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Up-Regulation of 150-kDa Oxygen-Regulated Protein by Celecoxib in Human Gastric Carcinoma Cells

Takushi Namba, Tatsuya Hoshino, Ken-ichiro Tanaka, Shinji Tsutsumi, Tomoaki Ishihara, Shinji Mima, Keitarou Suzuki, Satoshi Ogawa, and Tohru Mizushima

Graduate School of Medical and Pharmaceutical Sciences, Kumamoto University, Kumamoto, Japan (T.N., T.H., K.-i.T., S.T., T.I., S.M., K.S., T.M.); and Department of Neuroanatomy, Kanazawa University Medical School, Kanazawa, Japan (S.O.)

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ABSTRACT

Induction of apoptosis by nonsteroidal anti-inflammatory drugs, such as celecoxib, is involved in their antitumor activity. An endoplasmic reticulum chaperone, 150-kDa oxygen-regulated protein (ORP150) is essential for the maintenance of cellular viability under hypoxia and is reported to be overexpressed in clinically isolated tumors. We here found that ORP150 was up-regulated by celecoxib in human gastric carcinoma cells. In conjunction with the suppression of tumor growth, orally administered celecoxib up-regulated ORP150 in xenograft tumors. Both the ATF4 and ATF6 pathways were activated by celecoxib, and suppression of ATF4 and ATF6 mRNA expression by small interfering RNA (siRNA) inhibited the celecoxib-dependent up-regulation of ORP150. Celecoxib administration led to an increase in the intracellular concentration of Ca^{2+}, whereas 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid-acetoxymethyl ester, an intracellular Ca^{2+} chelator, inhibited the up-regulation of ORP150 and the activation of the ATF4 and ATF6 pathways. These results suggest that these Ca^{2+}-activated pathways are involved in the celecoxib-mediated up-regulation of ORP150. Clones overexpressing ORP150 were less susceptible to celecoxib-induced, but not staurosporine-induced, apoptosis and displayed less up-regulation of C/EBP homologous transcription factor (CHOP), a transcription factor with apoptosis-inducing activity. In contrast, siRNA for ORP150 stimulated apoptosis and expression of CHOP in the presence of celecoxib but not staurosporine. These results suggest that up-regulation of ORP150 in cancer cells inhibits celecoxib-induced apoptosis, thereby decreasing the potential antitumor activity of celecoxib.

Nonsteroidal anti-inflammatory drugs (NSAIDs) are made up of a useful family of therapeutics, accounting for nearly 5% of all prescribed medications (Smalley et al., 1995). In addition to their anti-inflammatory effects, recent epidemiological studies have revealed that prolonged NSAID use reduces the risk of cancer (such as colonic, rectal, and stomach cancer), and preclinical and clinical studies have indicated that some NSAIDs, in particular celecoxib, are effective in the treatment and prevention of cancer (Wang et al., 2003). The antitumor activity of NSAIDs involves various mechanisms, including cell growth suppression, inhibition of angiogenesis, and inhibition of metastasis. In particular, NSAID-induced apoptosis in cancer cells is thought to play an important role in the antitumor action of this class of drugs (Gupta and Dubois, 2001; Kismet et al., 2004).

Together with the anti-inflammatory action of NSAIDs, NSAID-induced apoptosis was only thought to be mediated through the NSAID-dependent inhibition of cyclooxygenase (COX), an enzyme essential for the synthesis of prostaglandins (PGs). This belief was based on the inhibition of cellular apoptosis by PGs, such as PGE_2, and the overexpression of COX-2 (a subtype of COX) in various types of clinically iso-

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ABBREVIATIONS: NSAID, nonsteroidal anti-inflammatory drug; COX, cyclooxygenase; PG, prostaglandin; AGS, apoptosis in human gastric carcinoma; ER, endoplasmic reticulum; IRE1, protein-kinase and site-specific endoribonuclease; PERK, eukaryotic translation initiation factor 2 kinase; ATF, activating transcription factor; eIF2α, eukaryotic initiation factor-2α; CHOP, C/EBP homologous transcription factor; ORP150, 150-kDa oxygen-regulated protein; GRP, glucose-regulated protein; AM, acetoxymethyl ester; BAPTA, 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid; PARP, poly(ADP-ribose)polymerase; siRNA, small interfering RNA; PCR, polymerase chain reaction; FITC, fluorescein isothiocyanate; FACS, fluorescence activated cell sorting; PI, propidium iodide; PIPES, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]propanesulfonate; RT-PCR, reverse transcription-polymerase chain reaction; ERSE, endoplasmic reticulum stress response element; XBP-1, X box binding protein; ROSE, reactive oxygen species; VEGF, vascular endothelial growth factor.

lated tumors and cancer cell lines (Eberhart et al., 1994; Ristimaki et al., 1997). However, a derivative of the NSAID sulindac (sulindac sulfone), which has no COX-inhibitory activity, has subsequently been shown to induce apoptosis in tumor cells, whereas it has been demonstrated that some NSAIDs induce apoptosis in COX-null fibroblasts and in tumor cells without COX expression (Hanif et al., 1996; Elder et al., 1997; Zhang et al., 1999). Therefore, COX-independent mechanisms are also clearly involved in NSAID-induced apoptosis.

To investigate this COX-independent mechanism, we systematically searched for genes whose expression is up-regulated by NSAIDs in concert with induction of apoptosis in human gastric carcinoma (AGS) cells. This study revealed that various endoplasmic reticulum (ER) stress response-related genes are up-regulated by NSAIDs (Mima et al., 2005). The ER stress response is induced by accumulation of unfolded protein in the ER, a process involving three types of ER transmembrane proteins: protein-kinase and site-specific endoribonuclease (IRE1), protein kinase R-like ER kinase (PERK), and activating transcription factor (ATF) 6 (Yoshida et al., 2000; Kaufman, 2002; Ron, 2002). ER stressors phosphorylate PERK, which in turn phosphorylates eukaryotic initiation factor-2α (eIF2α), leading to activation of ATF4 expression (ATF4 pathway) (Luo et al., 2003). ER stressors also cause cleavage of p90-ATF6 into p50-ATF6, which translocates to the nucleus (ATF6 pathway) (Yoshida et al., 2000). Both ATF4 and p50-ATF6 specifically activate transcription of ER stress response-related genes. ER stress response-related proteins contain not only ER chaperones (such as GRP78), which confer protection against stressors by refolding unfolded proteins in the ER, but also C/EBP homologous transcription factor (CHOP), a transcription factor with apoptosis-inducing activity (Zinszner et al., 1998). We have previously shown, using both CHOP-deficient mice and a dominant-negative form of CHOP, that this CHOP induction is important for NSAID-induced apoptosis (Tsutsumi et al., 2004). We have also recently reported that up-regulation of GRP78 by celecoxib protects cancer cells from celecoxib-induced apoptosis, decreasing the potential antitumor activity of the drug (Tsutsumi et al., 2006). Therefore, ER stress response seems to be important for elucidating the mechanism of NSAID-induced apoptosis.

