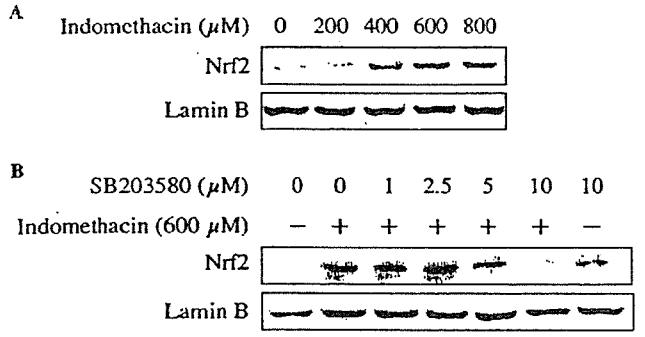

FIGURE 8. **p38 MAPK-dependent nuclear accumulation of Nrf2.** AGS cells were preincubated with or without an inhibitor of p38 MAPK (SB203580) for 0.5 h (*B*). Cells were incubated with the indicated concentrations of indomethacin for 3 h (*A* and *B*). Nuclear extracts were prepared and were analyzed by immunoblotting with an antibody against Nrf2 and lamin B.

was monitored by immunoblotting analysis. As shown in Fig. 8*A*, 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. 8*B*). 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.

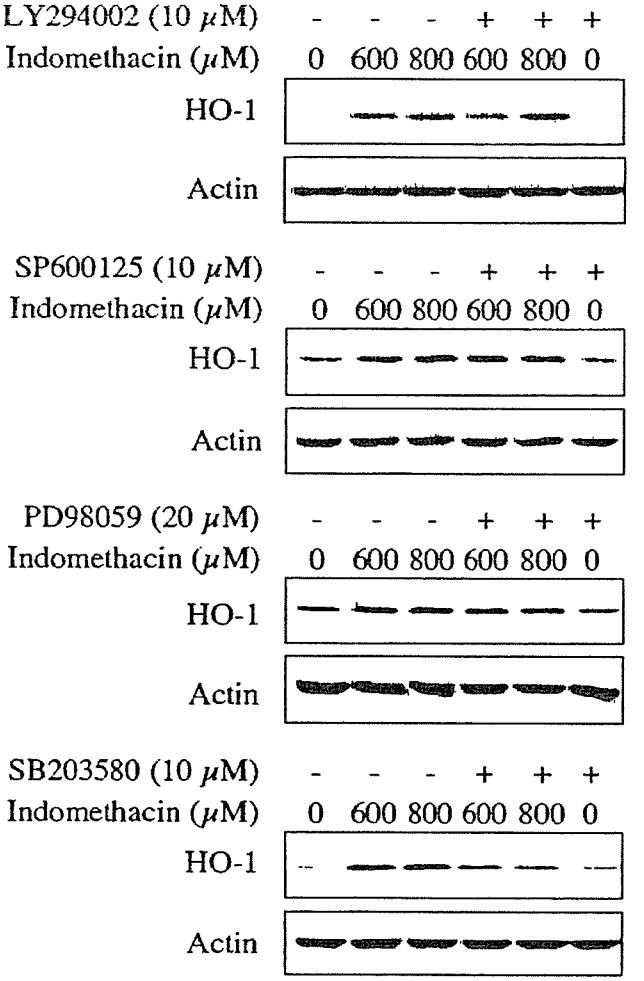

FIGURE 9. **Involvement of p38 MAPK in the up-regulation of HO-1 by indomethacin.** AGS cells were preincubated with indicated concentrations of each inhibitor (LY294002 for PI3K, SP600125 for JNK, PD98059 for ERK, SB203580 for p38 MAPK) for 0.5 h and further incubated with the indicated concentrations of indomethacin for 6 h. Up-regulation of HO-1 was monitored as described in the legend of Fig. 1.

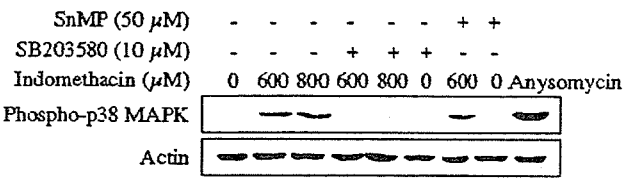

FIGURE 10. **Phosphorylation of p38 MAPK by indomethacin.** AGS cells were preincubated with the indicated concentrations of SB203580 or SnMP for 0.5 h and further incubated with the indicated concentrations of indomethacin or anysomycin (25 μg/ml) for 6 h. Whole cell extracts were prepared and analyzed by immunoblotting with an antibody to the phosphorylated form of p38 MAPK.

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

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 PGE_2, 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 PGE_2 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 Ca^{2+} by stimulating Ca^{2+} influx through permeabilization of cytoplasmic membranes, and BAPTA-AM, an intracellular Ca^{2+} chelator, suppressed NSAID-induced apoptosis (6). However, membrane permeabilization and the resulting increase in the intracellular Ca^{2+} 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 Ca^{2+} 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-Alzheimers 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 Alzheimers disease (64), although the mechanism for this activity is not fully understood. Tau protein plays a major role in the development of Alzheimers disease and high levels of reactive oxygen species are believed to promote the development of Alzheimers 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-Alzheimers 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.

Acknowledgment—We thank Dr. J. Alam (Alton Ochsner Medical Foundation) for the pHO15luc plasmid.

REFERENCES

1. Smalley, W. E., Ray, W. A., Daugherty, J. R., and Griffin, M. R. (1995) *Am. J. Epidemiol.* 141, 539–545
2. Hawkey, C. J. (2000) *Gastroenterology* 119, 521–535

3. Barrier, C. H., and Hirschowitz, B. I. (1989) *Arthritis Rheum.* **32,** 926–932
4. Fries, J. F., Miller, S. R., Spitz, P. W., Williams, C. A., Hubert, H. B., and Bloch, D. A. (1989) *Gastroenterology* **96,** 647–655
5. Lichtenberger, L. M. (2001) *Biochem. Pharmacol.* **61,** 631–637
6. Tanaka, K., Tomisato, W., Hoshino, T., Ishihara, T., Namba, T., Aburaya, M., Katsu, T., Suzuki, K., Tsutsumi, S., and Mizushima, T. (2005) *J. Biol. Chem.* **280,** 31059–31067
7. Tsutsumi, S., Gotoh, T., Tomisato, W., Mima, S., Hoshino, T., Hwang, H. J., Takenaka, H., Tsuchiya, T., Mori, M., and Mizushima, T. (2004) *Cell Death Differ.* **11,** 1009–1016
8. Tomisato, W., Tanaka, K., Katsu, T., Kakuta, H., Sasaki, K., Tsutsumi, S., Hoshino, T., Aburaya, M., Li, D., Tsuchiya, T., Suzuki, K., Yokomizo, K., and Mizushima, T. (2004) *Biochem. Biophys. Res. Commun.* **323,** 1032–1039
9. Tomisato, W., Tsutsumi, S., Rokutan, K., Tsuchiya, T., and Mizushima, T. (2001) *Am. J. Physiol. Gastrointest Liver Physiol.* **281,** G1092–G1100
10. Tomisato, W., Tsutsumi, S., Hoshino, T., Hwang, H. J., Mio, M., Tsuchiya, T., and Mizushima, T. (2004) *Biochem. Pharmacol.* **67,** 575–585
11. Mathew, A., and Morimoto, R. I. (1998) *Ann. N. Y. Acad. Sci.* **851,** 99–111
12. Gehrmann, M., Brunner, M., Pfister, K., Reichle, A., Kremmer, E., and Multhoff, G. (2004) *Clin. Cancer Res.* **10,** 3354–3364
13. Tsutsumi, S., Namba, T., Tanaka, K. I., Arai, Y., Ishihara, T., Aburaya, M., Mima, S., Hoshino, T., and Mizushima, T. (2006) *Oncogene* **25,** 1018–1029
14. Tomisato, W., Takahashi, N., Komoto, C., Rokutan, K., Tsuchiya, T., and Mizushima, T. (2000) *Dig. Dis. Sci.* **45,** 1674–1679
15. Takano, T., Tsutsumi, S., Tomisato, W., Hoshino, T., Tsuchiya, T., and Mizushima, T. (2002) *Dig. Dis. Sci.* **47,** 1546–1553
16. Tomisato, W., Tsutsumi, S., Tsuchiya, T., and Mizushima, T. (2001) *Biol. Pharm. Bull.* **24,** 887–891
17. Hirakawa, T., Rokutan, K., Nikawa, T., and Kishi, K. (1996) *Gastroenterology* **111,** 345–357
18. Ponka, P. (1999) *Am. J. Med. Sci.* **318,** 241–256
19. Maines, M. D. (1997) *Annu. Rev. Pharmacol. Toxicol.* **37,** 517–554
20. Tenhunen, R., Marver, H. S., and Schmid, R. (1969) *J. Biol. Chem.* **244,** 6388–6394
21. Brouard, S., Otterbein, L. E., Anrather, J., Tobiasch, E., Bach, F. H., Choi, A. M., and Soares, M. P. (2000) *J. Exp. Med.* **192,** 1015–1026
22. Chou, Y. H., Ho, F. M., Liu, D. Z., Lin, S. Y., Tsai, L. H., Chen, C. H., Ho, Y. S., Hung, L. F., and Liang, Y. C. (2005) *Int. J. Biochem. Cell Biol.* **37,** 604–615
23. Igarashi, K., Kataoka, K., Itoh, K., Hayashi, N., Nishizawa, M., and Yamamoto, M. (1994) *Nature* **367,** 568–572
24. Ishii, T., Itoh, K., Takahashi, S., Sato, H., Yanagawa, T., Katoh, Y., Bannai, S., and Yamamoto, M. (2000) *J. Biol. Chem.* **275,** 16023–16029
25. Cho, H. Y., Jedlicka, A. E., Reddy, S. P., Kensler, T. W., Yamamoto, M., Zhang, L. Y., and Kleeberger, S. R. (2002) *Am. J. Respir. Cell Mol. Biol.* **26,** 175–182
26. Elbirt, K. K., Whitmarsh, A. J., Davis, R. J., and Bonkovsky, H. L. (1998) *J. Biol. Chem.* **273,** 8922–8931
27. Lee, J. M., Hanson, J. M., Chu, W. A., and Johnson, J. A. (2001) *J. Biol. Chem.* **276,** 20011–20016
28. Buckley, B. J., Marshall, Z. M., and Whorton, A. R. (2003) *Biochem. Biophys. Res. Commun.* **307,** 973–979
29. Alcaraz, M. J., Habib, A., Creminon, C., Vicente, A. M., Lebret, M., Levy-Toledano, S., and Maclouf, J. (2001) *Biochim. Biophys. Acta* **1526,** 13–16
30. Hou, C. C., Hung, S. L., Kao, S. H., Chen, T. H., and Lee, H. M. (2005) *Ann. N. Y. Acad. Sci.* **1042,** 235–245
31. Nascimento-Silva, V., Arruda, M. A., Barja-Fidalgo, C., Villela, C. G., and Fierro, I. M. (2005) *Am. J. Physiol. Cell Physiol.* **289,** C557–563
32. Grosser, N., Abate, A., Oberle, S., Vreman, H. J., Dennery, P. A., Becker, J. C., Pohle, T., Seidman, D. S., and Schroder, H. (2003) *Biochem. Biophys. Res. Commun.* **308,** 956–960
33. Cantoni, L., Valaperta, R., Ponsoda, X., Castell, J. V., Barelli, D., Rizzardini, M., Mangolini, A., Hauri, L., and Villa, P. (2003) *J. Hepatol.* **38,** 776–783