Another ER chaperone, 150-kDa oxygen-regulated protein (ORP150), was originally identified in cultured astrocytes exposed to hypoxia (Kuwabara et al., 1996). Cellular expression of ORP150 confers resistance to apoptosis induced not only by hypoxia but also by glutamate and α-amino-3-hydroxy-5-methylisoxazole-propionate (Ozawa et al., 1999; Kitao et al., 2001; Tamatani et al., 2001; Asahi et al., 2002). Previous studies have reported that ORP150 is up-regulated under various pathological conditions, and this up-regulation has been implicated in the progression of diabetes, atherosclerotic plaque, and ischemia in brain (Tsukamoto et al., 1996; Matsushita et al., 1998; Asahi et al., 2002; Ozawa et al., 2005). Furthermore, recent papers have described the up-regulation of ORP150 in clinically isolated tumors and cancer cell lines (Tsukamoto et al., 1998; Miyagi et al., 2002). However, being different from well studied ER chaperones, such as GRP78, the mechanism underlying the up-regulation of ORP150 remains unclear. In this study, we demonstrate that celecoxib up-regulates ORP150, and examine its action in AGS cells. Our findings suggest that up-regulation of ORP150 decreases the antitumor activity of the drug by inhibiting apoptosis. We also provide evidence that both the ATF4 and ATF6 pathways are involved in this celecoxib-induced up-regulation of ORP150.

Materials and Methods

Chemicals, Plasmids, and Animals. RPMI 1640 medium was obtained from Nissui (Tokyo, Japan). Fetal bovine serum was purchased from Gibco Co. (Carlsbad, CA). Pluronic F127, fluo-3/AM and BAPTA-AM were obtained from Dojindo Co. (Kumamoto, Japan). Staurosporine was purchased from Sigma-Aldrich (St. Louis, MO). Indomethacin was obtained from Wako Pure Chemicals (Tokyo, Japan). Celecoxib was from LKT Laboratories Inc. (St. Paul, MN). Antibodies against ATF4, ATF6, lamin, pro-caspase-3, and actin were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). An antibody against poly(ADP-ribose)polymerase (PARP) was from Cell Signaling Technology Inc. (Beverly, MA). An antibody against ORP150 came from our laboratory stock (Tsukamoto et al., 1998). The RNeasy kit, siRNAs, and HiPerFect and RNAiFect transfection reagent were from QIAGEN (Valencia, CA). Acetyl-DEVD-methylcoumarin amide was from Peptide Institute Inc. (Osaka, Japan). A first-strand cDNA synthesis kit was purchased from Amersham (Little Chalfont, Buckinghamshire, UK). Lipofectamine (TM2000) and pcDNA3.1 plasmid were obtained from Invitrogen. SYBR GREEN PCR Master Mix was from Applied Biosystems (Foster City, CA). Annexin V-FITC apoptosis detection kit I was from BD Biosciences (San Jose, CA). Female ICR nude mice (5 weeks of age) were obtained from the Kyudoh Co. (Saga, Japan). 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 Institutes of Health, and they were approved by the Animal Care Committee of Kumamoto University.

Cell Culture and Overexpression of ORP150. AGS, MKN45, and Kato III are human carcinoma cell lines derived from stomach. Cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum, 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 dimethyl sulfoxide, and control experiments were performed in the same concentrations of dimethyl sulfoxide alone. 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 before use in experiments. Transfection of AGS cells with plasmids (pCI-neo containing the *ORP150* gene) was carried out using Lipofectamine (TM2000) according to the manufacturer's protocols. The stable transfectants expressing ORP150 were selected by immunoblotting analysis. Positive clones were maintained in the presence of 800 μg/ml Geneticin (G-418).

Annexin V binding by Fluorescence-Activated Cell Sorting. Experiments were done using Annexin V-FITC apoptosis detection kit I according to the manufacturer's protocols. Briefly, cells were gently washed with phosphate-buffered saline and the binding buffer and finally resuspended in the binding buffer. After addition of Annexin V-FITC and PI solutions, samples were incubated for 15 min at room temperature in the dark. Samples were scanned with a FACSCalibur (BD Biosciences) cell sorter and analyzed by CellQuest software (BD Biosciences). Plots in Annexin V-positive/PI-negative quadrant were counting as apoptotic cells.

Caspase Activity Assay. The caspase-3-like activity was determined as described previously (Hoshino et al., 2003). 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 was determined using a fluorescence spectrophotometer. One unit of protease activity was defined as the amount of enzyme required to release 1 pmol of aminomethylcoumarin per minute. For statistical analysis, we measured three different samples in the same experiment.

Real-Time Reverse Transcription-PCR Analysis. Total RNA was extracted from cells using an RNeasy kit according to the manufacturer's protocols. Samples 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 analyzed 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 finally 45 cycles each 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. For statistical analysis, we performed PCR reaction three times on the same sample. Furthermore, we confirmed results by performing at least two independent experiments.

Primers were designed using the Primer3 Web site (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). Primers are listed as follows (name: forward primer and reverse primer): ATF4: 5′-tcaaacctcatgggttctcc-3′ and 5′-gtgtcatccaacgtggtcag-3′; ATF6: 5′-ctccgagatcagcagaggaa-3′ and 5′-aatgactcagggatggtgct-3′; CHOP: 5′-tgcctttctcttcggacact-3′ and 5′-tgtgacctctgctggttctg-3′; GRP78: 5′-tagcgtatggtgctgctgtc-3′ and 5′-tttgtcaggggtctttcacc-3′; ORP150: 5′-gaagatgcagagcccatttc-3′ and 5′-tctgctccaggacctcctaa-3′; GRP94: 5′-tggatcttgctgtggttttg-3′ and 5′-tgaggcgaagcattctttct-3′; calnexin: 5′-tgaagaagatggtggcactg-3′ and 5′-cgtggctttctgtttcttgg-3′; and calreticulin: 5′-tcaccaacgatgaggcatac-3′ and 5′-tcctcgtcctgtttgtcctt-3′.

Northern Blotting. Total RNA was extracted by use of an RNeasy kit, according to the manufacturer's specifications. Samples were separated by agarose gel electrophoresis in the presence of 6.3% formaldehyde, and blotted onto nylon membranes (Amersham Bioscience). For obtaining RNA probe, PCR-amplified partial DNA fragments of *ORP150* (407 base pairs) were cloned into a pBluescript II SK (+) vector (Stratagene, La Jolla, CA), and RNA probe was prepared using DIG Northern Starter kit (Roche Diagnostics, Indianapolis, IN). After hybridization and washing, membranes were analyzed with LAS 1000 plus (FUJIX Ltd., Kyoto, Japan).

Immunoblotting Analysis. Whole-cell and nuclear extracts were prepared as described previously (Tsutsumi et al., 2002). The protein concentration of samples was determined by the Bradford method (Bradford, 1976). Samples were applied to polyacrylamide SDS gels, subjected to electrophoresis, and the resultant proteins then immunoblotted with respective antibodies.

Xenograft Tumor Growth. The effect of celecoxib on xenograft tumor growth was examined as described previously (Tsutsumi et al., 2006). Briefly, each nude mouse was inoculated s.c. in the right hind footpad with 2×10^6 cells of MKN45. When tumors reached a mean volume of 66 ± 14 mm^3, the mice began to receive a single daily oral dose of celecoxib in 1% methylcellulose, a protocol that continued for the duration of the study. Tumors were measured every 5 days, and their volumes were calculated. For examination of ORP150 expression in tumors, tumor xenografts (dissected into <1-mm pieces) were solubilized with buffer and subjected to immunoblotting analysis.