34. Alam, J., Wicks, C., Stewart, D., Gong, P., Touchard, C., Otterbein, S., Choi, A. M., Burow, M. E., and Tou, J. (2000) *J. Biol. Chem.* **275,** 27694–27702
35. Tomisato, W., Hoshino, T., Tsutsumi, S., Tsuchiya, T., and Mizushima, T. (2002) *Dig. Dis. Sci.* **47,** 2125–2133
36. Schreiber, E., Matthias, P., Muller, M. M., and Schaffner, W. (1989) *Nucleic Acids Res.* **17,** 6419
37. Tsutsumi, S., Tomisato, W., Takano, T., Rokutan, K., Tsuchiya, T., and Mizushima, T. (2002) *Biochim. Biophys. Acta* **1589,** 168–180
38. Tenhunen, R., Marver, H. S., and Schmid, R. (1968) *Proc. Natl. Acad. Sci. U. S. A.* **61,** 748–755
39. Hoshino, T., Tsutsumi, S., Tomisato, W., Hwang, H. J., Tsuchiya, T., and Mizushima, T. (2003) *J. Biol. Chem.* **278,** 12752–12758
40. Schroeder, C. P., Yang, P., Newman, R. A., and Lotan, R. (2004) *Cancer Biol. Ther.* **3,** 847–852
41. Ben-Chetrit, E., Fischel, R., Hinz, B., and Levy, M. (2005) *Rheumatol. Int.* **25,** 332–335
42. Kawai, S., Nishida, S., Kato, M., Furumaya, Y., Okamoto, R., Koshino, T., and Mizushima, Y. (1998) *Eur. J. Pharmacol.* **347,** 87–94
43. Tanaka, Y., Nishikawa, Y., and Hayashi, R. (1983) *Chem. Pharm. Bull. (Tokyo)* **31,** 3656–3664
44. Valaes, T., Petmezaki, S., Henschke, C., Drummond, G. S., and Kappas, A. (1994) *Pediatrics* **93,** 1–11
45. Ghattas, M. H., Chuang, L. T., Kappas, A., and Abraham, N. G. (2002) *Int. J. Biochem. Cell Biol.* **34,** 1619–1628
46. Nakahira, K., Takahashi, T., Shimizu, H., Maeshima, K., Uehara, K., Fujii, H., Nakatsuka, H., Yokoyama, M., Akagi, R., and Morita, K. (2003) *Biochem. Pharmacol.* **66,** 1091–1105
47. Tsutsumi, S., Mima, S., Tomisato, W., Hoshino, T., Tsuchiya, T., and Mizushima, T. (2003) *Exp. Biol. Med. (Maywood)* **228,** 1089–1095
48. Mima, S., Tsutsumi, S., Ushijima, H., Takeda, M., Fukuda, I., Yokomizo, K., Suzuki, K., Sano, K., Nakanishi, T., Tomisato, W., Tsuchiya, T., and Mizushima, T. (2005) *Cancer Res.* **65,** 1868–1876
49. Itoh, K., Wakabayashi, N., Katoh, Y., Ishii, T., Igarashi, K., Engel, J. D., and Yamamoto, M. (1999) *Genes Dev.* **13,** 76–86
50. Kietzmann, T., Samoylenko, A., and Immenschuh, S. (2003) *J. Biol. Chem.* **278,** 17927–17936
51. Lee, J. C., Kassis, S., Kumar, S., Badger, A., and Adams, J. L. (1999) *Pharmacol. Ther.* **82,** 389–397
52. Young, P. R., McLaughlin, M. M., Kumar, S., Kassis, S., Doyle, M. L., McNulty, D., Gallagher, T. F., Fisher, S., McDonnell, P. C., Carr, S. A., Huddleston, M. J., Seibel, G., Porter, T. G., Livi, G. P., Adams, J. L., and Lee, J. C. (1997) *J. Biol. Chem.* **272,** 12116–12121
53. Dudley, D. T., Pang, L., Decker, S. J., Bridges, A. J., and Saltiel, A. R. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92,** 7686–7689
54. Gong, P., Cederbaum, A. I., and Nieto, N. (2003) *J. Biol. Chem.* **278,** 29693–29700
55. Kiemer, A. K., Bildner, N., Weber, N. C., and Vollmar, A. M. (2003) *Endocrinology* **144,** 802–812
56. Rockwell, P., Martinez, J., Papa, L., and Gomes, E. (2004) *Cell Signal* **16,** 343–353
57. Zhang, X., Shan, P., Otterbein, L. E., Alam, J., Flavell, R. A., Davis, R. J., Choi, A. M., and Lee, P. J. (2003) *J. Biol. Chem.* **278,** 1248–1258
58. Linden, T., Doutheil, J., and Paschen, W. (1998) *Neurosci. Lett.* **247,** 103–106
59. Yachie, A., Niida, Y., Wada, T., Igarashi, N., Kaneda, H., Toma, T., Ohta, K., Kasahara, Y., and Koizumi, S. (1999) *J. Clin. Investig.* **103,** 129–135
60. Poss, K. D., and Tonegawa, S. (1997) *Proc. Natl. Acad. Sci. U. S. A.* **94,** 10925–10930
61. Hayashi, S., Takamiya, R., Yamaguchi, T., Matsumoto, K., Tojo, S. J., Tamatani, T., Kitajima, M., Makino, N., Ishimura, Y., and Suematsu, M. (1999) *Circ. Res.* **85,** 663–671
62. Lee, T. S., and Chau, L. Y. (2002) *Nat. Med.* **8,** 240–246
63. Lee, T. S., Tsai, H. L., and Chau, L. Y. (2003) *J. Biol. Chem.* **278,** 19325–19330
64. Akiyama, H., Barger, S., Barnum, S., Bradt, B., Bauer, J., Cole, G. M., Cooper, N. R., Eikelenboom, P., Emmerling, M., Fiebich, B. L., Finch, C. E., Frautschy, S., Griffin, W. S., Hampel, H., Hull, M., Landreth, G., Lue, L., Mrak, R., Mackenzie, I. R., McGeer, P. L., O'Banion, M. K., Pachter, J.,

Pasinetti, G., Plata-Salaman, C., Rogers, J., Rydel, R., Shen, Y., Streit, W., Strohmeyer, R., Tooyoma, I., Van Muiswinkel, F. L., Veerhuis, R., Walker, D., Webster, S., Wegrzyniak, B., Wenk, G., and Wyss-Coray, T. (2000) *Neurobiol. Aging* **21**, 383–421
65. Klein, J. A., and Ackerman, S. L. (2003) *J. Clin. Investig.* **111**, 785–793
66. Takeda, A., Perry, G., Abraham, N. G., Dwyer, B. E., Kutty, R. K., Laitinen, J. T., Petersen, R. B., and Smith, M. A. (2000) *J. Biol. Chem.* **275**, 5395–5399
67. Kwak, M. K., Wakabayashi, N., and Kensler, T. W. (2004) *Mutat. Res.* **555**, 133–148
68. Ramos-Gomez, M., Kwak, M. K., Dolan, P. M., Itoh, K., Yamamoto, M., Talalay, P., and Kensler, T. W. (2001) *Proc. Natl. Acad. Sci. U. S. A.* **98**, 3410–3415

BJ www.biochemj.org

Biochem. J. (2007) 402, 581–589 (Printed in Great Britain) doi:10.1042/BJ20061318

Endoplasmic reticulum chaperones inhibit the production of amyloid- peptides

Tatsuya HOSHINO*, Tadashi NAKAYA†, Wataru ARAKI‡, Keitarou SUZUKI*, Toshiharu SUZUKI† and Tohru MIZUSHIMA*[1]

*Graduate School of Medical and Pharmaceutical Sciences, Kumamoto University, Kumamoto 862-0973, Japan, †Graduate School of Pharmaceutical Sciences, Hokkaido University, Sapporo 060-0812, Japan, and ‡Department of Demyelinating Disease and Ageing, National Institute of Neuroscience, Kodaira 187-8502, Japan

A (amyloid- peptides) generated by proteolysis of APP (-amyloid precursor protein), play an important role in the pathogenesis of AD (Alzheimer's disease). ER (endoplasmic reticulum) chaperones, such as GRP78 (glucose-regulated protein 78), make a major contribution to protein quality control in the ER. In the present study, we examined the effect of overexpression of various ER chaperones on the production of A in cultured cells, which produce a mutant type of APP (APPsw). Overexpression of GRP78 or inhibition of its basal expression, decreased and increased respectively the level of A 40 and A 42 in conditioned medium. Co-expression of GRP78's co-chaperones ERdj3 or ERdj4 stimulated this inhibitory effect of GRP78. In the case of the other ER chaperones, overexpression of some (150 kDa oxygen-regulated protein and calnexin) but not others (GRP94 and calreticulin) suppressed the production of A . These results indicate that certain ER chaperones are effective suppressors of A production and that non-toxic inducers of ER chaperones may be therapeutically beneficial for AD treatment. GRP78 was co-immunoprecipitated with APP and overexpression of GRP78 inhibited the maturation of APP, suggesting that GRP78 binds directly to APP and inhibits its maturation, resulting in suppression of the proteolysis of APP. On the other hand, overproduction of APPsw or addition of synthetic A 42 caused up-regulation of the mRNA of various ER chaperones in cells. Furthermore, in the cortex and hippocampus of transgenic mice expressing APPsw, the mRNA of some ER chaperones was up-regulated in comparison with wild-type mice. We consider that this up-regulation is a cellular protective response against A .

Key words: amyloid- peptides (A), -amyloid precursor protein (APP), endoplasmic reticulum (ER) chaperone, glucose-regulated protein 78 (GRP78), protein maturation.

INTRODUCTION

AD (Alzheimer's disease) is the leading cause of adult onset dementia, with a dramatic increase in the incidence of AD apparent in our rapidly aging society. AD is pathologically characterized by the accumulation of tangles and senile plaques. Senile plaques are composed of the A (amyloid- peptides), A 40 and A 42 [1,2]. A is generated by secretase-dependent proteolysis of APP (-amyloid precursor protein). Prior to proteolysis, APP undergoes modification [for example, by N-glycosylation in the ER (endoplasmic reticulum) and *O*-glycosylation in the Golgi apparatus]. In order to generate A 40 and A 42, APP is first cleaved by -secretase and then by -secretase. For the cleavage of APP, -secretase competes with -secretase, which produces non-amyloidogenic peptides [3,4]. -Secretase is an aspartyl protease complex composed of four core components, including PS1 (presenilin 1) and PS2 [5]. The early onset familial form of AD (FAD) is linked to three genes, *APP*, *PS1* and *PS2* [5,6], strongly suggesting that the production of A is a key factor in the pathogenesis of AD. Therefore, cellular factors that suppress the generation of A provide important drug targets for the treatment of AD.