Measurement of Intracellular Ca^{2+} Levels. The intracellular Ca^{2+} levels were monitored as described previously (Tanaka et al., 2005). Cells were incubated with 4 μM fluo-3/AM in assay buffer (115 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgCl_2$, 20 mM HEPES, 13.8 mM glucose, 0.1% bovine serum albumin, 0.04% Pluronic F127, and 2 mM probenecid) for 40 min at 37°C. After washing, cells were suspended in assay buffer, again containing 2 mM probenecid. Fluo-3 fluorescence of cells in a water-jacketed cuvette was measured with a Hitachi F-4500 spectrofluorophotometer. 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. For statistical analysis, we measured three different samples in three independent experiments.

siRNA Targeting of Genes. We used siRNA of 5′-cagugauguugaaggagaadTdT-3′ and 5′-uucuccuucaacaucacugdTdT-3′, 5′-gccuaggucucuuagaugadTdT-3′ and 5′-ucaucuaagagaccuaggcdTdT-3′ or 5′-

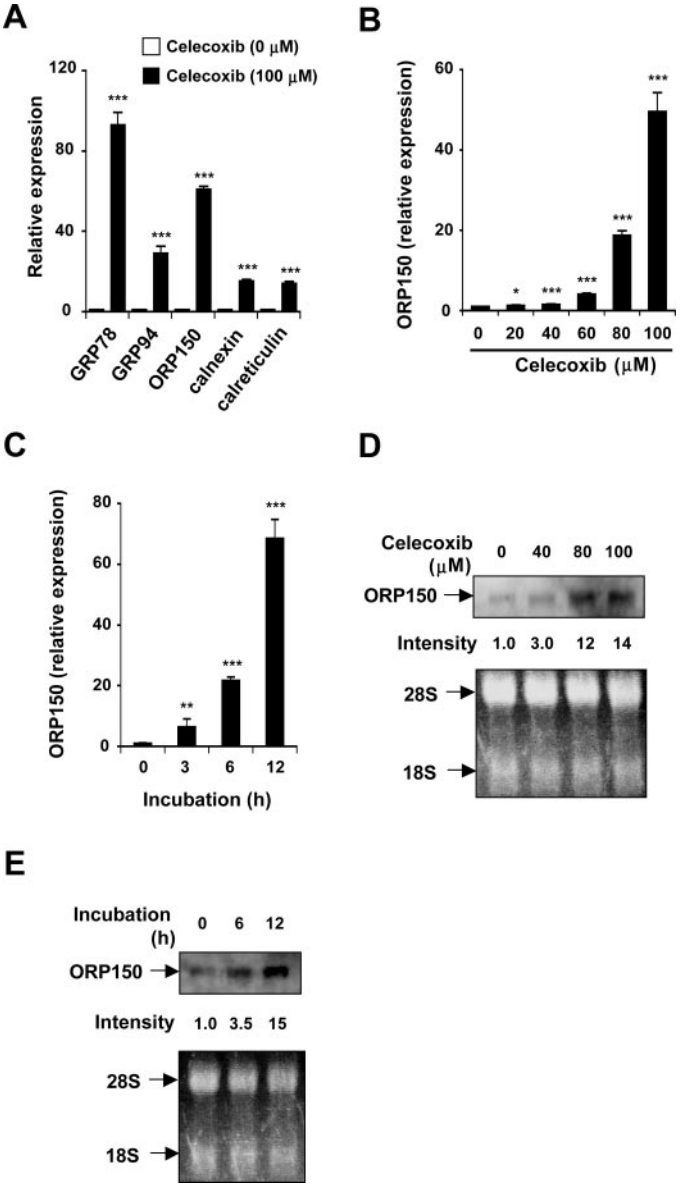


Fig. 1. Up-regulation of mRNA of various ER chaperone genes by celecoxib. AGS cells were incubated with either the indicated concentrations (A, B, and D) or 100 μM of celecoxib (C and E) for 12 h (A, B, and D) or the time periods indicated (C and E) and total RNA extracted. Samples were subjected to real-time RT-PCR using a specific primer for each gene. Values were normalized to actin gene expression and expressed relative to the control sample (i.e., without celecoxib). Values are given as mean ± S.D. ($n = 3$). ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$ (A–C). Samples were also analyzed by Northern blotting analysis. Bottom panels show ribosomal RNA (18S and 28S) stained with ethidium bromide (D and E).

gcaaccaauuaucaguuuadTdT-3′ and 5′-uaaacugauaauugguugcdTdT-3′ as annealed oligonucleotides for repressing ORP150, ATF4, or ATF6 expression, respectively. AGS cells were transfected with siRNA using HiPerFect or RNAiFect transfection reagent according to the manufacturer's instructions. Nonsilencing siRNA (5′-uucuccgaacgugucacgudTdT-3′ and 5′-acgugacacguucggagaadTdT-3′) was used as a negative control.

Statistical Analysis. All values are expressed as the mean ± S.D. One-way analysis of variance followed by Scheffé'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

Celecoxib Up-Regulates Various ER Chaperones. In a previous report, we showed that NSAIDs (such as celecoxib, indomethacin, and diclofenac) up-regulate GRP78 expression in primary cultures of guinea pig gastric mucosal cells (Tsutsumi et al., 2004) and AGS cells (Tsutsumi et al., 2006). Here, we used real-time RT-PCR techniques to examine the effect of celecoxib on mRNA expression of various ER chaperone genes in AGS cells. As shown in Fig. 1A, celecoxib up-regulated GRP78 mRNA, as described previously (Tsutsumi et al., 2006). A similar result was obtained with all of the other ER chaperones tested, i.e., GRP94, ORP150, calnexin, and calreticulin (Fig. 1A). Of these, we focused on ORP150 in the following experiments. The dose-response and time-course properties of celecoxib-dependent up-regulation of ORP150 mRNA expression are shown in Fig. 1, B and C. Both reflect similar results to those obtained using GRP78 mRNA, as reported in our previous article (Tsutsumi et al., 2006). We also confirmed celecoxib-dependent up-regulation of ORP150 mRNA expression by Northern blotting analysis (Fig. 1, D and E).

Immunoblotting experiments revealed that celecoxib also up-regulates ORP150 at the protein level (Fig. 2A). A similar response was observed with another NSAID, indomethacin, suggesting that we were not observing a celecoxib-specific phenomenon.

COX exists as two subtypes, COX-1 and COX-2, for which celecoxib is COX-2-selective. We examined the celecoxib-dependent up-regulation of ORP150 in Kato III cells, in which COX-1 but not COX-2 mRNA is expressed (Saukkonen et al., 2001). This phenotype was confirmed by RT-PCR (data not shown). As shown in Fig. 2B, celecoxib up-regulated ORP150 even in Kato III cells; thus, a COX-2-selective NSAID up-regulated ORP150 in cells lacking COX-2 expression, suggesting that up-regulation of ORP150 by NSAIDs is independent of COX inhibition. For further confirmation of this point, we examined the effect of PGE_2 on the up-regulation of ORP150 and found that PGE_2 did not affect the expression of ORP150 in the presence or absence of celecoxib (data not shown).

We also examined the effect of celecoxib on ORP150 expression in tumors in vivo. Tumors were developed in nude mice by inoculation (s.c.) of MKN45 cells in which celecoxib-dependent up-regulation of ORP150 was confirmed in vitro (data not shown). Oral administration of celecoxib clearly inhibited the growth of xenograft tumors (Fig. 3C), this being consistent with our previous report (Tsutsumi et al., 2006).