Proteins, including APP, first translocate into the ER where they undergo modification. N-glycosylation of APP in the ER is essential for the generation of A [4]. The ER is also proposed to be important for A -induced apoptosis of neuronal cells; for example, a potential intracellular target of A in mediating apoptosis, ERAB (ER-associated -binding protein), is an ER membrane protein [7,8]. Accumulation of unfolded protein in the ER induces the ER stress response, a process involving three types of ER transmembrane protein: IRE1 (protein-kinase and site-specific endoribonuclease), PERK (protein kinase R-like ER kinase) and ATF 6 (activating transcription factor 6) [9–11]. ER stressors phosphorylate PERK, which in turn phosphorylates eIF2 (eukaryotic initiation factor-2), leading to activation of ATF4 expression (ATF4 pathway) [12,13]. ER stressors also cause cleavage of $p90^{ATF6}$ into $p50^{ATF6}$, which translocates to the nucleus (ATF6 pathway) [11]. Both ATF4 and $p50^{ATF6}$ specifically activate transcription of ER stress response-related genes, including those genes that encode ER chaperones. A close relationship between the ER stress response and A has been suggested; mutations in the *PS1* or *PS2* genes increase cellular sensitivity to ER stressors by suppressing the activation of IRE1, PERK and ATF6 [14–18]. These observations suggest that the ER is an important cellular compartment for the pathogenesis of AD.

ER chaperones, such as GRP78 (glucose-regulated protein 78), GRP94, ORP150 (150 kDa oxygen-regulated protein), CRT (calreticulin) and CNX (calnexin), contribute greatly to protein quality control in the ER by assisting the refolding of unfolded proteins [19–21]. Therefore, it is reasonable to speculate that ER

Abbreviations used: A , amyloid- peptides; AD, Alzheimer's disease; AICD, -amyloid precursor protein intracellular domain; APP, -amyloid precursor protein; APPsw, Swedish mutant of APP; APPwt, wild-type APP; ATF6, activating transcription factor 6; CNX, calnexin; Co-IP, co-immunoprecipitation; CRT, calreticulin; CTF ,C-terminal fragment ; DAPT, *N*-[*N*-(3,5-difluorophenacetyl-L-alanyl)]-S-phenylglycine *t*-butyl ester; DMEM, Dulbecco's modified Eagle's medium; EDEM, endoplasmic reticulum degradation-enhancing -mannosidase I-like protein; eIF2 , eukaryotic initiation factor-2 ; ER, endoplasmic reticulum; ERAD, ER-associated degradation; ERdj4 J, J domain-deleted ERdj4; FAD, familial AD; GRP, glucose-regulated protein; HEK-293, human embryonic kidney 293; HSP, heat shock protein; imAPP, immature APP; IRE1, protein-kinase and site-specific endoribonuclease; mAPP, mature APP; ORP150, 150 kDa oxygen-regulated protein; PERK, protein kinase R-like ER kinase; PS1, presenilin 1; RT, reverse transcriptase; sELISA, sandwich ELISA; siRNA, small interfering RNA.

[1] To whom correspondence should be addressed (email mizu@gpo.kumamoto-u.ac.jp).

chaperones affect the generation of A and the pathogenesis of AD. In fact, some ER chaperones have been shown to physically interact with APP, and overexpression of GRP78 in cells decreases the level of both mature APP and secreted A [22,23]. Furthermore, the accumulation of GRP78 in senile plaques, the up-regulation of ER chaperones in the brains of AD patients and the co-localization of ER chaperones with A have all been reported [24–26]. In the present study, we systematically examined the effect of overexpression of various ER chaperones and found that some, but not all, suppress the generation of A *in vitro*. We propose that this suppression is due to inhibition of the secretase-dependent proteolytic processing of APP through direct interaction between ER chaperones and APP, resulting in the inhibition of APP maturation. Furthermore, we found that ER chaperones are up-regulated not only in cultured neuronal cells overproducing mutant forms of APP or treated with synthetic A 42, but also in the cortex and hippocampus of transgenic mice expressing mutant APP.

MATERIALS AND METHODS

Cell culture and overexpression of ER chaperones

HEK-293 (human embryonic kidney 293) or SH-SY5Y cells were cultured in DMEM (Dulbecco's modified Eagle's medium) or DMEM/Ham's-F12 medium respectively, supplemented with 10 % (v/v) fetal bovine serum, 100 units/ml penicillin and 100 g/ml streptomycin in a humidified atmosphere of 95 % air with 5 % CO_2 at 37 C. SH-SY5Y cells expressing APPsw (Swedish mutant of APP) or APPwt (wild-type APP) were described previously [27]. For transient expression of each gene, cells were seeded 24 h before the transfection in 24-well plates at a density of 1.5×10^5 cells/well. The transfection was carried out using Lipofectamine™ 2000 (Invitrogen) according to the manufacturer's instructions. Cells were used for experiments after a 24 h recovery period. Transfection efficiency was determined in parallel plates by transfection of the pEGFP-N1 control vector. Transfection efficiency was greater than 90 % in all experiments. The stable transfectants expressing each gene were selected by immunoblotting or real-time RT (reverse transcriptase)-PCR analyses. Positive clones were maintained in the presence of 800 g/ml G418, 100 g/ml zeocin or 200 g/ml hygromycin.

Immunoblotting analysis

Whole cell extracts were prepared as described previously [28]. For detection of CTF (C-terminal fragment) and CTF , membrane fractions were prepared as described previously [29]. The protein concentration of samples was determined by the Bio-Rad protein assay kit (Bio-Rad Laboratories), according to the manufacturer's instructions. Samples were applied to 7 % (for APP), 8 % (for GRP78), 10 % (for actin), 12 % (for His-tagged ERdj3), 15 % (for Myc-tagged ERdj4) or 16.5 % (for CTF and CTF ; v/v) polyacrylamide gels and subjected to SDS/PAGE, after which proteins were immunoblotted with respective antibodies.

sELISA (sandwich ELISA) assay for A

Cells were cultured for 24 h and the conditioned medium was subjected to sELISA using three types of specific monoclonal antibody, as described previously [30].

Co-IP (co-immunoprecipitation) assay

Co-IP was carried out as described previously [31], with some modifications. Cells were harvested, lysed with buffer (10 mM Hepes/KOH, pH 7.4, 150 mM NaCl and 0.5 % Triton X-100) and centrifuged at 20000 *g*. The antibody against the C-terminal fragment of APP was added to the supernatant, and the sample incubated for 12 h at 4 C with rotation. Dynabeads® coated with Protein A were then added and the samples were incubated for 2 h at 4 C and rotated, after which they were centrifuged at 20000 *g*. The beads were then washed four times with the same buffer and the proteins were eluted by boiling the beads in SDS sample buffer [62.5 mM Tris/Hcl, pH 6.8, 2 % (w/v) SDS, 5 % (v/v) 2-mercaptoethanol, 10 % (v/v) glycerol and 0.001 % Bromophenol Blue).

Real-time RT-PCR analysis

Total RNA was extracted from cells and mouse brain using an RNeasy kit according to the manufacturer's protocols (Qiagen). Samples were reverse-transcribed using a first-strand cDNA synthesis kit according to the manufacturer's instructions (Amershan Biosciences). Synthesized cDNA was subjected to real-time RT-PCR using SYBR® Green PCR Master Mix (Applied Biosystems) and analysed with ABI PRISM 7500 Sequence Detection software according to the manufacturer's instructions (Applied Biosystems). Real-time cycle conditions were 2 min at 50 C, followed by 10 min at 90 C and then 45 cycles at each of 95 C for 30 s and 63 C for 60 s. Specificity was confirmed by electrophoretic analysis of the reaction products and by inclusion of template- or RT-free controls. To normalize the amount of total RNA present in each reaction, the actin gene was used as an internal standard.

Statistical analysis

All values are expressed as the means _ S.D. Student's *t* test for unpaired results was used for the evaluation of differences between the two groups. Differences were considered to be significant for P 0.05.

RESULTS

ER chaperones inhibit the generation of A

We based our investigations on HEK-293 cells that stably express APP with the double mutations, K651N/M652L, known as the 'Swedish' mutations (APPsw) [30]. These mutations elevate cellular and secreted levels of A [32]. The effect of ER chaperones on the generation of A 40 and A 42 was monitored by determining the amount of these peptides in conditioned medium by sELISA after transient transfection of the cells with expression plasmid for each ER chaperone. Transient overexpression of GRP78 (Figure 1A) caused a decrease in the level of A 40 and A 42 (Figures 1B and 1C) suggesting that GRP78 inhibits the production of A . This was confirmed using siRNA (small interfering RNA). Transfection of siRNA against GRP78 not only caused a decrease in the background cellular expression of GRP78 (Figure 1D), but also led to a weak increase in the level of A 40 and A 42 in the conditioned medium (Figures 1E and 1F). The weakness of the induction may be due to other ER chaperones compensating for the function of GRP78 under the experimental conditions (see Supplementary Figure S2 at http://www.BiochemJ.org/bj/402/bj4020581add.htm).

GRP78 belongs to the HSP70 (heat shock protein 70) family of proteins for which co-chaperones have also been identified [19]. For example, HSP40 binds to HSP70, stimulating its ATPase and chaperone activities [33]. Various co-chaperones have been suggested for GRP78, among which ERdj3 and ERdj4 have been

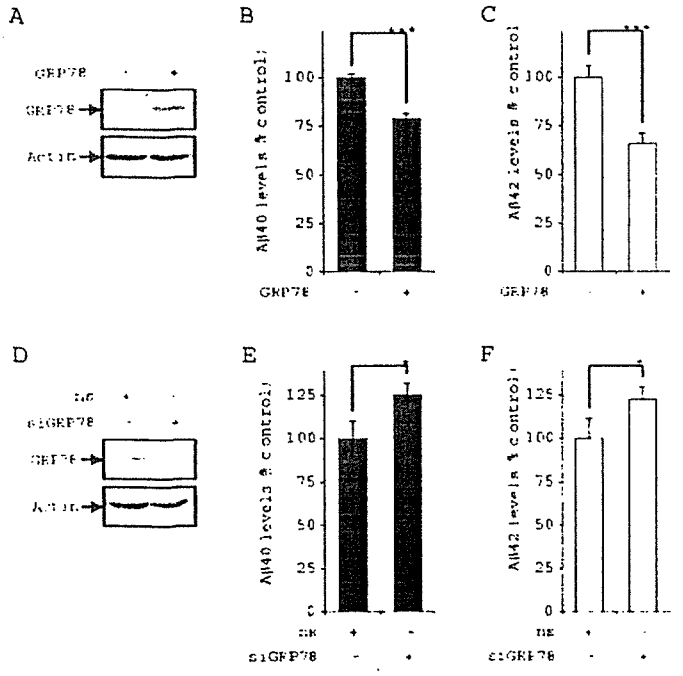

Figure 1 Inhibition of A generation by transient expression of GRP78

HEK-293 cells expressing APPsw were transiently transfected either with expression plasmid for GRP78 or control vector (**A–C**), or with siRNA against GRP78 (siGRP78) or non-silencing (ns) siRNA (**D–F**), after which they were cultured for 24 h. Whole cell extracts (10 g protein) were analysed by immunoblotting with an antibody against GRP78 (**A**) or actin (**D**). To detect GRP78 in the control sample, a relatively longer exposure was used in (**D**) than in (**A**). The amount of A 40 and A 42 in the conditioned medium was determined by sELISA and expressed relative to the control. Results are means _ S.D. (n 3). ***, P 0.001; *, P 0.05 (**B**, **C**, **E** and **F**).