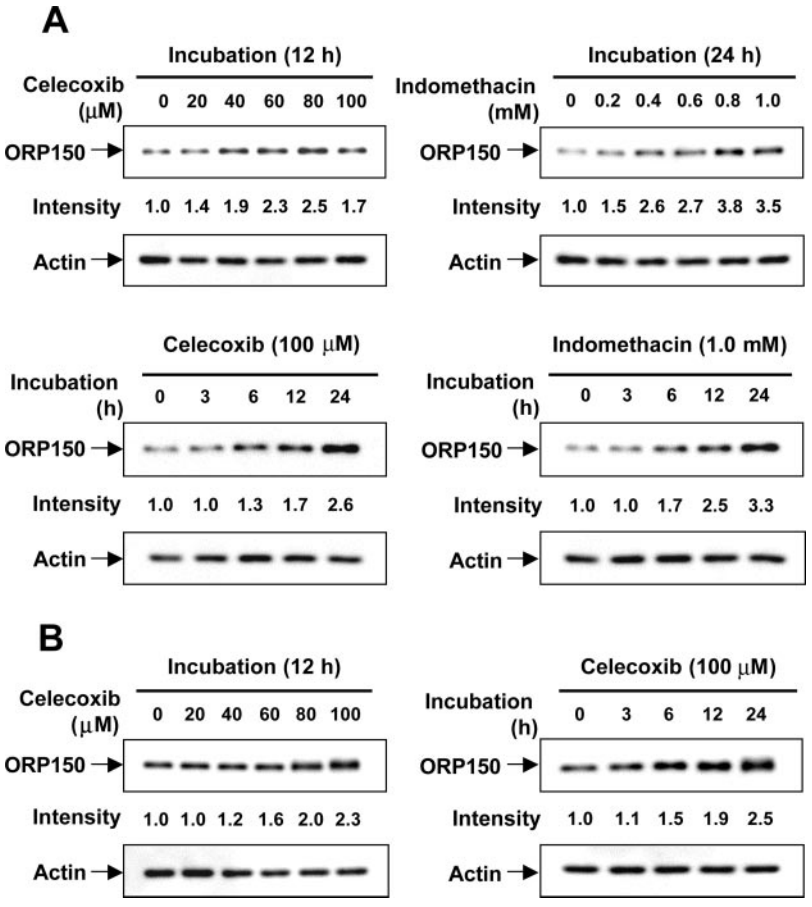


Fig. 2. Up-regulation of ORP150 by NSAIDs. AGS (A) or Kato III (B) cells were incubated with various concentrations of celecoxib or indomethacin for the indicated periods. Whole-cell extracts were analyzed by immunoblotting with an antibody against ORP150 or actin. The band intensity of ORP150 was determined by densitometric scanning. Gel-loading levels were compensated against the band intensity of actin and expressed relative to the control sample (i.e., without NSAIDs).

However, as shown in Fig. 3, A and B, the level of ORP150 in these tumors was also increased, indicating that celecoxib exerts this in vivo effect while simultaneously suppressing tumor growth.

Mechanism for Up-Regulation of ORP150 by Celecoxib. As outlined above, the mechanism underlying up-regulation of ORP150 by ER stressors is still unclear. Here, we used siRNA for ATF4 and ATF6 to examine the contribution of these transcription factors to celecoxib-dependent up-regulation of ORP150. As shown in Fig. 4, A and E, ATF4 mRNA and ATF4 protein was up-regulated by celecoxib as described previously (Tsutsumi et al., 2006), but, surprisingly, so, too, was ATF6 mRNA (Fig. 4B). The amount of p90 ATF6 or p50 ATF6 were decreased or increased, respectively, by celecoxib (Figs. 4E and 5F), suggesting that cleavage of p90-ATF6 into p50-ATF6 was stimulated by celecoxib as described previously (Tsutsumi et al., 2006). Transfection of a given siRNA decreased mRNA and protein levels of its target gene, but it had no effect on those of the other gene in both absence and presence of celecoxib (Fig. 4, A, B, and E). Furthermore, double transfection of siRNAs for both ATF4 and ATF6 resulted in suppression of mRNA levels of these genes to a similar extent to that seen with single transfection of each siRNA alone (Fig. 4, A and B). Celecoxib-dependent up-regulation of ORP150 mRNA was partially suppressed by siRNA for either ATF4 or ATF6 (Fig. 4C). However, interestingly, double transfection exerted a stronger suppressive effect than transfection of either siRNA alone (Fig. 4C). Similar results were obtained for celecoxib-dependent up-regulation of GRP78 mRNA (Fig. 4D). None of the transfections illustrated in Fig. 4 affected the baseline cell viability (data not shown). We also confirmed that both PERK and eIF2α were phosphorylated under the same conditions as in Fig. 4 (data

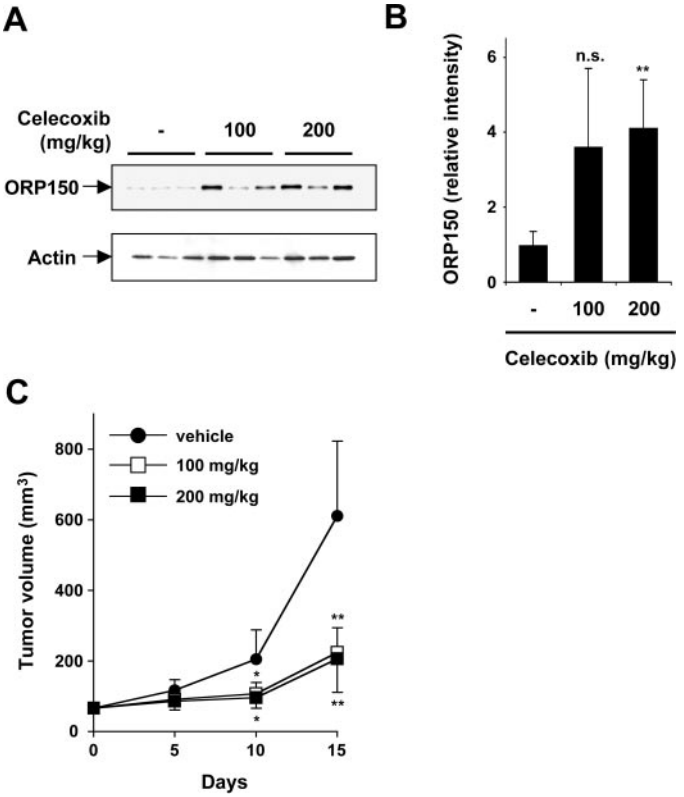


Fig. 3. Effect of celecoxib on growth of xenograft tumor and expression of ORP150 in nude mice. Each nude mouse ($n = 3$) was inoculated s.c. with MKN45 cells, leading to tumor development. Celecoxib was then administered as a single daily oral dose for the duration of the study. Four days after celecoxib administration commenced, cell lysates prepared from tumors were analyzed by immunoblotting as described in the legend of Fig. 2 (A and B). Tumors were measured every 5 days and their volumes calculated (C). Values given are mean ± S.D. ($n = 5$). **, $P < 0.01$; *, $P < 0.05$ (B and C).