shown to bind to GRP78, enhancing its ATPase and chaperone activities [34,35]. Here we examined the effect of overexpression of ERdj3 and ERdj4, or their co-expression with GRP78, on A generation. Overexpression of ERdj3 or ERdj4 was confirmed by immunoblotting (see Supplementary Figure S1 at http://www.BiochemJ.org/bj/402/bj4020581add.htm) and real-time RT-PCR (results not shown) and found that the level of overexpression of GRP78 (or ERdj3 and ERdj4) was not affected by simultaneous overexpression of ERdj3 and ERdj4 (or GRP78; see Supplementary Figure S1). As illustrated in Figures 2(A) and 2(B), transfection of an expression plasmid for ERdj3 decreased the level of both A 40 and A 42. Furthermore, co-transfection of expression plasmids for both GRP78 and ERdj3 produced an even greater decrease (Figures 2A and 2B). Although the transfection of an expression plasmid for ERdj4 significantly decreased the level of A 40 but not A 42 (Figures 2C and 2D), co-transfection of expression plasmids for both GRP78 and ERdj4 caused a clear decrease in both peptides; co-overexpression of both GRP78 and ERdj4 decreased the level of A 42 to about 30% of the control level (Figure 2D). These results show that ERdj3 and ERdj4 stimulate the inhibitory effect of GRP78 on the generation of A and suggest that this effect of GRP78 involves its ATPase and chaperone activities. The slight inhibitory effect of overexpression of ERdj3 or ERdj4 alone on the generation of A may be due to the activation of endogenous GRP78 by these co-chaperones.

The J domain of the HSP40 family of proteins is responsible for their interaction with the HSP70 family of proteins [33]. It has been shown that the J domain of ERdj4 is essential for its interaction with GRP78 [35]. As shown in Figures 2(E) and 2(F), and in comparison with the results obtained with the wild-type

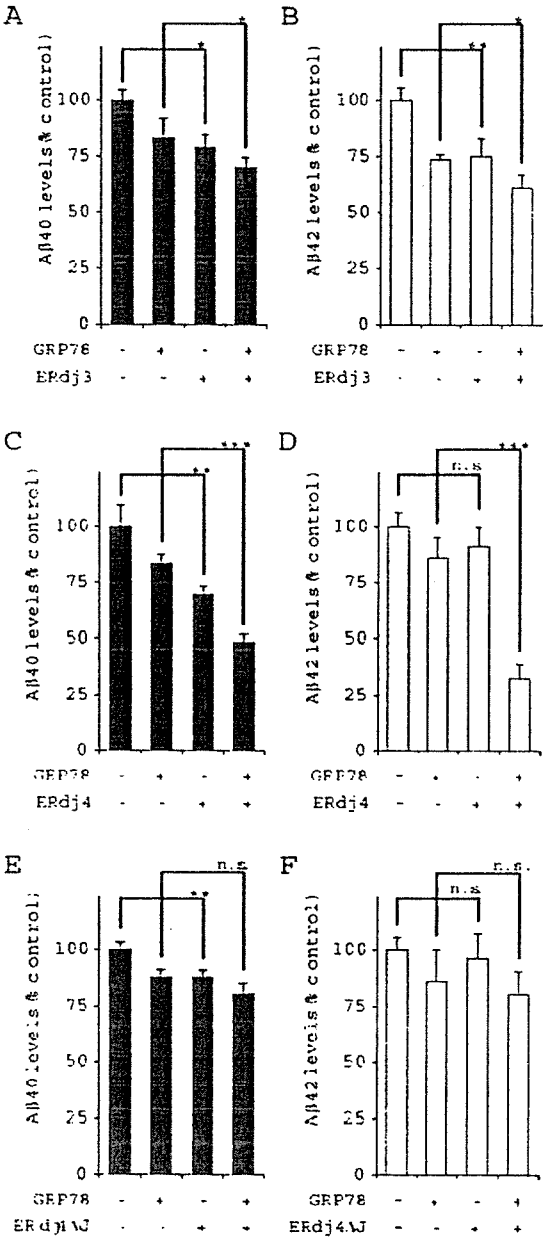

Figure 2 Stimulation by ERdj3 or ERdj4 of inhibitory effect of GRP78 on A generation

HEK-293 cells expressing APPsw were transiently transfected with expression plasmid for GRP78 (**A–F**), ERdj3 (**A** and **B**), ERdj4 (**C** and **D**), ERdj4 J (**E** and **F**) and/or control vector (**A–F**), with total DNA amounts fixed at 1 g, and cultured for 24 h. The amount of A 40 and A 42 in the conditioned medium was determined and expressed as described in the legend for Figure 1. Results are means _ S.D. (n 3). ***, P 0.001; **, P 0.01; *, P 0.05. n.s., not significant.

ERdj4 (Figures 2C and 2D), transfection of an expression plasmid for ERdj4 J (J domain-deleted ERdj4) had less activity in the stimulation of the effect of GRP78 on A generation. A similar level of overexpression between wild-type ERdj4 and ERdj4 J was confirmed by immunoblotting (see Supplementary Figure S1) and real-time RT-PCR (results not shown). These findings suggest that the inhibitory effect of ERdj4 (and maybe ERdj3) on A production seems to be achieved via its interaction with GRP78.

We also examined the effect of ER chaperones other than GRP78 (ORP150, GRP94, CNX and CRT). Overexpression of