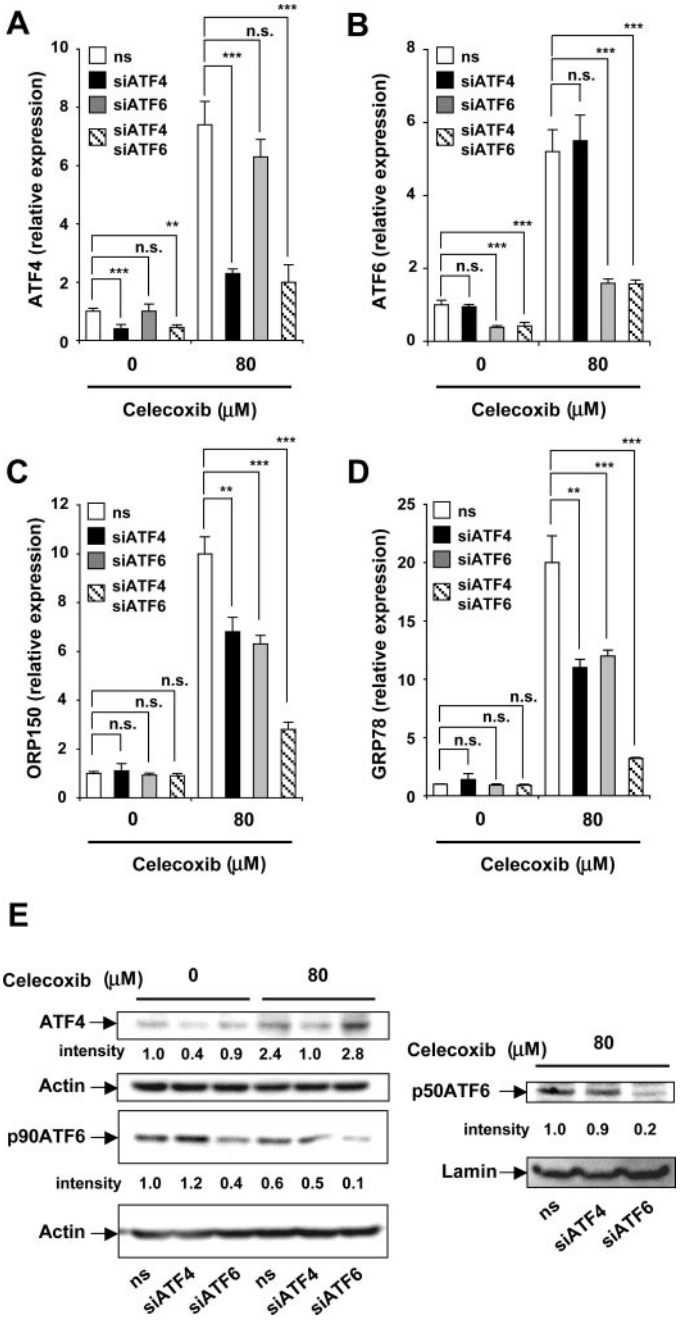


Fig. 4. Effect of siRNA for ATF4 and/or ATF6 on the celecoxib-dependent up-regulation of ORP150. AGS cells transfected with siRNA for ATF4 (siATF4), ATF6 (siATF6), and/or nonsilencing (ns) siRNA (the total amount of siRNA is fixed at 10 μg) were incubated with or without 80 μM celecoxib for 6 h. Total RNA was extracted and subjected to real-time RT-PCR by use of a specific primer for *ATF4* (A), *ATF6* (B), *ORP150* (C), and *GRP78* (D). Values were analyzed and expressed as described in the legend of Fig. 1. Values are shown as mean ± S.D. ($n = 3$). ***, $P < 0.001$; **, $P < 0.01$. E, whole-cell extracts (for ATF4, actin, and p90-ATF6) or nuclear extracts (for p50-ATF6 and lamin) were analyzed by immunoblotting as described in the legend to Fig. 2.

not shown). These results suggest that both the PERK-eIF2α-ATF4 and the ATF6 pathways are involved in the up-regulation of ORP150 by celecoxib.

We have previously reported that NSAIDs increase intracellular Ca^{2+} concentrations, leading us to suggest that this increase is involved in the NSAID-induced ER stress response (Tomisato et al., 2004; Tanaka et al., 2005; Tsutsumi et al., 2006). Here, we tested whether an increase in intracellular Ca^{2+} is involved in the celecoxib-dependent up-regulation of ORP150. First, we confirmed that celecoxib increases intracellular Ca^{2+} in a dose-dependent manner in AGS cells (Fig. 5A), this increase being consistent with our previous results (Tsutsumi et al., 2006). BAPTA-AM, an intracellular Ca^{2+} chelator, partially inhibited the celecoxib-dependent up-regulation of ORP150, GRP78, ATF4, and ATF6 mRNA and the cleavage of p90-ATF6 into p50-ATF6 (Fig. 5, B–F). At the concentrations used, BAPTA-AM did not affect cell viability (data not shown). These results suggest that an increase in intracellular Ca^{2+} is involved in the up-regulation of ORP150 through activation of both the ATF4 and ATF6 pathways.

Role of Up-Regulation of ORP150 in the in Vitro Antitumor Activity of Celecoxib. As described above, various mechanisms have been proposed for the chemopreventive and chemotherapeutic action of NSAIDs; these include inhibition of cell growth and stimulation of apoptosis. Here, we examined the role of celecoxib-dependent up-regulation of ORP150 in the antitumor activity of the drug in vitro. This was achieved by constructing stable transfectants of AGS cells that continuously overexpressed ORP150 (clones 3 and 5) (Fig. 6, A and B).

Figure 6C shows the cell growth curve for each clone; these curves were indistinguishable from that of the mock transfectant control. Therefore, up-regulation of ORP150 by celecoxib does not seem to be involved in its inhibition of cell growth.

We recently reported that celecoxib induces apoptosis but that up-regulation of GRP78 contributes to suppression of this apoptosis in AGS cells (Tsutsumi et al., 2006). We therefore examined the role of up-regulation of ORP150 in apoptosis using ORP150-overexpressing clones and siRNA for ORP150. Figure 6D shows the time course of celecoxib-dependent induction of apoptosis. Significant apoptosis was observed 3 h after the addition of celecoxib. Since treatment of cells with 80 μM celecoxib for more than 12 h caused lower recovery of mRNA and protein (data not shown), we choose 6 h as a condition for observing celecoxib-dependent apoptosis. We examined the role of up-regulation of ORP150 in apoptosis by FACS analysis (counting annexin V-positive/PI-negative cells). As shown in Fig. 6, E and F, apoptotic cells (annexin V-positive/PI-negative cells) increased after the treatment of cells with celecoxib, and this increase was partially inhibited in ORP150-overexpressing clones. We also examined the effect of ORP150 overexpression on celecoxib-induced apoptosis by measuring caspase-3-like activity using fluorogenic peptide substrates and by monitoring cleavage of pro-caspase-3 and cleavage of PARP (a substrate of caspase-3) and obtained results similar to those from FACS analysis (Fig. 6, G and H). Furthermore, compared with the mock transfectant control, up-regulation of CHOP mRNA by celecoxib was partially suppressed in ORP150-overexpressing clones (Fig. 6I), suggesting that overexpression of ORP150 protects AGS cells from apoptosis through inhibition of *CHOP* expression. To examine the specificity of this antiapoptotic effect of ORP150, the apoptosis induced by staurosporine, a chemotherapy drug that lacks any ER stress response-inducing ability, was compared between ORP150-overexpressing clones and the mock transfectant control. As shown in Fig. 6K, staurosporine did not up-regulate ORP150

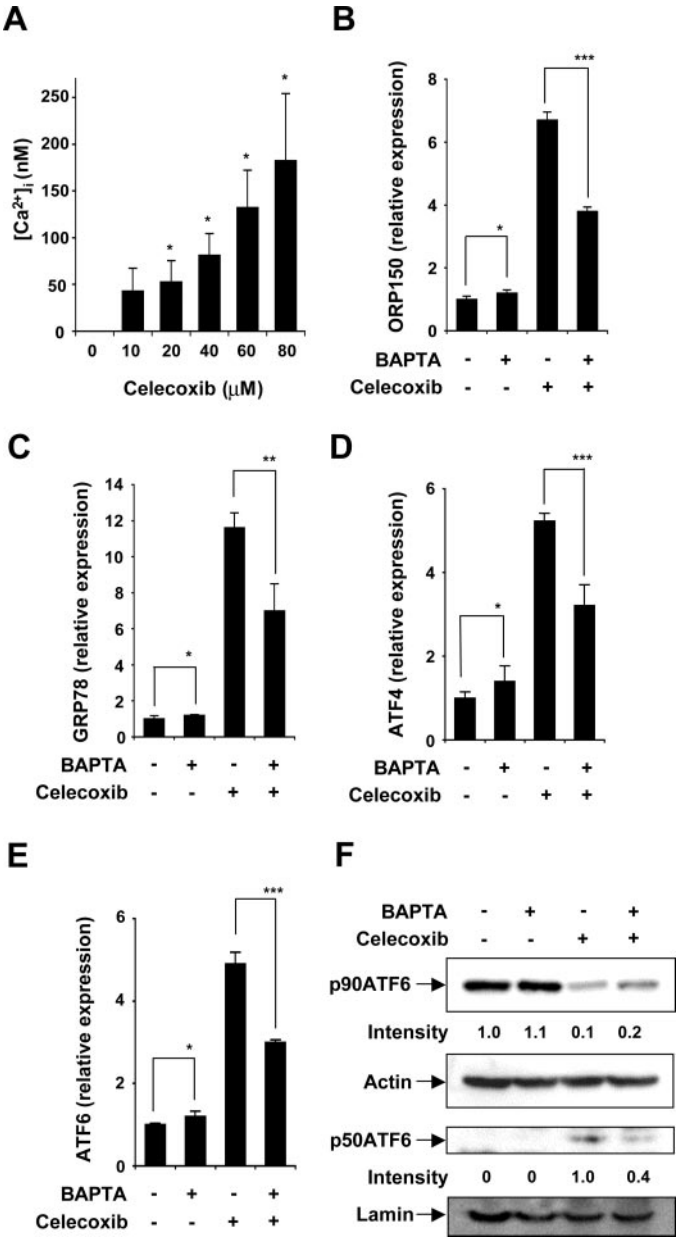
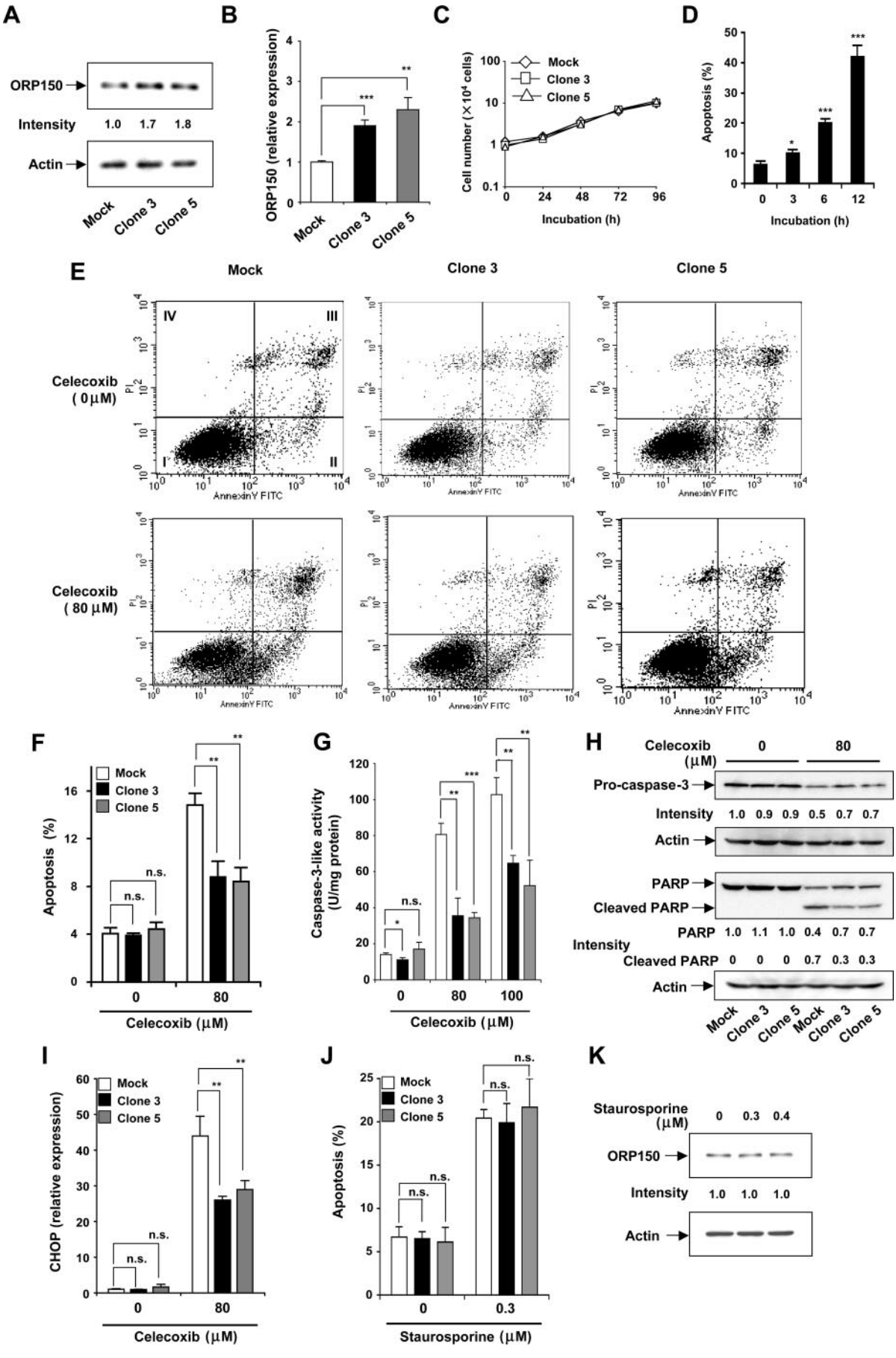


Fig. 5. Changes in intracellular Ca^{2+} concentration and its role in the celecoxib-dependent up-regulation of ORP150. The intracellular Ca^{2+} concentration was monitored using a fluo-3/AM assay system. The indicated concentrations of celecoxib were added to fluo-3/AM-loaded cells, and the time course of fluo-3 fluorescence change was monitored. The maximum value for the increase in the intracellular Ca^{2+} level ($\Delta[Ca^{2+}]_i$) is shown (A). AGS cells were preincubated with or without 2 μM BAPTA-AM for 1 h and further incubated with or without 80 μM celecoxib in the presence or absence of 2 μM BAPTA-AM for 6 h (B–F). The levels of ORP150 mRNA (B), GRP78 mRNA (C), ATF4 mRNA (D), ATF6 mRNA (E), and p50- and p90-ATF6 protein (F) were estimated by real-time RT-PCR or immunoblotting experiments as described in the legends of Figs. 1 and 4. Values are shown as mean ± S.D. ($n = 3$). ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$ (A–E).



at concentrations that were sufficient to induce apoptosis (Fig. 6J), and there was no difference in the level of staurosporine-induced apoptosis between ORP150-overexpressing clones and the mock transfectant control (Fig. 6J). These results suggest that the suppression of apoptosis by overexpression of ORP150 is specific for apoptosis induced by chemotherapy drugs that induce an ER stress response.

Transfection of siRNA for ORP150 decreased the expression of ORP150 protein (Fig. 7A) and ORP150 mRNA (Fig. 7B) in the presence or absence of celecoxib. FACS analysis and analysis on caspase-3 showed that this transfection stimulated celecoxib-induced apoptosis (Fig. 7, C–F). Furthermore, this transfection stimulated celecoxib-induced CHOP mRNA expression (Fig. 7G). Together, these results support the idea that celecoxib-induced up-regulation of ORP150 protects cells from apoptosis induced by the drug. In contrast, as illustrated in Fig. 7H, transfection of siRNA for ORP150 had no effect on apoptosis induced by staurosporine, further supporting the idea that the antiapoptotic effect of ORP150 is specific for chemotherapy drugs that induce an ER stress response.