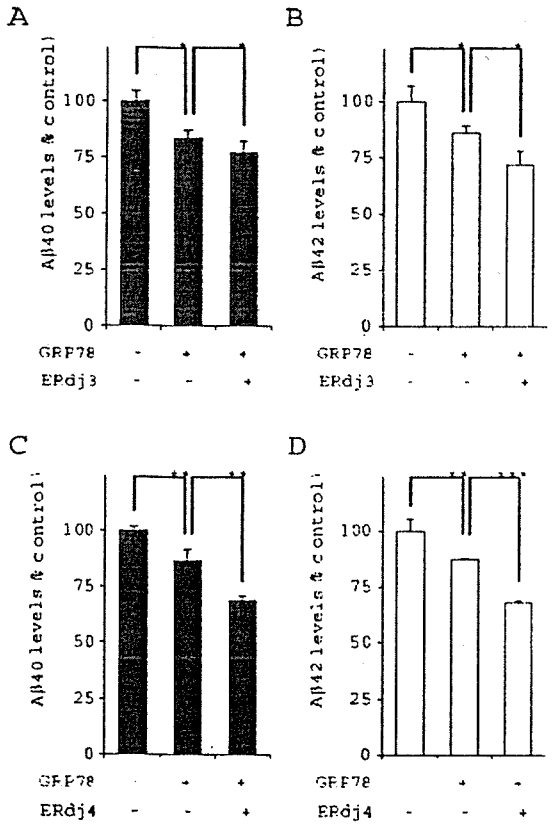

Figure 3 Inhibition of A generation in clones stably expressing ER chaperones

HEK-293 clones expressing APPsw and the indicated ER chaperones were cultured for 24 h. The amount of A 40 and A 42 in the conditioned medium was determined and expressed as described in the legend for Figure 1. Results are means _ S.D. (*n* 3). ***, *P* 0.001; **, *P* 0.01; *, *P* 0.05.

each ER chaperone was confirmed by immunoblotting and/or real-time RT-PCR analyses (results not shown). The results revealed that these ER chaperones can be classified into three groups. As well as GRP78, overexpression of ORP150 decreased the level of both A 40 and A 42. Overexpression of CNX decreased the level of A 42 but not that of A 40. On the other hand, the expression of GRP94 and CRT had no effect on the level of either A 40 or A 42 (see Supplementary Figure S2). Thus, while the suppression of A production is not specific to GRP78, neither is it a general feature of all ER chaperones.

Mechanism for inhibitory effect of GRP78 on A generation

In order to examine the molecular mechanism governing the inhibitory effect of GRP78 on A production, we produced HEK-293 cells that stably expressed not only APPsw, but also GRP78, His-tagged ERdj3 and/or Myc-tagged ERdj4. Expression of each ER chaperone was confirmed by immunoblotting (which are shown in Figures 5A and 5C). First, we examined the production of A in each clone. As shown in Figure 3, the level of both A 40 and A 42 in the conditioned medium was decreased for the GRP78-overexpressing clone. A further decrease was observed for clones overexpressing GRP78 and ERdj3 or ERdj4, which was consistent with the results obtained from our transient-expression experiments (Figure 2). However, the difference in the effects of ERdj4 and ERdj3, as illustrated in Figure 2, was not observed in stable-expression experiments (Figure 3).

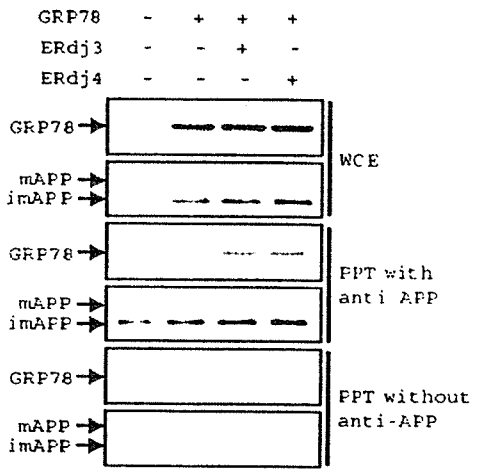

Figure 4 Co-IP of GRP78 with APP

Whole cell extracts prepared from HEK-293 clones expressing APPsw and the indicated ER chaperones were precipitated with or without antibody against the CTF of APP. Whole cell extracts (WCE) and the precipitates (PPT) with or without the antibody were analysed by immunoblotting with antibodies against GRP78 or APP.

Using these clones, the physical interaction of GRP78 with APP was estimated by co-IP. The mature (N- and *O*-glycosylated) and immature (N-glycosylated alone) forms of APP (mAPP and imAPP respectively) can be separated by SDS/PAGE on the basis of their molecular masses [36]. As shown in Figure 4, GRP78 was co-precipitated with APP in a manner that was dependent on the antibody against APP, which showed that GRP78 physically interacted with APP. Furthermore, co-expression of either ERdj3 or ERdj4 slightly stimulated this interaction (Figure 4).

We then examined the maturation of APP, an essential step in the production of A that ocuured in ER. As shown in Figures 5(A) and 5(B), the amount of mAPP relative to imAPP was decreased in the GRP78-overexpressing clone, with a further decrease being observed in the clone expressing both GRP78 and ERdj3. Similar results were obtained with ERdj4 (Figures 5C and 5D). These results suggest that GRP78 inhibits the maturation of APP and that this action is stimulated by its co-chaperones.

We also performed pulse-chase labelling experiments. Proteins were pulse-labelled with [^{35}S]methionine, chased with excess amounts of cold methionine, precipitated with antibody against APP and then examined by autoradiography. Compared with the controls, the conversion of labelled imAPP to mAPP and the disappearance of labelled imAPP and mAPP were retarded in clones expressing GRP78/ERdj3 or GRP78/ERdj4 (see Supplementary Figure S3 at http://www.BiochemJ.org/bj/402/bj4020581add.htm). These results show that the maturation of APP was inhibited, whereas the half-life of APP was prolonged, by the overexpression of GRP78/ERdj3 or GRP78/ERdj4.

Based on the results described above, we speculated that the inhibition of A production by ER chaperones is due to the inhibition of secretase-dependent APP proteolysis as a result of inhibition of APP maturation. In order to assess this, we attempted to detect the CTFs of APP that are generated by -, - and -secretase (CTF , CTF and CTF respectively). CTF could not be detected using our experimental conditions (results not shown). However, as shown in Figure 6, the amount of CTF and CTF was decreased in the GRP78-overexpressing clone, with a further decrease being observed in clones overexpressing GRP78/ERdj3 or GRP78/ERdj4. Based on these results we consider that proteolysis of APP by - and -secretases is inhibited in cells overexpressing these ER chaperones.