We also examined the effect of siRNA for ORP150 on celecoxib-dependent cell growth inhibition. Since the growth inhibition was observed with lower concentrations of celecoxib than apoptosis induction, we used 40 μM celecoxib for this experiment. We confirmed that siRNA for ORP150 but not nonspecific siRNA suppressed the 40 μM celecoxib-dependent induction of ORP150 mRNA (Fig. 7I). As shown in Fig. 7J, the growth of cells transfected with siRNA for ORP150 in the presence of celecoxib was slower than that with nonspecific siRNA. In contrast, there was no clear difference in cell growth between siRNA for ORP150 and nonspecific siRNA in the absence of celecoxib (Fig. 7J). These results suggest that celecoxib-induced up-regulation of ORP150 protects cells not only from apoptosis but also from growth inhibition induced by the drug.

Discussion

In this study, we have shown that celecoxib up-regulates ORP150 not only in cultured human gastric carcinoma cells but also in xenograft tumors in nude mice. Given that celecoxib (a COX-2-selective NSAID) up-regulated ORP150 in cells lacking COX-2 expression (Kato III cells) and that endogenously added PGE_2 did not affect this up-regulation, the up-regulation of ORP150 by celecoxib seems to occur independently of COX inhibition, as does the up-regulation of GRP78 by the same drug (Tsutsumi et al., 2006).

Although various ER stressors have been reported to up-regulate ORP150, the underlying molecular mechanism has remained unclear. As far as we are aware, the only available information is that the promoter of the *ORP150* gene contains an ER stress response element (ERSE) to which p50-ATF6 specifically binds, activating transcription (Kaneda et al., 2000). In this study, we investigated the molecular mechanism responsible for celecoxib-dependent up-regulation of ORP150 using an siRNA technique. siRNA for either ATF4 or ATF6 partially suppressed celecoxib-dependent up-regulation of ORP150, whereas double transfection of the two together proved even more inhibitory. We have previously reported that celecoxib causes sequential activation of PERK, eIF2α, and ATF4 in AGS cells (Tsutsumi et al., 2006). Furthermore, we showed that p90-ATF6 (the inactive form of ATF6 for ERSE-dependent transcription) is cleaved into p50-ATF6 (the active form) in the presence of celecoxib. Together, these results suggest that both the PERK-eIF2$\tilde{\alpha}$-ATF4 and the ATF6 pathways are involved in the celecoxib-dependent up-regulation of ORP150, this being consistent with up-regulation of GRP78 by other ER stressors (Yoshida et al., 2000; Luo et al., 2003). However, unlike other ER stressors (Yoshida et al., 2000), celecoxib also up-regulates the expression of ATF6 mRNA. At present, the underlying mechanism and its contribution to celecoxib-induced up-regulation of ORP150 remain unclear. Another ER transmembrane protein, IRE1, may also be involved in the celecoxib-induced up-regulation of ORP150. IRE1 splices the mRNA of X box binding protein 1 (XBP-1), thereby converting it into a potent activator of transcription from ERSE (Kaufman, 2002). We have previously reported that exposure to the NSAID indomethacin decreases the unspliced (inactive) and increases spliced (active) forms of the XBP-1 protein, respectively (Tsutsumi et al., 2004). However, since none of the *IRE1* or *XBP-1* siRNAs tested here significantly suppressed the target gene (data not shown), we could not test the contribution of the IRE1-XBP-1 pathway to celecoxib-induced up-regulation of ORP150.

In terms of the mechanism upstream of activation of ER transmembrane proteins by celecoxib, we propose that an increase in the intracellular Ca^{2+} concentration plays an important role. It is well known that an increase in intracellular Ca^{2+} induces the ER stress response (Drummond et al., 1987; Wooden et al., 1991). Here, we showed that celecoxib administration leads to a rise in the concentration of intracellular Ca^{2+}, whereas the application of the intracellular Ca^{2+} chelator, BAPTA-AM, inhibits the celecoxib-dependent up-regulation of ORP150 mRNA. In a recent study, we also demonstrated that all of the NSAIDs tested could cause membrane permeabilization and the increase in the intracellular Ca^{2+} level induced by celecoxib was inhibited under Ca^{2+}-free conditions (Tanaka et al., 2005), suggesting that stimulation of the influx of extracellular Ca^{2+} by permeabilization of cytoplasmic membranes is responsible for the observed NSAID-induced rise in intracellular Ca^{2+}. Celecoxib-dependent inhibition of sarcoplasmic/endoplasmic reticulum Ca^{2+} ATPase (an ER-located Ca^{2+} pump that is responsible

Fig. 6. Effect of overexpression of ORP150 on cell growth and apoptosis in AGS cells. The extent of expression of ORP150 in each clone (stable transfectant of ORP150 expression plasmid) or staurosporine-treated (6 h) AGS cells was estimated by immunoblotting experiments (A and K) or real-time RT-PCR (B) as described in the legend of Figs. 1 and 2. Cells from each clone were cultured for the indicated periods, and cell numbers were determined by direct cell counting (C). AGS cells were treated with 80 μM celecoxib for indicated periods (D). Cells from each clone were cultured in the presence of the indicated concentrations of celecoxib (E–I) or staurosporine (J) for 6 h (E–H and J) or 3 h (I), and apoptotic cell numbers were determined by FACS (Annexin V-FITC and PI double staining) (D, E, F, and J). Caspase-3-like activities were measured (G). Total protein or RNA was extracted and subjected to immunoblotting with antibodies against pro-caspase-3 and PARP or real-time RT-PCR using a specific primer for CHOP, respectively (H and I). Values are given as mean ± S.E.M. ($n = 3$). ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$. One datum based on which we draw the F is shown (E). Cells contained in the quadrant of Annexin V-positive and PI-negative (shown as II in control sample in E) are counted as apoptotic cells (E and F).

for accumulation of Ca^{2+} in the ER) may also be involved in this process (Johnson et al., 2002). The mechanism how increase in the intracellular Ca^{2+} level induces ER stress response is unclear at present. One possibility mechanism is Ca^{2+}-dependent protease is involved in this process. NO is known to induce ER stress response. It was recently sug-

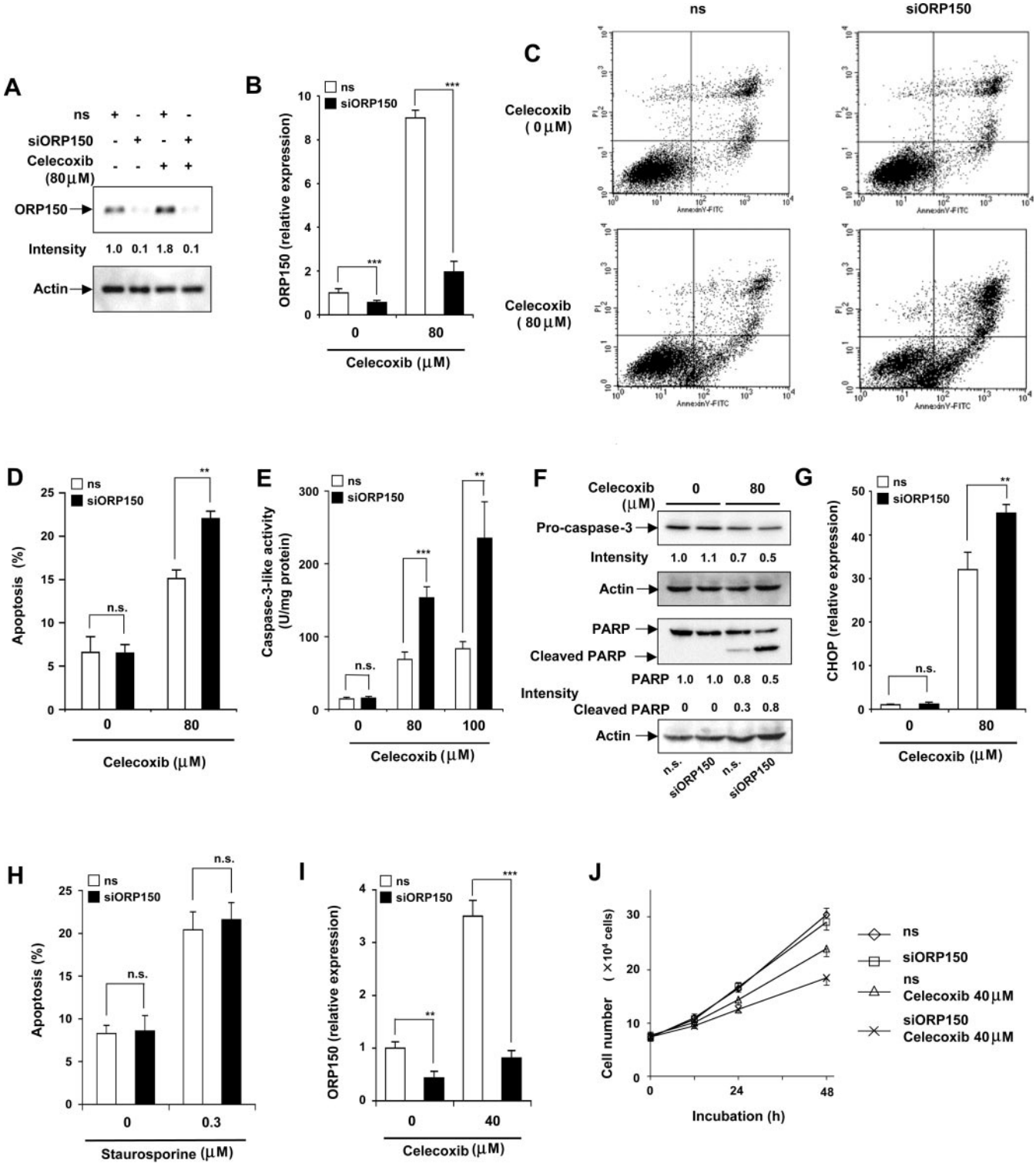


Fig. 7. Effect of siRNA for ORP150 on celecoxib-induced apoptosis. AGS cells were transfected with 5 μg of siRNA for ORP150 (siORP150) or nonsilencing siRNA (ns) (A–J). After 48 h (A–H), cells were incubated with or without indicated concentrations of celecoxib (A–G) or staurosporine (H) for 6 h (A–F and H) or 3 h (G). The levels of ORP150 protein (A), ORP150 mRNA (B), and CHOP mRNA (G) were estimated by immunoblotting or real-time RT-PCR experiments as described in the legends of Figs. 1 and 2. Apoptosis was monitored as described in the legend of Fig. 6. After 24 h (I and J), cells were incubated with or without 40 μM celecoxib for indicated periods and cell numbers were determined by direct cell counting (J). The level of ORP150 mRNA after 24-h incubation was estimated by RT-PCR experiments (I). Values shown are mean ± S.D. ($n = 3$). ***, $P < 0.001$; **, $P < 0.01$.

gested that this ER stress response is mediated by increase in the intracellular Ca^{2+} level and Ca^{2+}-dependent activation of site-1 protease involved in cleavage of p90-ATF6 into p50-ATF6 (Xu et al., 2004). This pathway may be involved in celecoxib-dependent activation of ATF6 and resulting induction of ER stress response. Another possibility is involvement of reactive oxygen species (ROS). It is known that ROS inhibited sarcoplasmic/endoplasmic reticulum Ca^{2+} ATPase (Downey, 1990; Suzuki and Ford, 1991), as is the case of thapsigargin, an inducer of ER stress response. It was recently reported that a Ca^{2+} ionophore A23187 enhances production of ROS (Przygodzki et al., 2005). Therefore, the increase in the intracellular Ca^{2+} level may induce ER stress response through stimulation of ROS production.

Although it was previously shown that the expression of ORP150 in cells renders them resistant to apoptosis induced by hypoxia, glutamate, and α-amino-3-hydroxy-5-methylisoxazole-propionate (Ozawa et al., 1999; Kitao et al., 2001, 2004; Tamatani et al., 2001; Asahi et al., 2002), this is the first demonstration that the expression of ORP150 protects cancer cells from apoptosis induced by chemotherapy and chemoprevention drugs. Stimulation of ORP150 expression inhibited celecoxib-induced apoptosis, whereas its inhibition had the opposite effect. In contrast, staurosporine, a chemotherapy drug that lacks ORP150-inducing activity, had no effect, suggesting that the up-regulation of ORP150 induced by celecoxib decreases its potential as a chemotherapy and chemoprevention drug through inhibition of apoptosis. We also suggested that the up-regulation of ORP150 induced by celecoxib suppresses celecoxib-dependent growth inhibition of tumor cells, which may also decreases its potential as a chemotherapy and chemoprevention drug. Although siRNA for ORP150 almost completely inhibited the expression of ORP150, this siRNA caused a modest increase in apoptosis (Fig. 7). This may be due to that not only ORP150 but also GRP78 is involved in inhibition of celecoxib-induced apoptosis (Tsutsumi et al., 2006). Given that the expression of ORP150 has been shown to suppress glutamate-dependent increases in intracellular Ca^{2+} levels in cultured neurons (Kitao et al., 2001) and that the Ca^{2+}-dependent up-regulation of CHOP is involved in NSAID-induced apoptosis (Tsutsumi et al., 2004; Tanaka et al., 2005), the antiapoptotic effect of ORP150 may be mediated through changes in intracellular Ca^{2+} levels.

Solid tumors usually exist under conditions of glucose starvation and hypoxia, which causes induction of the ER stress response. Moreover, ORP150 was reported to be up-regulated in various clinically isolated tumors and cancer cell lines (Miyagi et al., 2002; Tsukamoto et al., 1998). Furthermore, stronger expression of ORP150 in bladder cancer was reported to reflect a more advanced stage of the disease (Asahi et al., 2002), with the suppression of ORP150 expression by antisense RNA causing inhibition of tumor formation in vivo (Miyagi et al., 2002). Results in this study suggest that not only celecoxib-induced ORP150 but also constitutively overproduced ORP150 in tumors may render them resistant to chemotherapy regimes involving celecoxib or other chemotherapy drugs with ER stress response-inducing activity. We consider that the antiapoptotic activity of ORP150 may partially explain the close relationship between ORP150 expression and tumor progression. Another mechanism that may underpin this relationship seems to be mediated by vascular endothelial growth factor (VEGF), which is representative of the angiogenic factors. Several previous studies have shown that VEGF is deeply involved in tumor progression (Ferrara et al., 1996; Machein et al., 1999). Overexpression of ORP150 stimulates secretion of VEGF and therefore seems to play an important role in tumor-mediated angiogenesis (Ozawa et al., 2001). Based on the evidence outlined above, we propose that an inhibitor of ORP150 (and chemicals that inhibit both ORP150 and GRP78 may be more beneficial) may offer considerable clinical benefit as a chemotherapy and chemoprevention drug.

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Address correspondence to: Dr. Tohru Mizushima, Graduate School of Medical and Pharmaceutical Sciences, Kumamoto University, 5-1 Oe-honmachi, Kumamoto 862-0973, Japan. E-mail: mizu@gpo.kumamoto-u.ac.jp